IPCS

INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY

Environmental Health Criteria 180

Principles and Methods for Assessing Direct Immunotoxicity Associated with Exposure to Chemicals

Published under the joint sponsorship of the United Nations Environment Programme, the International Labour Organisation, and the World Health Organization

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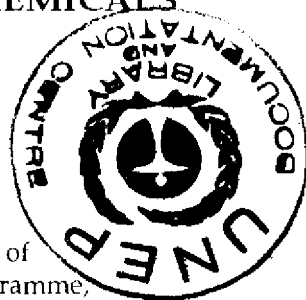
Environmental Health Criteria 180

PRINCIPLES AND METHODS FOR ASSESSING DIRECT IMMUNOTOXICITY ASSOCIATED WITH EXPOSURE TO CHEMICALS



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World Health Organization
Geneva, 1996

The International Programme on Chemical Safety (IPCS), established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), the International Labour Organisation (ILO), and the World Health Organization (WHO). The overall objectives of the IPCS are to establish the scientific basis for assessment of the risk to human health and the environment from exposure to chemicals, through international peer-review processes, as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

The Inter-Organization Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, the Food and Agriculture Organization of the United Nations, WHO, the United Nations Industrial Development Organization and the Organisation for Economic Co-operation and Development (Participating Organizations), following recommendations made by the 1992 UN Conference on Environment and Development to strengthen cooperation and increase coordination in the field of chemical safety. The purpose of the IOMC is to promote coordination of the policies and activities pursued by the Participating Organizations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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NOTE TO READERS OF THE CRITERIA MONOGRAPHS

Every effort has been made to present information in the Criteria monographs as accurately as possible without unduly delaying their publication. In the interest of all users of the Environmental Health Criteria monographs, readers are requested to communicate any errors that may have occurred to the Director of the International Programme on Chemical Safety, World Health Organization, Geneva, Switzerland, in order that they may be included in corrigenda.

* * *

A detailed data profile and a legal file can be obtained from the International Register of Potentially Toxic Chemicals, Case postale 356, 1219 Châtelaine, Geneva, Switzerland (Telephone No. 979 9111).

* * *

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Environmental Health Criteria

PREAMBLE

Objectives

The WHO Environmental Health Criteria Programme was initiated in 1973, with the following objectives:

(i) to assess information on the relationship between exposure to environmental pollutants and human health and to provide guidelines for setting exposure limits;
(ii) to identify new or potential pollutants;
(iii) to identify gaps in knowledge concerning the health effects of pollutants;
(iv) to promote the harmonization of toxicological and epidemiological methods in order to have internationally comparable results.

The first Environmental Health Criteria (EHC) monograph, on mercury, was published in 1976; numerous assessments of chemicals and of physical effects have since been produced. Many EHC monographs have been devoted to toxicological methods, e.g. for genetic, neurotoxic, teratogenic, and nephrotoxic effects. Other publications have been concerned with e.g. epidemiological guidelines, evaluation of short-term tests for carcinogens, biomarkers, and effects on the elderly.

Since the time of its inauguration, the EHC Programme has widened its scope, and the importance of environmental effects has been increasingly emphasized in the total evaluation of chemicals, in addition to their health effects.

The original impetus for the Programme came from resolutions of the World Health Assembly and the recommendations of the 1972 United Nations Conference on the Human Environment. Subsequently, the work became an integral part of the International Programme on Chemical Safety (IPCS), a cooperative programme of UNEP, ILO, and WHO. In this manner, with the strong support of the new partners, the importance of occupational health and environmental effects was fully recognized. The EHC monographs have become widely established, used, and recognized throughout the world.

The recommendations of the 1992 United Nations Conference on Environment and Development and the subsequent establishment of the Intergovernmental Forum on Chemical Safety, with priorities for action in the six programme areas of Chapter 19, Agenda 21, lend further weight to the need for EHC assessments of the risks of chemicals.

The Criteria monographs are intended to provide critical reviews of the effect on human health and the environment of chemicals, combinations of chemicals, and physical and biological agents. They include reviews of studies that are of direct relevance for the evaluation and do not describe every study that has been carried out. Data obtained worldwide are used, and results are quoted from original studies, not from abstracts or reviews. Both published and unpublished reports are considered, and the authors are responsible for assessing all of the articles cited; however, preference is always given to published data, and unpublished data are used only when relevant published data are absent or when the unpublished data are pivotal to the risk assessment. A detailed policy statement is available that describes the procedures used for citing unpublished proprietary data, so that this information can be used in the evaluation without compromising its confidential nature (WHO, 1990).

In the evaluation of human health risks, sound data on humans, whenever available, are preferred to data on experimental animals. Studies of animals and in-vitro systems provide support and are used mainly to supply evidence missing from human studies. It is mandatory that research on human subjects be conducted in full accord with ethical principles, including the provisions of the Helsinki Declaration.

The EHC monographs are intended to assist national and international authorities in making risk assessments and subsequent risk management decisions. They represent a thorough evaluation of risks and are not in any sense recommendations for regulation or setting standards. The latter are the exclusive purview of national and regional governments.

Content

The layout of EHC monographs for chemicals is outlined below.

- Summary: a review of the salient facts and the risk evaluation of the chemical
- Identity: physical and chemical properties, analytical methods
- Sources of exposure
- Environmental transport, distribution, and transformation
- Environmental levels and human exposure
- Kinetics and metabolism in laboratory animals and humans
- Effects on laboratory mammals and in-vitro test systems
- Effects on humans
- Effects on other organisms in the laboratory and the field
- Evaluation of human health risks and effects on the environment
- Conclusions and recommendations for protection of human health and the environment
- Further research

- Previous evaluations by international bodies, e.g. the International Agency for Research on Cancer, the Joint FAO/WHO Expert Committee on Food Additives, and the Joint FAO/WHO Meeting on Pesticide Residues

Selection of chemicals

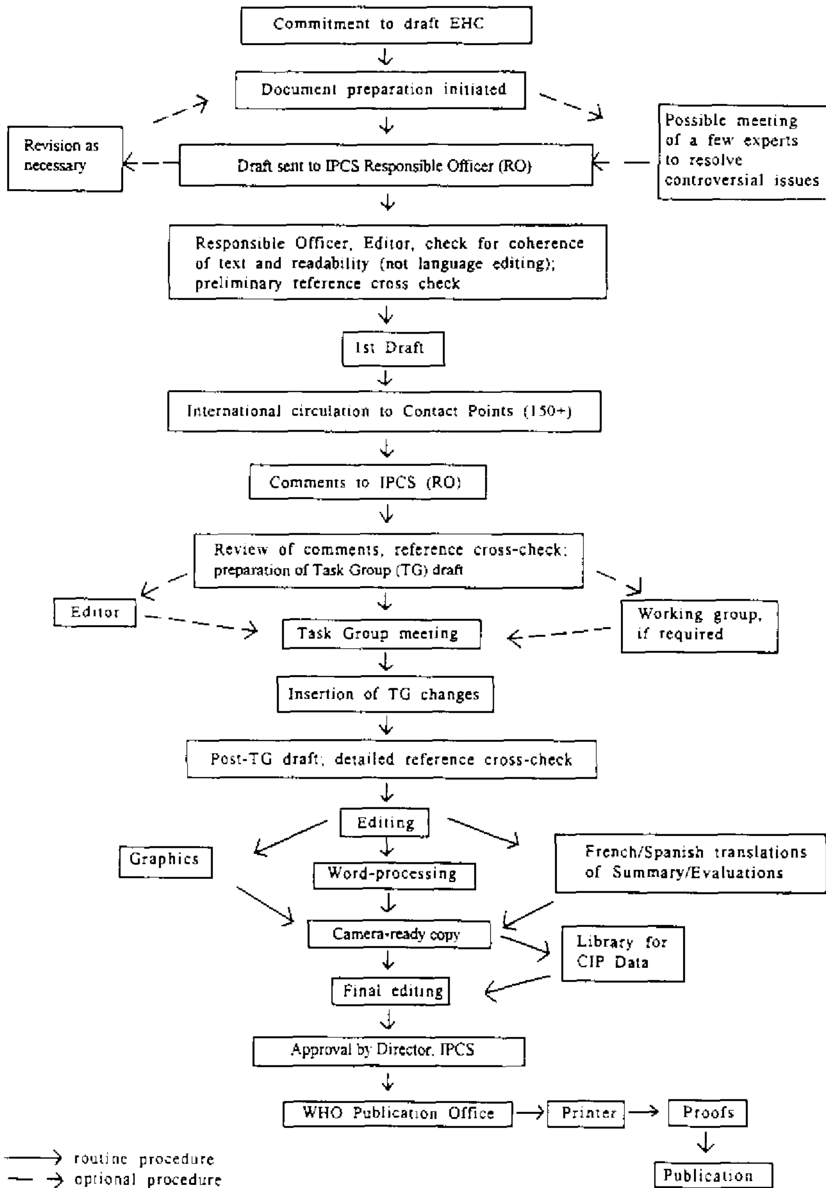
Since the inception of the EHC Programme, the IPCS has organized meetings of scientists to establish lists of chemicals that are of priority for subsequent evaluation. Such meetings have been held in Ispra, Italy (1980); Oxford, United Kingdom (1984); Berlin, Germany (1987); and North Carolina, United States of America (1995). The selection of chemicals is based on the following criteria: the existence of scientific evidence that the substance presents a hazard to human health and/or the environment; the existence of evidence that the possible use, persistence, accumulation, or degradation of the substance involves significant human or environmental exposure; the existence of evidence that the populations at risk (both human and other species) and the risks for the environment are of a significant size and nature; there is international concern, i.e. the substance is of major interest to several countries; adequate data are available on the hazards.

If it is proposed to write an EHC monograph on a chemical that is not on the list of priorities, the IPCS Secretariat first consults with the cooperating organizations and the participating institutions.

Procedures

The order of procedures that result in the publication of an EHC monograph is shown in the following flow chart. A designated staff member of IPCS, responsible for the scientific quality of the document, serves as Responsible Officer (RO). The IPCS Editor is responsible for the layout and language. The first draft, prepared by consultants or, more usually, staff at an IPCS participating institution is based initially on data provided from the International Register of Potentially Toxic Chemicals and reference data bases such as Medline and Toxline.

The draft document, when received by the RO, may require an initial review by a small panel of experts to determine its scientific quality and objectivity. Once the RO finds the first draft acceptable, it is distributed in its unedited form to over 150 EHC contact points throughout the world for comment on its completeness and accuracy and, where necessary, to provide additional material. The contact points, usually designated by governments, may be participating institutions, IPCS focal points, or individual scientists known for their particular expertise. Generally, about four months are allowed before the comments are considered by the RO and author(s). A second draft incorporating the comments received and approved by the Director,


EHC PREPARATION FLOW CHART
Commitment to draft EHC
Document preparation initiated
Possible meeting of a few experts to resolve controversial issues
Revision as necessary
Draft sent to IPCS Responsible Officer (RO)
Responsible Officer, Editor, check for coherence of text and readability (not language editing); preliminary reference cross check
1st Draft
International circulation to Contact Points (150+)
Comments to IPCS (RO)
Review of comments, reference cross-check; preparation of Task Group (TG) draft
Editor
Task Group meeting
Working group, if required
Insertion of TG changes
Post-TG draft; detailed reference cross-check
Editing
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IPCS, is then distributed to Task Group members, who carry out a peer review at least six weeks before their meeting.

The Task Group members serve as individual scientists, not as representatives of any organization, government, or industry. Their function is to evaluate the accuracy, significance, and relevance of the information in the document and to assess the risks to health and the environment from exposure to the chemical. A summary and recommendations for further research and improved safety are also drawn up. The composition of the Task Group is dictated by the range of expertise required for the subject of the meeting and by the need for a balanced geographical distribution.

The three cooperating organizations of the IPCS recognize the important role played by nongovernmental organizations, so that representatives from relevant national and international associations may be invited to join the Task Group as observers. While observers may provide valuable contributions to the process, they can speak only at the invitation of the Chairperson. Observers do not participate in the final evaluation of the chemical, which is the sole responsibility of the Task Group members. The Task Group may meet *in camera* when it considers that to be appropriate.

All individuals who participate in the preparation of an EHC monograph as authors, consultants, or advisers must, in addition to serving in their personal capacity as scientists, inform the RO if at any time a conflict of interest, whether actual or potential, could be perceived in their work. They are required to sign a statement to that effect. This procedure ensures the transparency and probity of the process.

When the Task Group has completed its review and the RO is satisfied as to the scientific correctness and completeness of the document, it is edited for language, the references are checked, and camera-ready copy is prepared. After approval by the Director, IPCS, the monograph is submitted to the WHO Office of Publications for printing. At this time, a copy of the final draft is also sent to the Chairperson and Rapporteur of the Task Group to check for any errors.

It is accepted that the following criteria should initiate the updating of an EHC monograph: new data are available that would substantially change the evaluation; there is public concern about health or environmental effects of the agent because of greater exposure; an appreciable time has elapsed since the last evaluation.

All participating institutions are informed, through the EHC progress report, of the authors and institutions proposed for the drafting of the documents. A comprehensive file of all comments received on drafts of each EHC monograph is maintained and is available on request. The chairpersons of task groups are briefed before each meeting on their role and responsibility in ensuring that these rules are followed.

WHO TASK GROUP MEETING ON PRINCIPLES AND METHODS FOR ASSESSING DIRECT IMMUNOTOXICITY ASSOCIATED WITH EXPOSURE TO CHEMICALS

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ENVIRONMENTAL HEALTH CRITERIA PRINCIPLES AND METHODS FOR ASSESSING DIRECT IMMUNOTOXICITY ASSOCIATED WITH EXPOSURE TO CHEMICALS

A WHO Task Group on Principles and Methods for Assessing Direct Immunotoxicity Associated with Exposure to Chemicals met at the World Health Organization, Geneva, from 10 to 14 October 1994. Dr E. Smith, IPCS, welcomed the participants on behalf of Dr M. Mercier, Director IPCS, and the cooperating organizations. The Task Group reviewed and revised the draft monograph and prepared the final text.

The first draft of the monograph was prepared by a group of authors (listed below) under the coordination of Dr J.G. Vos and Dr H. Van Loveren of the Dutch National Institute for Public Health and Environmental Protection (RIVM), an IPCS Collaborating Centre for Immunotoxicology and Allergic Hypersensitization. The second draft, incorporating comments received after international circulation to national experts of the first draft to IPCS contact points for *Environmental Health Criteria* monographs, was prepared by Dr J.G. Vos and Dr H. Van Loveren of the Netherlands and Dr Kimber White, USA.

Dr E. Smith of the IPCS Unit for the Assessment of Risk and Methods was responsible for the scientific content of the monograph and Mrs E. Heseltine for the editing.

The efforts of all who helped in the preparation and finalization of the monograph are gratefully acknowledged.

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ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
Ah	aromatic hydrocarbon
AIDS	acquired immunodeficiency syndrome
B	bursa-dependent
CALLA	common acute lymphoblastic leukaemia antigen
CD	cluster of differentiation
CEC	Commission of the European Communities
CH50	haemolytic complement
CML	cell-mediated lympholysis
DMBA	7,12-dimethylbenz[*a*]anthracene
DNCB	dinitrochlorobenzene
ELISA	enzyme-linked immunosorbent assay
EPO	erythrocyte lineage differentiation factor
FACS	fluorescence activated cell sorter
GALT	gut-associated lymphoid tissue
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte–macrophage colony-stimulating factor
GVH	graft-versus-host
HCB	hexaclorobenzene
HEV	high endothelial venule
HIV	human immunodeficiency virus
HPCA	human progenitor cell antigen
HSA	heat-stable antigen
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPCS	International Programme on Chemical Safety
LFA	lymphocyte function-related antigen
LIF	leukaemia inhibitory factor
LOAEL	lowest-observed-adverse-effect level
LOEL	lowest-observed-effect level
M	microfold
MALT	mucosa-associated lymphoid tissue
MARE	monoclonal anti-rat immunoglobulin E
MARK	monoclonal antibody anti-κ
M-CSF	macrophage colony-stimulating factor
MED	minimal erythemal dose
MHC	major histocompatibility complex
NCAM	neural cell adhesion molecule

NK	natural killer
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
NTP	National Toxicology Program
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PG	prostaglandin
QCA	quiescent cell antigen
RIVM	Dutch National Institute of Public Health and Environmental Protection
S9	9000 x *g* supernatant
SCF	stem-cell factor
SCID	severe combined immunodeficiency
SIS	skin immune system
STM	*Salmonella typhimurium* mitogen
TBTO	tri-*n*-butyltin oxide
Tc	cytotoxic T cell
TCDD	2,3,7,8-tetrachlorodibenzo-*para*-dioxin
TCR	T-cell receptor
Tdth	delayed-type hypersensitivity T cell
TGF	transforming growth factor
Th	T helper–inducer cell
THAM	T-cell activation molecule
THI	2-acetyl-4(5)-tetrahydroxybutylimidazole
O,O,S-TMP	*O,O,S*-trimethylphosphorothiate
TNF	tumour necrosis factor
UVB	ultraviolet B
UVR	ultraviolet radiation
VCAM	vascular cell adhesion molecule
VLA	very late antigen

SUMMARY

1 . The immune system has evolved to counter challenges to the integrity of self from either microorganisms or cells that have escaped the organism's control mechanisms. Recognition that xenobiotics can impair the function of the immune system has led to progress in immunotoxicology over the last two decades. Experimental approaches (mainly in rodent species) have been developed and validated in multilaboratory studies. In this monograph, the function and histophysiology of the immune system are reviewed, and the information necessary to understand and interpret the pathological changes caused by immunotoxic insults is provided. Emphasis is laid on the immune systems of humans and rodent species, but reference is made to other species, including fish, that have been the object of immunotoxicological studies. The pathophysiology of the immune system, including the variable susceptibility of its components, alterations to the lymphoid organs, and the reversibility of changes are important for understanding the impact of immunotoxicity.

2. Immunosuppression and immunostimulation both have clinical consequences. Immunodeficiency states and severe immunosuppression, such as can occur during transplantion and cytostatic therapy, have both been associated with increased incidences of infectious diseases (particularly opportunistic ones) and cancer. Exposure to immunotoxic chemicals in the environment, however, may be expected to result in more subtle forms of immunosuppression which may be difficult to detect, leading to increased incidences of infections such as influenza and the common cold. Studies of experimental animals and humans have shown that many environmental chemicals suppress the immune response. Immunotoxic xenobiotics are not restricted to a particular chemical class. Compounds that adversely affect the immune system are found among drugs, pesticides, solvents, halogenated and aromatic hydrocarbons, and metals; ultraviolet radiation can also be immunotoxic. Therapeutic administration of immunostimulating agents can have adverse effects, and a few environmental chemicals that have immunostimulating properties (beryllium, silica, hexachlorobenzene) can have clinical consequences.

3. The complexity of the immune system results in multiple potential target sites and pathological sequelae. The initial strategies devised by immunotoxicologists working in toxicology and safety assessment were to select and apply a tiered panel of assays to identify immunosuppressive and immunostimulatory agents in laboratory

animals. Although the configuration of these testing panels may vary depending on which agency or laboratory is conducting the test and on the animal species employed, they all include measurement of one or more of the following: altered lymphoid organ weights and histology; changes in the cellularity of lymphoid tissue, peripheral blood leukocytes, and/or bone marrow; impairment of cell function at the effector or regulatory level; and altered susceptibility to challenge with infectious agents or tumour cells.

The original test guideline No. 407 of the Organisation for Economic Co-operation and Development, published in 1981, was not designed to detect potential immunotoxicity, and modifications have been proposed to make the guideline more useful for identifying immunotoxicants. Tiered testing systems have been designed for more extensive investigation of potential immunotoxicity, by the US National Toxicology Program, the Dutch National Institute of Public Health and Environmental Protection, the US Environmental Protection Agency Office of Pesticides, and the US Food and Drug Administration Center for Food Safety and Applied Nutrition.

Studies have been conducted in mice, and to a lesser extent in rats, to investigate the specificity, precision (reproducibility), sensitivity, accuracy, and relevance for the assessment of risk to human health of a variety of measures of immune status. International, interlaboratory validations of methods have been carried out within the International Collaborative Immunotoxicity Study of IPCS and the European Union, the Bundesinstitut für Gesundheitlichen Verbraucherschutz, und Veterinärmedizin, and in studies of cyclosporin A in Fischer 344 rats.

4. The tests used in the tiered testing schemes are described in Section 3, which indicates the rationale for their selection and the complexities involved in their performance. Although these protocols were designed for studies of rats and mice, some have been applied successfully for studying immunotoxicity in other animal species, including non-human primates, marine mammals, dogs, birds, and fish.

A variety of factors must be considered in evaluating the potential of an environmental agent or drug to influence the immune system of experimental animals adversely. These include selection of the appropriate animal models and exposure variables, inclusion of general toxicological parameters, an understanding of the biological relevance of the end-points being measured, use of validated measures, and quality assurance. The experimental conditions should take into account the potential route and level of human exposure and any available information on toxicodynamics and toxicokinetics. The doses and sample sizes should be selected so as to generate clear dose–response

curves, in addition to no-observed-adverse-effect or no-observed-effect levels. The strategies are continually refined to allow better prediction of conditions that may lead to disease. In addition, techniques should be developed that would help to identify mechanisms of action; these might include methods *in vitro*, examination of local immune responses (such as in the skin, lung, and intestines), and use of the techniques of molecular biology and genetically modified animals.

5. The detection of immune changes after exposure to potentially immunotoxic compounds is more complicated in humans than in experimental animals. The testing possibilities are limited, levels of exposure to the agent (i.e. dose) are difficult to establish, and the immune status of populations is extremely heterogeneous. Age, race, gender, pregnancy, acute stress and the ability to cope with stress, coexistent disease and infections, nutritional status, tobacco smoke, and some medications contribute to this heterogeneity.

An important factor in assessing the usefulness of a particular study for risk assessment is epidemiological study design. The commonest design used in immunotoxicity is the cross-sectional study, in which exposure status and disease status are measured at one time or over a short period. The immune function of 'exposed' subjects is then compared with that of a comparable group of 'unexposed' individuals. There are possible pitfalls in this study design.

Because many of the immune changes seen in humans after exposure to a chemical may be sporadic and subtle, recently exposed populations must be studied and sensitive tests be used for assessing the immune system. Conclusions about immunotoxic effects should be based on changes not in a single parameter but in the immune profile of an individual or population.

Most of the tests for specific immunity (cell-mediated and humoral), nonspecific immunity and inflammation were developed to detect immune alterations in patients with immunodeficiency disease and are not always adequate to detect subtle alterations induced by environmental chemicals. IPCS, the Centers for Disease Control, and the US National Academy of Sciences have each described procedures for evaluating changes in the human immune system resulting from exposure to immunotoxicants, but the tests described require evaluation for this purpose.

6. Risk assessment is a process in which relevant data on the biological effects, dose–response relationships, and exposure for a particular agent are analysed in an attempt to establish qualitative and quantitative estimates of adverse outcomes. Typically, risk assessment comprises

four major steps: hazard identification, dose–response assessment, exposure assessment, and risk characterization. Up until now, immunotoxicology has focused mainly on hazard identification, and to some extent on dose–response assessment, and very few studies have included exposure assessment or risk characterization.

As in other areas of toxicology, uncertainties exist which may affect the interpretation of data on immunotoxicity with regard to human health risk. The two most problematic issues—extrapolating effects from individual cells to a whole organ or beyond and extrapolating data from experimental animals to humans—are common to most non-cancer end-points. The first issue is due to uncertainties associated with establishing a quantitative relationship between changes in individual immune function and altered resistance to infections and neoplastic disease. The second issue is due to uncertainties associated with assessing risk to human health on the basis of studies in laboratory animals.

The ultimate purpose of risk assessment is to protect human health and the environment. Suitable model systems must therefore be chosen. The toxicokinetics of the test material and the nature and magnitude of the immune response generated in the model should be comparable to that of humans.

Conventionally, empirical uncertainty factors are used in risk assessment to derive an acceptable exposure limit from experimental results. This approach does not take into account the functional reserve or redundancy of the immune system. A more recent development in risk assessment is use of in-vitro models as an adjunct to studies of experimental animals. The advantages of this approach are that it improves the accuracy of extrapolation of data from animals to man and minimizes the use of animals; it also bridges the gap between those data, particularly when human experimentation is limited for ethical considerations. Chapter 6 cites two examples in which in-vitro data make it possible to reduce the uncertainties in risk assessment associated with exposure to ozone and ultraviolet radiation. The difficulty in establishing quantitative relationships between immunosuppression and clinical disease has limited the use of immunotoxicological data in risk assessment.

RECOMMENDATIONS

Recommendations for the protection of human health

1. Chemicals should be screened to determine if they are potentially immunotoxic to humans. If immunotoxicity is detected, the chemicals should be investigated further as part of the risk assessment process.

2. Chemicals for which little or no information is available on toxicity should be screened for potential immunotoxicity following a protocol based on, for example, the revised OECD guideline No. 407. When some information is available on the test material (e.g. physicochemical properties, toxicokinetics, structure–activity relationships), a flexible approach to testing is recommended which permits a rational selection of test procedures.

3. The immunotoxic risk of mixtures of environmental pollutants, in, for example, fish, to certain human consumer groups (e.g. fishermen) should be assessed.

Recommendations for protection of the environment

1. Chemicals should be screened to determine if they are potentially immunotoxic to wildlife species. If immunotoxicity is detected, the chemicals should be investigated further as part of the risk assessment process.

2. The immunotoxic risk of environmental pollution to the health of the ecosystem should be assessed in laboratory, semi-field, and field studies of the wildlife occupying high trophic levels or those species judged to be sensitive.

Recommendations for further research

1. The panels of tests suggested for evaluating xenobiotic-induced immunotoxicity in humans should be investigated to determine their ability to detect subtle alterations in immune status.

2. The relationships between alterations in immune function and human health should be established for use in immunotoxic risk assessment. Epidemiological studies should be carried out that include assessment of exposure, in order to establish dose–response relationships.

3. The relationship between immunotoxicity and the development of neoplasia should be investigated.

4. Baseline immunological data should be established for the general population and for subpopulations such as ethnic minorities, children, the aged, and pregnant and lactating women in order to assess their immune status.

5. Immunotoxicological assessment should be conducted for subpopulations potentially susceptible to the effects of immunotoxic compounds, including those at the extremes of age and those with deficient nutritional status.

6. Biomarkers of exposure, effect, and susceptibility should be identified, developed, and validated for use in epidemiological studies of immunotoxicity in both humans and wildlife.

7. The quantitative relationship between immune function and host resistance in animal models, including the nature, magnitude, and significance of functional reserve and redundancy, should be explored for risk assessment.

8. Since chemicals and biological agents enter the body via the respiratory and alimentary tracts and the skin, more research should be carried out on local immunity.

9. Preliminary observations in laboratory animals that suggest that primary immunization does not compromise testing for subacute toxicity should be substantiated by further research, so that functional testing can be incorporated into toxicology testing.

10. Methods and reagents should be developed in order to characterize the immune system of wildlife species and to assess their immune status for immunotoxicological studies.

11. The mechanisms of the immunotoxic action of xenobiotics in humans should be elucidated by a combination of studies in laboratory animals *in vivo* and experiments with human and animal tissues and cell lines *in vitro*.

12. In view of the sensitivity of the developing immune system to immunotoxic injury, more emphasis should be placed on studies involving perinatal exposure to a chemical or mixture of chemicals.

13. Studies should be conducted to establish whether exposure to xenobiotics that are not themselves sensitizing adds to the risk of allergic disease in general.

14. Autoimmune models in laboratory animals should be used to assess whether xenobiotics can modulate autoimmune disease in humans.

15. The effects on immune function of confounding factors in humans and animals, including age, race, sex, gender, nutritional status, acute stress, and underlying disease, should be evaluated further in order to determine their effects in tests for the immunotoxicity of environmental chemicals.

16. Methods for assessing cytokines and their production in different body compartments, including plasma, bronchoalveolar lavage fluid, and nasal lavage fluid, and by cells isolated from various anatomical sites should be validated for humans and laboratory animals, and their applicability for assessing the risk of chemicals should be established.

17. Data from clinical trials should be made more widely available; and patients undergoing therapy with immunomodulatory drugs should be monitored clinically and immunologically in a systematic way.

18. The toxicokinetics of immunotoxic chemicals should be further investigated, particularly with regard to whether their concentrations in human biological fluids indicate levels of environmental exposure.

19. The interactions between the immune system, the nervous system, and the endocrine system should be further investigated, with particular emphasis on how xenobiotics adversely affect them.

20. The significance of ultraviolet radiation-induced immunosuppression for public health and the health of ecosystems should be evaluated.

1. INTRODUCTION TO IMMUNOTOXICOLOGY

1.1 Historical overview

It is well established that each individual has an intrinsic capacity to defend itself against pathogens in the environment, with a defence known as the immune system. By general definition, the immune system serves the body by neutralizating, inactivating, or eliminating potentially pathogenic invaders such as microorganisms (bacteria and viruses); it also guards against uncontrolled growth of cells into neoplasms, or tumours. The major features of the structure and function of the immune system have been elucidated over the last three decades; in parallel, awareness grew of toxicological manifestations after exposure to xenobiotic chemicals. Recognition of the interplay between toxicology and immunology is relatively recent: A comprehensive review, published in 1977 (Vos, 1977), was the first survey of a large series of xenobiotics that affect immune reactivity in laboratory animals and hence may influence the health of exposed individuals. Most research groups focusing on toxicity to the immune system started their activities during the last decade. Textbooks of immunotoxicology date only from the early 1980s (Gibson et al., 1983; Dean et al., 1985; Descotes, 1986), while one on clinical immunotoxicology is more recent (Newcombe et al., 1992).

Immunotoxicology is the study of the interactions of chemicals and drugs with the immune system. A major focus of immunotoxicology is the detection and evaluation of undesired effects of substances by means of tests on rodents. The prime concern is to assess the importance of these interactions in regard to human health. Toxic responses may occur when the immune system is the target of chemical insults, resulting in altered immune function; this in turn can result in decreased resistance to infection, certain forms of neoplasia, or immune dysregulation or stimulation which exacerbates allergy or autoimmunity. Alternatively, toxicity may arise when the immune system responds to the antigenic specificity of the chemical as part of a specific immune response (i.e. allergy or autoimmunity). Certain drugs induce autoimmunity (Kammüller et al., 1989; Kammüller & Bloksma, 1994). The differentiation between direct toxicity and toxicity due to an immune response to a compound is to a certain extent artificial. Some compounds can exert a direct toxic action on the immune system as well as altering the immune response. Heavy metals like lead and mercury, for instance, manifest immunosuppressive activity, hypersensitivity, and autoimmunity (Lawrence et al., 1987).

This monograph is concerned mainly with one aspect of immunotoxicology: the direct or indirect effect of xenobiotic compounds (or their biotransformation products) on the immune system. This effect is usually immunosuppression, or the induction of a state of deficiency or unresponsiveness. Allergy and autoimmunity will be dealt with in a future *Environmental Health Criteria* monograph.

Toxicological research over the past decade has indicated that the immune system is a potential 'target organ' for toxic damage. This finding was the basis for a number of large scientific conferences on immunotoxicology and sparked the active interest of national and international organizations in this field. One of the milestones in the development of the discipline was the international seminar on 'The Immunological System as a Target for Toxic Damage', held in Luxembourg in 1984 and organized by the International Programme on Chemical Safety (IPCS), and the Commission of the European Communities (CEC). At the seminar, immunotoxicology was defined as 'the discipline concerned with the study of the events that can lead to undesired effects as a result of interaction of xenobiotics with the immune system. These undesired events may result as a consequence of: (1) a direct and/or indirect effect of the xenobiotic (and/or its biotransformation product) on the immune system; or, (2) an immunologically-based host response to the compound and/or its metabolite(s) or host antigens modified by the compound or its metabolites' (Berlin et al., 1987). Recommendations were made concerning the significance to public health of immunotoxicology, immunotoxicity testing, research and development in immunotoxicology, the development of databases, and training and education. A subsequent workshop on 'Immunotoxicity of Metals and Immunotoxicology', organized by IPCS and the CEC, in collaboration with the International Union for Pure and Applied Chemistry and German governmental agencies, was held in Hanover, Germany, in 1989 (Dayan et al., 1990). A meeting on risk assessment in immunotoxicology was organized by the United States National Institute for Environmental Health Sciences in 1990. A meeting on human immunotoxicology tests was organized by the Agency for Toxic Substances and Disease Registry and the Centers for Disease Control, in Atlanta, Georgia, United States of America, in 1992. In 1994, two meetings were held: one in Oxford, United Kingdom, organized by IPCS, on risk assessment in human imunotoxicity, and one in Washington DC, United States, organized by the International Life Sciences Institute, on methods in immunotoxicology.

In parallel to these meetings, activities were started within IPCS for the development and validation of methods for assessing toxicity to the

immune system. In this regard, a hallmark event was the meeting in 1986 of a technical review and working group, in London, United Kingdom (IPCS, 1986).

A number of tiered approaches to immunotoxicity testing have been proposed, in rats (Vos, 1980; Van Loveren & Vos, 1989) and subsequently in mice (Luster et al., 1988). These approaches have been evaluated for their capacity to identify chemicals as immunosuppressive. Of a group of 18 pesticides evaluated in rats, six were identified as inducing immunotoxicity at doses similar to those that cause other toxic effects, and five were immunotoxic at lower doses (Vos & Krajnc, 1983; Vos et al., 1983a). Effects were seen on different parameters with different compounds and included lymphocytopenia, reduced thymic and spleen weights, and increased levels of serum immunoglobulin (Ig) G. One of the compounds identified was hexachlorobenzene (Vos, 1986), which is further described in Section 2. In mice, the tiered approach was used to assess the immunotoxicity of 51 chemicals, selected on the basis of factors including structure–activity relationships with previously identified immunotoxic substances, and use (Luster et al., 1992). Of the spectrum of assays applied, the strongest associations with immunotoxic potential were observed with the splenic IgM antibody plaque-forming cell response and cell surface marker analysis; somewhat weaker associations were found for natural killer (NK) cell activity, cytotoxic T-lymphocyte cytolytic activity, lymphocyte proliferation *in vitro* after mitogen stimulation, and thymus:body weight ratio. The tiered approach in immunotoxicity testing is further described in Section 3.

Multi-laboratory studies have been initiated to validate the screening of immunotoxic compounds, including the IPCS–European Union International Collaborative Immunotoxicity Study, a study in Fischer 344 rats, and the international study of the Bundesinstitut für Gesundheitlichen Verbraucherschutz, und Veterinärmedizin, which were designed to determine interlaboratory reproducibility. The experimental animal used in these studies is the rat, and some functional tests are included. Test methods are also being developed and validated within the National Toxicology Program (NTP) in the United States. This programme includes studies of carcinogenicity in rats and mice, but because the immune system of mice is better characterized than that of rats, the NTP chose the mouse as the experimental animal for immunotoxicity assessment. The immunotoxicity database of the NTP has been evaluated to determine the predictability (sensitivity and specificity) of the assays. In the Netherlands, a Committee for Immunotoxicology of the Dutch Health Council reviewed methods that could be used to assess the immunotoxic properties of a compound and for

deriving information about risks to humans on the basis of the results of laboratory experiments. The Committee also examined the relationship between the immunotoxic properties of a substance and its mutagenic and carcinogenic properties (Dutch Health Council, Committee for Immunotoxicology, 1991).

The immune system was reviewed by the United States National Research Council in order to identify the kinds of basic research that might reveal markers of environmental exposure and disease. Major emphasis was placed on biological markers of three types: those originating from the immune system, those related to exposure to immunosuppressive toxicants, and those of effects of environmental pollutants. Markers of susceptibility to environmental materials were also considered to be important, especially if they are of a genetic nature and can be used to identify individuals susceptible to autoimmune diseases. The National Research Council subcommittees on pulmonary toxicology and on immunotoxicology, have published their reports (US National Research Council, 1989, 1992).

Interest in immunotoxicology within the scientific community is reflected by the existence of a special section on immunotoxicology within the Society of Toxicology. An immunotoxicology discussion group initiated in the United States has an international composition. The European Union has a programme on science and technology for environmental protection that includes immunotoxicology as an important aspect.

There is growing concern in society about the effects of xenobiotics, such as environmental pollutants, on public health; the immune system is one of the targets of such effects. Some chemicals present in the environment that have been reported to influence the immune system are listed in Table 1 (IPCS, 1986). Immunotoxicity can result in e.g. reduced resistance towards infection or generation of tumours that escape immune surveillance. A number of substances affect immunological parameters; these include halogenated hydrocarbons such as polychlorinated biphenyls, polybrominated biphenyls, polychlorinated dibenzo-*para*-dioxins, and polychlorinated dibenzofurans (Elo et al., 1985; Lu & Wu, 1985; Bekesi et al., 1987; Kimbrough, 1987; Hoffman 1992); pesticides and precursors (Fiore et al., 1986; Deo et al., 1987; Nigam et al., 1993); organic solvents (Capurro, 1980; Denkhaus et al., 1986); asbestos (Lew et al., 1986); silica (Uber & McReynolds, 1982); and metals like lead (Ewers et al., 1982; Reigart & Graber, 1976). Oxidant air pollutants, like sulfur dioxide, nitrogen dioxide, and ozone, and particles in airborne dust may affect immune function (Koren et al., 1989; Van Loveren et al., 1994).

Table 1. Examples of compounds that are immunotoxic for humans or rodents

Chemical	Immune toxicity	
	Rodent	Human
2,3,7,8-Tetrachlorodibenzo-*para*-dioxin	+	+
Polychlorinated biphenyls	+	+
Polybrominated biphenyls	+	+
Hexachlorobenzene	+	Unknown
Lead	+	Unknown
Cadmium	+	Unknown
Methyl mercury compounds	+	Unknown
7,12-Dimethylbenz[*a*]anthracene	+	Unknown
Benzo[*a*]pyrene	+	Unknown
Di-*n*-octyltindichloride	+	Unknown
Di-*n*-butyltindichloride	+	Unknown
Benzidine	+	+
Nitrogen dioxide and ozone	+	+
Benzene, toluene, and xylene	+	+
Asbestos	+	+
N-Nitrosodimethylamine	+	Unknown
Diethylstilboestrol	+	+
Vanadium	+	+

From IPCS (1986)

Immunotoxicity in humans is further discussed in Section 2. Few epidemiological data have been published that indicate suppression or altered resistance to infection and tumours. In general, the usefulness of the epidemiological studies that have been published is limited by the following: exposure is usually uncontrolled, mainly occurring during accidents; the magnitude and pattern of exposure are not known, and the exposure is often too low to alter the immune system measurably; exposure is often not to one xenobiotic but to a mixture; it is almost impossible to control for confounding parameters, such as age, sex, genetic background, health status, and nutritional status; and it is not always possible to define and analyse appropriate control groups (US National Research Council, 1992). Environmental pollution and its effect on health status are currently subjects of concern in eastern European countries and have generated much interest in the worldwide environmental health science community. Recent epidemiological studies have compared the possible relationship between exposure to air pollutants and health effects in the former German Democratic Republic and Federal Republic of Germany, and some of these studies included immunological data or end-points. For instance, von Mutius et al. (1992) found a higher prevalence of asthma among schoolchildren

in western than eastern Germany, and Behrendt et al. (1993) observed, surprisingly, that total serum IgE levels were higher in schoolchildren in eastern than in western Germany. Several factors were found to influence total IgE: history of parasitic disease, number of persons per dwelling, and passive smoking. Sex and passive smoking were the only variables that had a significant effect in western German children. Air pollutants and parasitic infections were suggested to be the major contributing factors to increased IgE production in children in eastern Germany. Remarkable differences in air quality were seen between eastern and western Germany, and Behrendt et al. (1995) distinguished two types of air pollution: type I, composed of sulfur dioxide particles and dust, occurring predominantly in eastern Europe, is associated with respiratory infections and other chronic inflammatory airway reactions; type II, occurring both indoors and outdoors in the environment in industrialized western countries, is composed mainly of nitric oxide, nitrogen dioxide, ozone, volatile organic compounds, and fine particles. The latter type of air pollution is associated with allergic diseases and allergic sensitization, indicating that air pollutants interfere with parameters of allergy at the level of sensitization, elicitation of symptoms, and exacerbation of disease.

Until further epidemiological studies are conducted in humans, assessment of immunotoxicity in rodents, with subsequent extrapolation to the human situation, is still a good indicator of toxicity and can serve as a basis for subsequent decisions and regulations by authorities to reduce or prevent the risk of human exposure. This aspect is discussed further in Section 5.

Humans are exposed to environmental contaminants mainly via food, water, and air. Open water (e.g. rivers, lakes, and coastal areas) and sediments often act as sinks for environmental pollution. This global problem can be deduced from disease manifestations in fish that live in coastal areas, especially those species that live in close contact with contaminated silt. High levels of contaminants and the diseases associated with them are not only of economic importance (i.e. to fisheries) but also affect people who consume the fish, as seen in studies showing increased levels of contaminants in people eating fish from the contaminated Baltic Sea (Svensson et al., 1991) and in Inuit and Indian populations in Canada who consume large quantities of fish and marine mammals (DeWailly et al., 1992). There is now some evidence that wildlife aquatic species have decreased resistance and enhanced incidences of infection and tumours that may be linked to environmental pollution (Vos et al., 1989; Wester et al., 1994). Because the immune system of fish has not been characterized in such detail as that of mammals, immunotoxicological studies have not been extensively

included in ecotoxicology, although a number of reports of direct toxic actions of xenobiotics on fish species have been published in this developing field (Wester & Canton, 1987; Payne & Fancey, 1989; Anderson, 1990; Wester et al., 1990; Khangarot & Tripathi, 1991; Secombes et al., 1992; Anderson & Brubacher, 1993; Faisal & Hugget, 1993).

1.2 The immune system: functions, system regulation, and modifying factors; histophysiology of lymphoid organs

1.2.1 Function of the immune system

In order to interpret pathological alterations of the immune system in terms of altered function, the physiology of the system must be understood. Since knowledge of the structure and function of the immune system is growing rapidly, a review of this subject, focusing on histophysiology, is presented. This section is not meant to serve as a textbook on immunology but to provide sufficient information for an understanding of pathological changes due to immunotoxic action. For general textbooks on immunology, reference may be made to Sell (1987), Klein (1990), Brostoff et al. (1991), Roitt (1991), Paul (1993), and Roitt et al. (1993). The section covers mainly humans and rodents, but reference is made to other species that are relevant in immunotoxicity assessment, e.g. fish in ecotoxicology. It should be noted that species differences can be large, despite fundamental similarities between the immune systems of animals. It is therefore difficult to conduct immunotoxicological studies in immunologically less well characterized animal species, although comparative studies that are under way may lessen the problems. Zapata and Cooper (1990) have written a comprehensive textbook on phylogenetic aspects of immunology. Phylogenetic data, from primitive fish to mammals, are presented in Table 2 (Cooper, 1982; Klein, 1986; Du Pasquier, 1989; Zapata & Cooper, 1990; Sima & Vetvicka, 1992). Relevant phylogenetic aspects of the immune system are described below.

In mammals, the immune system and its reactions consist of a finely tuned, complex interplay between various cell types and soluble mediators secreted by those cells (Figure 1), some of which are listed in Section 7.

Immune responses can be classified roughly as innate (natural and nonspecific) and acquired (adaptive) responses, in which the reaction is directed to a specific determinant (antigenic determinant or epitope). The nonspecific response involves effector cells such as macrophages (Vetvicka & Fornusek, 1992), NK cells (Herberman & Ortaldo, 1981), granulocytes (Ross, 1992), and mediator systems including the complement system (Tomlinson, 1993). Specificity is based on

Table 2. Evolution of immunologically important traits among vertebrates

A. Major histocompatibility complex (MHC) and transplantation

Species	Graft rejection	MLR and/or GVH	CML	MHC control of immune response	Serologically detectable MHC antigens
Tunicata (sea squirts)	+	?	?	?	?
Agnatha					
Hagfish (Hyperotreti)	+	?	-	?	?
Lamprey (Hyperoartii)	+	?	-	?	?
Chondrichthyes (cartilaginous fish)					
Shark, ray	+	?	+	?	+
Osteichthyes (bony fish)					
Sturgeon (Chondrostei)	+	?	?	?	?
Bony fish (Teleostei)	+	?	+	?	+
Lungfish (Dipnoi)	+	?	?	?	?
Amphibia (amphibians)					
Salamanders (Urodela)	+	?	?	?	+
Frogs, toads (Anura)	+	+	+	+	+
Reptilia (reptiles)					
Turtles (Chelonia)	+	+	?	?	?
Lizards, snakes (Squamata)	+	+	+	?	?
Crocodiles, alligators (Crocodilia)	+	?	?	?	?
Aves (birds)	+	+	+	+	+
Mammalia (mammals)	+	+	+	+	+

Table 2 (contd)

B. Complement and immunoglobulins (Ig)

Species	Complement	Immunoglobulins				
		IgM	IgG-like	IgA	IgD	IgE
Tunicata (sea squirts)	?	-	-	-	-	-
Agnatha						
Hagfish (Hyperotreti)	?	?	-	-	-	-
Lamprey (Hyperoartii)	?	?	-	-	-	-
Chondrichthyes (cartilaginous fish)						
Shark, ray	+	+	+	-	-	-
Osteichthyes (bony fish)						
Sturgeon (Chondrostei)	+	+	+	-	-	-
Bony fish (Teleostei)	+	+	+	-	-	-
Lungfish (Dipnoi)	+	+	+	-	-	-
Amphibia (amphibians)						
Salamanders (Urodela)	+	+	?	-	-	-
Frogs, toads (Anura)	+	+	+	+	-	-
Reptilia (reptiles)						
Turtles (Chelonia)	+	+	+	-	-	-
Lizards, snakes (Squamata)	+	+	+	-	-	-
Crocodiles, alligators (Crocodilia)	+	+	+	?	-	-
Aves (birds)	+	+	?	+	-	-
Mammalia (mammals)	+	+	+	+	+	+

Table 2 (contd)

C. Leukocytes

Species	Lymphocytes			Plasma cells	Macrophages
	Small	T	B		
Tunicata (sea squirts)	+	-	-	-	+
Agnatha					
Hagfish (Hyperotreti)	+	-	-	+	+
Lamprey (Hyperoartii)	+	-	-	+	+
Chondrichthyes (cartilaginous fish)					
Shark, ray	+	-	?	+	+
Osteichthyes (bony fish)					
Sturgeon (Chondrostei)	+	-	?	+	+
Bony fish (Teleostei)	+	+	+	+	+
Lungfish (Dipnoi)	+	?	?	+	+
Amphibia (amphibians)					
Salamanders (Urodela)	+	+	+	+	+
Frogs, toads (Anura)	+	+	+	+	+
Reptilia (reptiles)					
Turtles (Chelonia)	+	+	+	+	+
Lizards, snakes (Squamata)	+	+	+	+	+
Crocodiles, alligators (Crocodilia)	+	+	+	+	+
Aves (birds)	+	+	+	+	+
Mammalia (mammals)	+	+	+	+	+

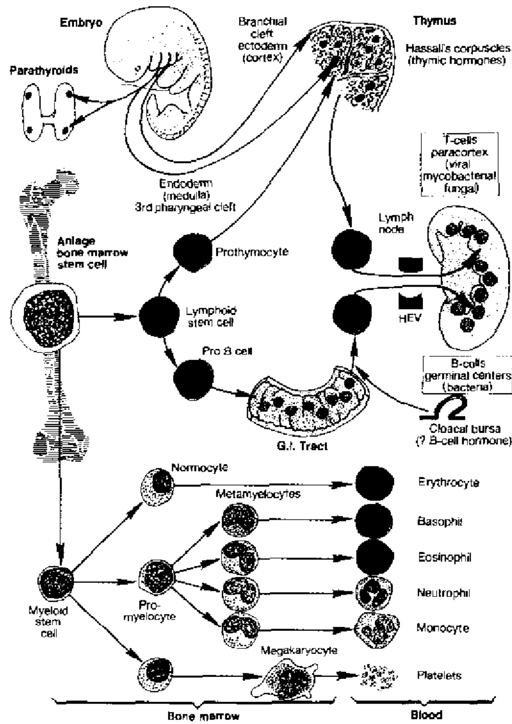
Table 2 (contd)

D. Lymphoid organs

Species	Bone marrow	Thymus	Spleen	Lymph glands or nodes
Tunicata (sea squirts)	-	-	-	-
Agnatha				
Hagfish (Hyperotreti)	-	-	-	GALT
Lamprey (Hyperoartii)	-	-	-	GALT
Chondrichthyes (cartilaginous fish)				
Shark, ray	-	+	+	GALT
Osteichthyes (bony fish)				
Sturgeon (Chondrostei)	-	+	+	GALT
Bony fish (Teleostei)	-	+	+	GALT
Lungfish (Dipnoi)	-	+	+	GALT
Amphibia (amphibians)				
Salamanders (Urodela)	+	+	+	-
Frogs, toads (Anura)	+	+	+	?
Reptilia (reptiles)				
Turtles (Chelonia)	+	+	+	?
Lizards, snakes (Squamata)	+	+	+	?
Crocodiles, alligators (Crocodilia)	+	+	+	?
Aves (birds)	+	+	+	+
Mammalia (mammals)	+	+	+	+

+, positive; ±, to be confirmed; -, negative; ?, not investigated.
MLR, mixed leukocyte response; GVH, graft-versus-host; CML, cell-mediated lympholysis; GALT, gut-associated lymphoid tissue

Figure 1. Maturation and differentiation phases in the haematopoietic system



All blood-cell populations originate from one pluripotent haematopoietic stem cell, which is located in the bone marrow in higher vertebrates. In the first maturation phase, a myeloid stem cell 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. The origin of the thymic epithelial anlage is also shown. The differentiation pathway for mammalian B cells through the gastrointestinal tract is not a prerequisite for becoming immunocompetent; in birds, B cells must pass through the bursa of Fabricius. The route of entry into lymph nodes via high endothelial venules (HEV) in the paracortex is indicated. Also shown are sites of immune reactions by T lymphocytes in the paracortex and by B lymphocytes in follicular germinal centres, after antigen stimulation. Reproduced with permission from Sell (1987)

recognition by specific receptors on lymphocytes or by antibodies: The classical reaction to bacterial infection, resulting in antibacterial antibody formation and antibody-mediated destruction of the pathogen, is only one part of the intrinsic capacity of the system. Further attributes of the system (Nossal, 1987) are summarized below.

1.2.1.1 *Encounter and recognition*

The initiation of an immune response requires adequate recognition of the pathogen. This recognition often occurs immediately after entry, e.g. during or after passage through the epithelial barrier of the body (skin or mucus-secreting epithelia in the respiratory and gastrointestinal tract). The first defence includes nonspecific inactivation, e.g. by nonspecific killer cells, neutrophilic granulocytes, and cells of the mononuclear phagocyte system (formerly called the reticuloendothelial system). It also includes antigen processing and presentation to cells such as lymphocytes of the T-helper–inducer type (Th) which can generate a specific response.

The importance of an epithelial barrier for primitive defence mechanisms is clear in fish, in which a specific mucosal immune system with local production of antibodies associated with mucus secretion has been recognized. The gills are the main entry for both antigens and pathogens living in water, and both migrating macrophages and gill epithelial cells are involved in the process.

1.2.1.2 *Specificity*

The immune system can distinguish one particular determinant in an immense spectrum of determinants. The discrimination between 'self' and 'non-self' (i.e. the avoidance of autoreactivity) is an example of this specificity.

Antigens can be polypeptides, carbohydrates, or lipids (lipopoly-saccharides and lectins). A polypeptide antigen epitope is made up of about 10 amino acids. Lymphocytes are central to antigen specificity, as they express receptors for a single, distinct antigenic determinant on their surface. On B lymphocytes, this antigen receptor is essentially an antibody (immunoglobulin) molecule (Hasemann & Capra, 1989). The antigen-binding fragment of the surface receptor and the antibodies produced after differentiation of B cells into plasma cells have virtually identical structures—a quaternary structure comprising the dual heavy and light chains of the immunoglobulin molecule (the so-called variable part of these protein chains). B-Cell surface immunoglobulin and the immunoglobulin product of the plasma cell progeny may differ in the constant part of the heavy chain. Like the T-cell receptor (TCR), the B-

cell receptor has a hetero-oligomeric structure. After the antigen is bound to the surface immunoglobulin, signal transduction occurs, in which one α–β chain is linked to the surface immunoglobulin. Biological responses involving tyrosine phosphorylation and calcium mobilization are then induced, including activation, tolerance, and differentiation, depending on the differentiation stage of the B cell (Pleiman et al., 1994). On virgin B cells, the surface receptor is an IgM or IgD molecule (with μ or δ heavy chains, respectively). After so-called immunoglobulin class switching (Vercelli & Geha, 1992; Harriman et al., 1993), IgG (γ chain), IgA (α chain), and IgE (ε chain) molecules can be synthesized. Associated with the immunoglobulin molecule on the cell surface is a dimeric transmembrane molecule, the Igα and Igβ chain, which functions in signal transduction and intracellular activation of kinases of the *src* family (Pleiman et al., 1994). This α–β dimeric molecule is now used in identifying B cells.

For T cells, the antigen receptor is a heterodimeric molecule (either the α–β or the γ–δ heterodimer), which has a constant and a variable part, like those of immunoglobulin molecules (Hedrick, 1989). Transmembrane signalling (tyrosine phosphorylation) after antigen contact occurs when this heterodimer is linked on the cell surface to the T3 (or CD3) molecule, which consists of at least five invariant chains (Clevers et al., 1988; Chan et al., 1992).

The structural differences between the TCR for antigens and the B-cell receptor (i.e. antibody) arise from differences in the gene segments that encode the receptors. It is thus not surprising that T cells recognize other determinants on the antigenic compound than those recognized by B cells. For large antigens such as proteins, distinct T-cell and B-cell epitopes can be identified, as illustrated by the fact that the α–β heterodimeric receptor on T cells recognizes the antigenic determinant in the context of polymorphic determinants of the major histocompatibility complex (MHC) antigens. The antigenic determinant is either a small peptide, produced during processing of antigen in the antigen-presenting cell and located in a 'groove' formed by the quaternary structure of the MHC molecule (Adorini, 1990; Rothbard & Gefter, 1991; Germain & Margulies, 1993), or a larger molecule associated with the MHC molecule outside the groove. The latter is found for so-called 'superantigens', like *Staphylococcus* enterotoxin A (Herman et al., 1991). Thus, with either type of antigen, Th cells and delayed-type hypersensitivity T cells (see below) recognize the antigenic determinant only when it is presented together with the individual's own (self) determinant of class II MHC. This phenomenon is called MHC class II restriction. In contrast, T cells of the suppressor (Ts) and cytotoxic (Tc) populations are MHC class I restricted. The processing of antigen by

antigen-presenting cells and subsequent complexing with MHC molecules occur intracellularly, but with different pathways for MHC class I-associated and MHC class II-associated complexing. In addition, MHC class II-associated complexing may occur with proteins present at very high concentrations in the extracellular environment (Neefjes & Momburg, 1993; Engelhard, 1994). MHC restriction does not necessarily apply to T-cell subsets within the pool expressing the α–β heterodimeric TCR (Haas et al., 1993). B Lymphocytes do not function in an MHC restricted manner but recognize nominal antigen with their surface immunoglobulin receptor. Therefore, recognition of antigens by B cells and by most γ–δ T cells, does not require antigen presentation on cells carrying their own MHC class I or class II determinants. The total repertoire of antigen recognition specificities is about 10^7 for antibodies and somewhat less for the TCR (about 10^6) (Roitt et al., 1993)

Phylogenetically, the capacity to reject an allograft is acquired very early (e.g. sponges), and this has been interpreted as evidence for an MHC complex. Information on the molecular features of MHC antigens (Hughes & Nei, 1993) is available, however, only for the toad *Xenopus* (Flajnik et al., 1991; Sato et al., 1993) and for fish species (Hashimoto et al., 1990; Kasahara et al., 1992; Ono et al., 1992; Hordvik et al., 1993). All vertebrate species produce specific antibodies and typical cell-mediated immunity indicative of specific responses, demonstrating that both T and B cells exist, as in birds and mammals. Few data are available, however, because there are virtually no reagents for lymphocyte identification and classification. In chickens, monoclonal antibodies recognize α–β and γ–δ TCR and a third TCR with a configuration β–β′; various T-cell subsets have also been identified in this species with appropriate antibodies (CD3, CD4, CD8; see below). MHC restriction of antigen recognition by T cells has been demonstrated in *Xenopus*. In fish, as in mammals, antigens are processed and presented by accessory antigen-presenting cells, such as monocytes, to specific lymphocytes in a seemingly alloantigen (presumably MHC or MHC-like) restricted fashion (Vallejo et al., 1990; Stet & Egberts, 1991; Vallejo et al., 1992). 'B-like' cells expressing immunoglobulins occur in all of the fish and amphibian species that have been studied so far; however, there is no IgD molecule in lower vertebrates. Presumably, all B-like cells express on their surface an immunoglobulin molecule of high relative molecular mass, similar to IgM in mammals.

All non-mammalian vertebrates produce immunoglobulins of high relative molecular mass (Fellah et al., 1992; Wilson & Warr, 1992; Wilson et al., 1992; Litman et al., 1993; Marchalonis et al., 1993). There is probably also an IgG-like immunoglobulin of low relative molecular mass that is functionally but not structurally equivalent to mammalian

IgG, but it has been observed in only a few species of bony fish. There is also evidence for the presence of an IgA-like immunoglobulin in some lower vertebrates. Non-mammalian vertebrates have no IgD or IgE. The total repertoire of antigen recognition specificities for antibodies is smaller in non-mammalian vertebrates than in mammals.

1.2.1.3 *Choice of effector reaction; diversity of the answer*

After activation of Th cells, an immune response develops in order to eliminate the antigen; in practical terms, the response results in inactivation of the pathogen. The response is humoral (antibody-mediated) and/or cellular (cell-mediated).

In the humoral response, Th cells together with antigen activate specified B cells to become antibody-producing plasma cells. The antibodies produced mediate the subsequent inactivation of the foreign substance in a number of ways. When present in the form of immune complexes, IgG and IgM either activate the complement system and induce complement-mediated cytotoxicity (Hansch, 1992; Tomlinson, 1993) or activate secondary effects, which include: (i) vasodilatation, increased vascular permeability, and attraction of granulocytes, with subsequent release of lysosomal proteolytic enzymes (i.e. components of acute inflammation); (ii) IgG antibody-mediated cellular cytotoxicity, in which the antibody forms the antigen-specific bridge between the killer cell (macrophage, binding of the Fc fragment of IgG) and the target; (iii) IgG- and IgM-induced opsonization and ingestion by phagocytic cells (granulocytes, macrophages), with involvement of receptors for immunoglobulin Fc and the complement split product C3d; (iv) binding of IgE to IgE receptors on the surface of mast cells and basophilic granulocytes, which induces degranulation with release of mediators after antigen binding.

Complement is phylogenetically very old, as genes that encode complement components and complement proteins have been identified in hagfish (Hanley et al., 1992; Ishiguro et al., 1992), and C3-like activity exists in invertebrates and in all vertebrates. Complement C3 has been purified from all classes of vertebrates, including fish (e.g. hagfish); in primitive fish like lampreys, it shows 30% homology with human C3, whereas that in rodents is about 80% homologous with that in humans (Lambris, 1993). Complement activation by immune complexes occurs in most primitive vertebrates, but the secondary effects emerged later in phylogeny. Antibody-mediated cellular cytotoxicity involving NK cells has been documented in some bony fish. Little is known about inflammatory reactions in lower vertebrates, except for some histopathological data obtained in fish.

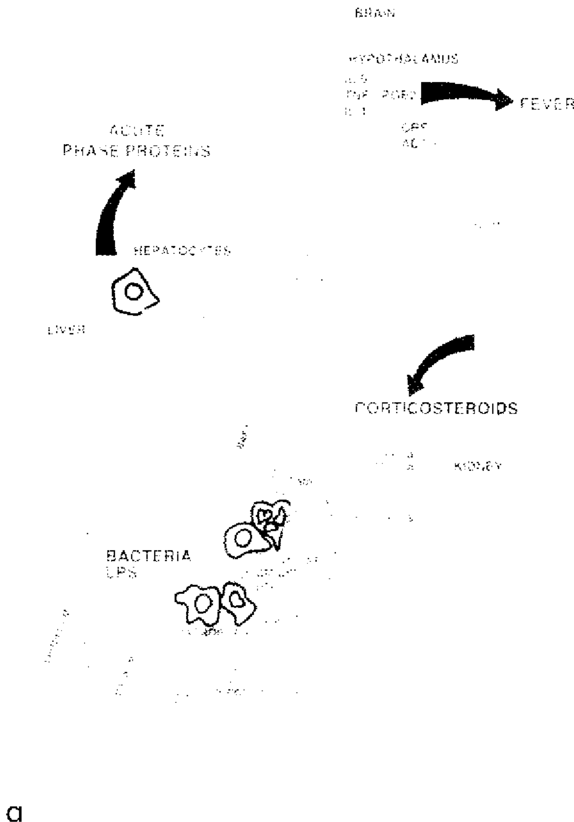
In the cellular response, Th cells activate precursors of Tc cells, which subsequently kill the target after antigen-specific recognition. Furthermore, precursors of lymphokine-producing cells (for example, delayed-type hypersensitivity T cells) can be activated, and the lymphokines thus secreted subsequently activate macrophages to kill the target. Studies of Th clones *in vitro* have revealed the existence of different types (Mosmann & Coffman, 1989). Th1 cells synthesize interleukin (IL)-2, IL-3, tumour necrosis factors α and β, and interferon (IFN)γ (cytokines are discussed below), provide help to B cells (especially in IgG2a synthesis), activate macrophages, and initiate delayed-type hypersensitivity reactions. Th2 cells secrete IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, and tumour necrosis factor α, providing extensive help to B cells (for IgM, IgG1, IgA, and IgE synthesis), and activate eosinophilic granulocytes. Th2 cells do not play a role in the initiation of delayed-type hypersensitivity. Various cytokines are involved in the generation of these Th populations, with dominant effects of IL-4 (Th2) and IL-12 (Th1). Cytokines produced by each type of Th cell subpopulation appear to downregulate the activity of the others. The choice of effector reaction is determined in part by cooperation between the various populations of Th cells: IFNγ from Th1 cells can downregulate Th2 cells, while IL-10 from Th2 cells can downregulate Th1 cells (Mossman & Coffman, 1989).

Finally, it should be noted that the immune system exerts other types of responses that do not involve activation of Th cells. These include T cell-independent activation of B cells, usually when the antigens consist of repeating polysaccharide units (present on bacteria such as *Escherichia coli* and *Pneumococcus*). They also include direct activation of T killer cells which bear antigen-specific receptors comprising the γ–δ heterodimer (Bell, 1989; De Weger et al., 1989; Raulet, 1989; Haas et al., 1993).

1.2.1.4 *Immunoregulation*

The effector reaction is induced by a finely tuned interplay between cells and soluble mediators (Figure 2) (Van Deuren et al., 1992). On the one hand, cell–cell contact is required; for instance, between the antigen-presenting cell with (processed) antigen on its surface and the Th cell. On the other hand, mediators (cytokines or interleukins) influence cell function at a distance, after binding to specific receptors on the target cell and subsequent signal transduction, resulting in cell activation (Foxwell et al., 1992; Taga & Kishimoto, 1992). New interleukins and their surface and soluble receptors are being identified and characterized rapidly: at least 15 different interleukins are known at present, in addition to various growth factors. As a reflection of their function,

Figure 2. Activity of various cytokines in the immune system, (a) in inflammation, (b) in immune responses, and (c) in haematopoiesis



a

Abbreviations: IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; SIS, skin immune system (protein family, chemoattractants for monocytes and lymphocytes); LIF, leukaemia inhibitory factor; SCF, stem-cell factor; TGFb, transforming growth factor b; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; EPO, erythrocyte lineage differentiation factor; PGE2, prostaglandin E2; ACTH, adrenocorticotrophic hormone

Figure 2 (contd)

ANTIGEN
MHC I
MHC II
ANTIGEN PRESENTING CELL
ANTIGEN PRESENTING CELL
IL - 1
MHC I
TCR
CD3
MHC I
TCR
CD2
PRECYTOTOXIC T CD8
HELPER T CD4
IL - 2R
IL - 2R
IL - 2R
IL - 2R
LGL (NK)
TH CD4
TH CD4
MEMBRANE Ig
IL - 2R
B Lymphocyte
CYTOTOXIC TC CD8
IFN
IL - 12
IFN
IL - 2
IL - 2R
TC CD8 TCR
TC CD8
TCR
MHC I
SECRETED Ig
B
LAK
?
TARGET CELL KILLING
PLASMA CELL

b

Figure 2 (contd)

STROMA

EPO

EPO

SCF

Myeloid
Progenitor

ERYTHROBLAST

RED CELLS

Hematopoietic
stem cell

PMN BASOPHIL
TISSUE MAST CELL

IL - 7

CFU - GM

PMN EOSINOPHIL

MEGAKARYOCYTE

G - CSF

M - CSF

Lymphoid
progenitor

PMN NEUTROPHIL

PLATELETS

MONOCYTE

MACROPHAGE

C

these factors are designated as chemokines (attracting cells) or cytokines (influencing cell function, such as stimulation). Factors synthesized by lymphocytes are called lymphokines; those synthesized by monocytes or macrophages are called monokines. Among the consequences of cytokine–cell interactions are chemotaxis (Miller & Krangel, 1992), production of subsequent mediators in the cascade, and down-regulation of cellular function (Kimchi, 1992). The role of mediators in cell adhesion to vascular endothelium is described below.

Examples of mediators that influence immune reactions positively are IL-1 (Dinarello, 1992), which is secreted by antigen-presenting cells and stimulates Th cells; IL-2, which is secreted by Th cells and stimulates a variety of T cells to amplify the response; and a number of B-cell stimulatory cytokines (Figure 2). Surface receptors for these interleukins are present on certain immune cells (and also in neuroendocrine tissue, mentioned below) or are expressed during activation. For instance, IL-2 receptors and HLA-DR molecules are expressed on T cells as part of the activation process, and their expression is used to assess the state of cell activation (Shabtai et al., 1991).

In down-regulation, the immune response manifests active processes. A number of mediators, such as prostaglandin E2, IL-10, and transforming growth factor β (Brigham, 1989; Fontana et al., 1992; Larrick & Wright, 1992), can suppress subsets of T and B lymphocytes. After stimulation of Th cells in the initiation of the response, precursors of Ts cells are activated which subsequently inhibit Th cells from further amplifying the response, in direct cell–cell contact or by secretion of soluble inhibitors. The existence of Ts cells has been disputed, as part of the function of these cells is cytotoxicity, which is exerted by the closely related MHC class I-restricted Tc population (Bloom et al., 1992). Immunoregulatory circuits have also been documented at the antibody level, where a first antibody generates a second one directed to itself. The relevance of this antibody–anti-antibody network, the so-called 'anti-idiotype' network, remains a subject of speculation.

The immune system is in a continuous state of homoeostatic equilibrium. The introduction of an antigen (pathogen) disturbs that balance by activating antigen-specific cell clones of T and B lymphocyte origin. The system not only allows the proliferation and amplification of relevant clones to cope with the antigen, but it also searches for (and reaches) a state of newly defined homeostasis.

Little is known about the phylogenetic development of mechanisms for regulating immune responses in vertebrates. Helper, cytotoxic, and even suppressor lymphoid functions have been reported in ectothermic vertebrates, but the existence of T-cell subpopulations has not been demonstrated. T and B cells cooperate in teleost fish and in amphibians,

and there is indirect evidence that they do so in reptiles; however, MHC restriction of these cell interactions has been demonstrated only in *Xenopus*. The existence of clusters consisting of lymphocytes, macrophages, and plasma cells has been documented in various lower vertebrate species, which indicates the importance of cell–cell interactions in evoking immune responses. Cytokine activity resembling that of IL-1 and interferon has been identified in various fish species. A T-cell growth factor was first characterized in *Xenopus*. An idiotype–anti-idiotype network has been proposed to explain the regulation of antibody production in some bony fish (Zapata & Cooper, 1990).

1.2.1.5 *Modifying factors outside the immune system*

Communication with other homeostatic mechanisms in the body is an important aspect of immunoregulation; mediators of the response have effects not only on the internal regulatory network but also on systems outside the immune system. Communication with the clotting and kallikrein systems by complement components is one example; communication with the central nervous system is another (Ader et al., 1990). For instance, IL-1 generated by antigen-presenting cells affects the temperature regulatory centre (induction of fever) and the sleep regulatory centre (induction of slow-wave sleep) in the hypothalamus (Dinarello, 1992).

One example of interaction between the immune system and the central nervous system is the profound influence of stress on immune reactivity (Khansari et al., 1990). Both stressful events and the way in which individuals cope with stress are involved (Bohus et al., 1991), as documented in studies mainly in rats but also in other species, including fish (Faisal et al., 1989). In humans, conditions of acute psychological stress include bereavement (Bartrop et al., 1977), marital disruption (Kiecolt-Glaser et al., 1987), and examination periods (Kiecolt-Glaser et al., 1986), and these can be associated with a decrease in immune status (Kiecolt-Glaser & Glaser, 1986) which can result in increased risk for infection, including common respiratory tract infections, e.g. influenza (Boyce et al., 1977; Clover et al., 1989), infectious mononucleosis (Kasl et al., 1979), and herpes virus reactivation (Glaser et al., 1985).

The hypothalamus–pituitary–adrenal axis is an important pathway in the communication between the central nervous system and the immune system, resulting in synthesis of glucocorticosteroid hormone by the adrenal gland induced by adrenocorticotrophic hormone from the pituitary gland (Buckingham et al., 1992). Other mechanisms are those mediated by the direct action of neuropeptides, such as opioid peptides (van den Bergh et al., 1991), on immune cells; these are either stimulatory or down-regulatory, depending in part on experimental

design and conditions. In addition, almost all lymphoid tissues are innervated (Bulloch, 1987; Felten et al., 1987; Kendall & Al-Shawaf, 1991), although the role of this neuroregulatory pathway is largely unknown (Freier, 1990). The immune system and the neuroendocrine system have a number of biologically active mediators in common, including cytokines and neuromediators (Fabry et al., 1994), and are strongly interrelated (Weigent & Blalock, 1987; Sibinga & Goldstein, 1988; Heijnen & Kavelaars, 1991; Heijnen et al., 1991; Knight et al., 1992). Drugs that act on the central nervous system, like some tranquillizers, antidepressants, benzodiazepines, antiepileptics, anaesthetics, and levodopa (an antiparkinson drug), may cause immunosuppression (Descotes, 1986). Thus, the principles of immunotoxicology may find application in neurotoxicology and endocrine toxicology (Snyder, 1989). Some examples of factors that modify the immune system, based on studies of the thymus of mice during pregnancy and of birds after hatching, are given below. Exogenous conditions may have pivotal influences on the structure and function of the immune system. In ectothermic vertebrates, these include seasonal changes like temperature and photoperiod and are governed by corticosteroid and sex hormones. In spring, during the mating period, there is low immune reactivity, with thymic involution; in the postmating period and the first part of summer, there is maximal development of lymphoid tissue and immune reactivity; at the end of summer, activity declines, and the lymphoid organs undergo pronounced involution, which persists throughout the autumn and winter (Zapata et al., 1992). The lymphoid system of ectothermic vertebrates therefore cannot be described in morpho-functional terms without taking into account the season in which studies were conducted. Seasonal variation in immunoresponsiveness is also seen in laboratory animals (Ratajczak et al., 1993).

1.2.1.6 *Immunological memory*

A special feature of the immune response is the generation of memory after initial contact with an antigen (Gray, 1993). The first response includes activation and amplification of antigen-specific T or B cells to exert effector reactions; it ends with the return of antigen-recognizing cells to the normal resting state (small lymphocytes). The second contact with the antigen results in recruitment of more antigen-specific cells to give a stronger signal and more efficient elimination of the antigen or pathogen. The reaction also occurs faster, and there is a stronger binding of antibody to antigen. The gradual increase in the binding capacity of antibodies during the immune response is known as 'affinity maturation'. The memory pool of lymphocytes mediates a faster response than the virgin (unprimed) pool.

The memory effect is most evident in the humoral arm of the immune response. The first response gives rise only to IgM class antibody, but antibodies of other immunoglobulin classes (especially IgG in the internal system) are generated after subsequent contact and immunoglobulin class switch. The antibodies thus formed may show increased affinity as a result of somatic mutation (Kocks & Rajewski, 1989).

The cellular basis of immunological memory is largely unresolved. It appears to be located within the T-cell population, because its presence within the B-cell population is apparently of short duration and is associated with the presence and stimulatory activity of the antigen in germinal centres of secondary lymphoid tissue. The generation of memory is the basis for vaccination, performed to prevent contracting infectious disease by bringing about contact with the pathogen in an attenuated or inactivated, nonpathogenic form.

It is not known whether immunological memory exists in primitive vertebrates. The classical differentiation into primary and secondary responses does not exist, as it is mainly immunoglobulin of high relative molecular mass that is present, and the repertoire of antigen-recognizing specificities is smaller than that in birds and mammals. Some fish species may have immunological memory.

1.2.2 *Histophysiology of lymphoid organs*

1.2.2.1 *Overview: structure of the immune system*

Components of the immune system are present throughout the body (Figure 3). The lymphocyte compartment is lodged in lymphoid organs, from which cells can move to sites of infection or inflammation. Phagocytic cells of the monocyte–macrophage lineage occur in lymphoid organs and also at extranodal sites, such as Kupffer cells in the liver, alveolar macrophages in the lung, mesangial macrophages in the kidney, and glial cells in the brain (Figure 4). Polymorphonuclear leukocytes are present mainly in blood and bone marrow as mature and progenitor cells. These cells accumulate at sites of inflammation.

The lymphoid organs can be classified roughly into two types: primary or central (antigen-independent) and secondary or peripheral (antigen-dependent). This classification is based on the antigen-dependence of cell proliferation and differentiation. It does not hold for lower vertebrates, including fish. The bone marrow in higher vertebrates is a primary organ, in which are found the pluripotent haematopoietic stem cells which differentiate into progenitors of myeloid cells (which in turn differentiate into granulocytes, monocytes, erythrocytes, and platelets) and lymphoid progenitors (Figure 1). The differentiation is

Figure 3. Lymphoid organs and tissues in the rat

From Clarke (1984)
Mucosal and skin lymphoid tissues are presented in green, specific mononuclear phagocyte systems in pink, and lymphoid organs in black. No data are available in the literature on the lymph nodes that drain the genital organs of the rat. In mice, the lumbar (also called iliac, caudal, or paraaortic) lymph nodes drain the lymph nodes of the uterus.

Figure 4. The mononuclear phagocytic system in the rat: (a) lung stained with antibody ED1, which labels alveolar macrophages (arrows); (b) liver stained with antibody ED2, which labels Kupffer cells (arrows); (c) intestine stained with antibody ED2, which labels interstitial macrophages (brown-stained cells) in the lamina propria (S, submucosa; L, luminal side)

Frozen tissue sections; micrographs (immunoperoxidase technique) kindly provided by C.D. Dijkstra, Department of Cell Biology, Medical Faculty, Free University, Amsterdam, Netherlands

not antigen-dependent or antigen-driven, but this does not exclude a role for antigens in the process. Factors secreted in the periphery during antigen-specific stimulation can promote haematopoiesis in the bone marrow (Figure 2) (Fletcher & Williams, 1992; Kincade, 1992; Saito 1992; Williams & Quesenberry, 1992) or inhibit haematopoietic activity (Wright & Pragnell, 1992). The bone marrow also functions as a secondary lymphoid organ, because terminal antigen-induced lymphoid cell differentiation can occur in its microenvironment. For instance, the bone-marrow cells include both the memory lymphocyte pool and the major plasma cell population, which contributes to the intravascular pool of immunoglobulins. Normally, plasma cell differentiation follows antigen presentation at peripheral sites, and stimulated cells subsequently migrate to the bone marrow for final differentiation. The secondary or peripheral lymphoid organs in the body include the lymph nodes, spleen, and lymphoid tissue along secretory surfaces like the gastrointestinal and respiratory tracts.

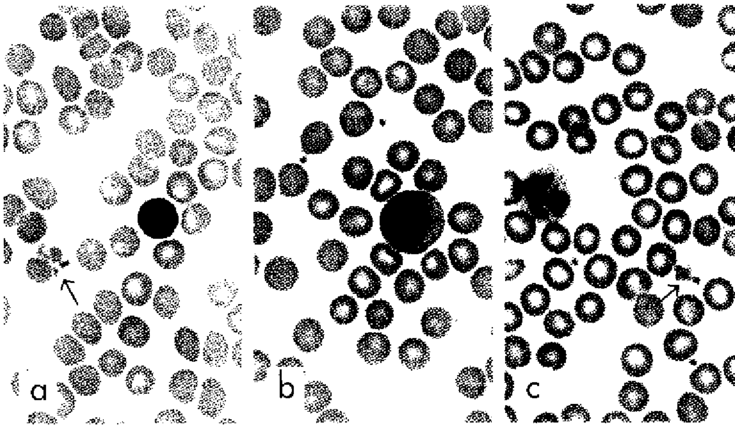
A second classification is based on the location of lymphoid organs, divided into internal organs (some lymph nodes and the spleen, in addition to thymus and bone marrow) and external organs (lymphoid tissue along secretory surfaces and the lymph nodes that drain the mucosa-associated lymphoid tissue (MALT)) (Figure 3). Lymphoid organs at these two locations behave somewhat independently in host defence, for instance in immunoglobulin synthesis. The main function of the external or secretory immune system is to produce (secretory) IgA antibody, whereas the internal immune system (mainly bone marrow) produces IgG or IgM antibody; the major site of IgE synthesis in the body is also along secretory surfaces. The extent of the secretory immune system should not be underestimated: About half of the body's lymphocytes are located in the secretory immune system, and its capacity for immunoglobulin synthesis is about 1.5 times that of the internal system. In all vertebrates, intraepithelial lymphocytes and nonencapsulated lymphoid infiltrates occur in the intestine. True lymphoid organs, such as the tonsils, Peyer's patches, and the appendix, have been reported only in higher vertebrates. In birds, the caecal tonsil, a blind appendix of the posterior intestine, becomes an important peripheral lymphoid organ after involution of the bursa of Fabricius.

Another organ that contributes to the immune system is the skin. It does not contain organized lymphoid tissue, but immune components in skin are interconnected with other immune organs, leading to the concept of the skin immune system, or skin-associated lymphoid tissue (Stingl & Steiner, 1989; Bos, 1990; Nickoloff, 1993; see section 1.2.2.7).

Immune cells and cellular products are transported between lymphoid organs by blood and lymph vessels (Duijvestijn & Hamann,

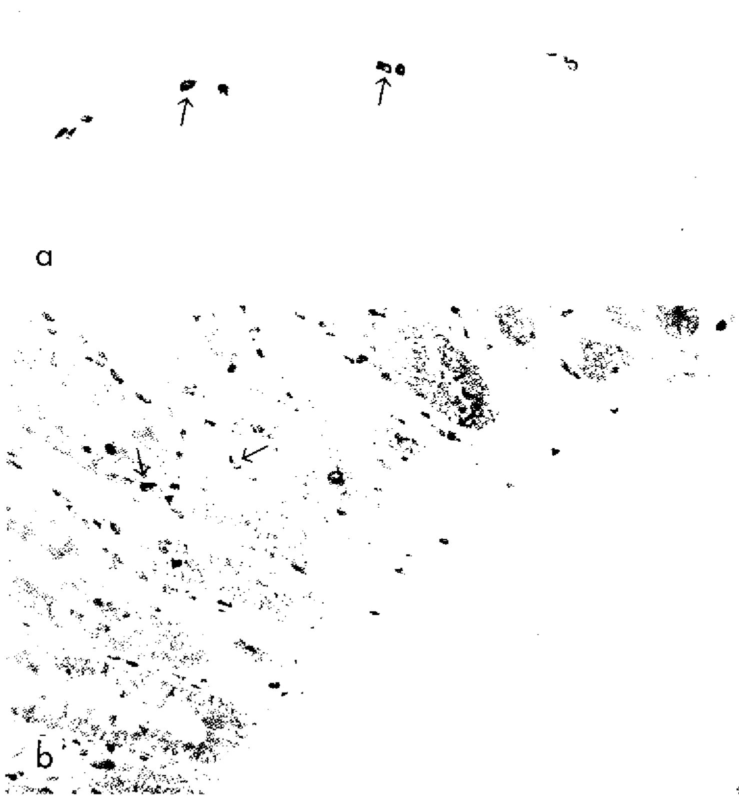
1989). For example, Langerhans cells on their way from the skin to a lymph node are present in lymph as 'veiled macrophages'. The blood circulation contains only a minor part of the body's total pool of lymphocytes (estimated at about 1%) and only a selected population, i.e. the recirculating lymphocyte pool (Figure 5). Therefore, assessment of only the blood lymphoid compartment does not give a complete inventory of the body's immune system, as it ignores the activities of the non-recirculating cells. In general terms, the blood lymphoid compartment does not include cells that are in a state of activation, proliferation, or differentiation; such cells are typically tissue-bound. This rule is not absolute. For instance, in the case of a highly activated B-cell system with hyperplasia of germinal centres in lymph nodes, activated B cells may occur in the circulation. These cells are normally not part of the recirculating pool. With regard to the non-lymphoid cells of the immune system, macrophages are tissue-bound (histiocytes), and monocytes are their counterpart in blood. Dendritic cells, which are present in very low proportions in blood, are the counterparts of Langerhans cells in skin, veiled macrophages in lymph, and interdigitating dendritic cells in lymphoid tissue. The follicular dendritic cells, i.e. the antigen-presenting cells in follicles in lymphoid tissue (see below), do not exist in the circulation. Within the myeloid series, the mast cell is typically tissue-bound (Figure 6).

Figure 5. Blood smear from a rat showing (a) small lymphocytes, (b) a monocyte, (c) a polymorphonuclear neutrophilic granulocyte, and platelets (arrow)



May Grünwald-Giemsa stain

Figure 6. Mast cells in the rat: (a) connective tissue mast cells (arrows), in which the metachromasia is resistant to formalin fixation, in septa of the thymus; (b) mucosal mast cells (arrows), in which the metachromasia is not resistant to formalin fixation, in lamina propria of small intestine



It is claimed that the mucosal mast cell develops under the influence of the thymus, whereas this is not the case for the connective tissue mast cell; toluidine blue stain

The endothelium lining the blood vasculature has a major role in the passage of cells from blood to tissue parenchyma. Adhesion molecules on circulating cells and endothelium are involved in this process (Patarroyo, 1991; Gahmberg et al., 1992; Gumbiner & Yamada, 1992). These glycoproteins belong to three families of molecules, the immunoglobulin supergene family, the integrins, and the selectins (Springer, 1990; Lasky, 1992; Bevilacqua, 1993). They may be present on endothelium in the resting state but often require alteration to become biologically active in inflammatory processes (e.g. under the influence of mediators like IFNγ). In lymphoid tissue, they are called addressins, to indicate their role in the selective homing of passenger lymphocytes into internal or external lymphoid tissue.

The structure and histophysiology of the bone marrow, thymus, lymph node, spleen, and MALT are presented in more detail in the following sections. The different microenvironments of these organs are summarized in Table 3. A detailed description of the histological and pathological aspects of rat lymphoid tissue is given by Jones et al. (1990).

Not only conventional histological features but also the expression of cell surface markers (immunological phenotype) are emphasized. Monoclonal antibodies to marker substances (differentiation antigens) on cell populations are used widely in the identification of leukocyte populations, especially in cell suspensions (flow cytometry) and tissue sections (immunohistochemistry). The wide range of monoclonal antibodies that currently exists have been grouped according to the 'cluster of differentiation' (CD) nomenclature, in which they are classified according to their reactivity to the same cell marker molecule (but not the same epitope on those molecules) (Clark & Lanier, 1989; Knapp et al., 1989; Schlossman et al., 1994, 1995). The CD nomenclature has been adapted for species other than man (Holmes & Morse, 1988; Jefferies, 1988; Schuurman et al., 1992a). Some monoclonal antibodies that can be used in the identification of leukocytes and stromal cells in tissue sections and cell suspension are described in section 4.1.2.

Typing of cells in the T lymphocyte lineage is a good illustration of immunological phenotyping. Subsets with other functions usually cannot be identified by conventional cytology but can be recognized by immunological phenotyping. For example, T cells with a helper–inducer function are labelled by antibodies to CD4 antigens, and cells with a cytotoxic function are labelled by antibodies to CD8. The reverse does not hold, however; for example, not all $CD4^+$ cells are helper–inducer cells, because T cells in the separate subset effecting delayed-type hypersensitivity and some macrophage populations are also $CD4^+$. $CD8^+$ T cells are in either the cytotoxic or the suppressor subset.

Table 3. Microenvironments in lymphoid tissue

Microenvironment	Cells present	Function
Bone marrow	Haematopoietic cells organized as islands within fatty tissue, mature leukocytes, plasma cells	Differentiation of stem cells into cells of the erythroid,myeloid/monocytoid, platelet and lymphoid lineage; antibody synthesis, memory cells
Thymus		
Cortex	Reticular epithelium, immature T cells	Generation of T-cell competence, T-cell receptor rearrangement, positive selection (MHC restriction), negative selection (autoreactive cells), phenotypic changes
Medulla	Reticular epithelium, dendritic cells, T lymphocytes	Final generation of T-cell competence (negative selection), thymic hormone synthesis, antigen presentation
Lymph node and spleen		
Paracortex (lymph node), lymphocyte sheath	Interdigitating cells, Th and Ts cells	Lymphocyte entry through high periarteriolar lymphoendothelial venules (lymph node) or central arteriole (spleen), antigen presentation to Th cells, T-cell proliferation, differentiation, and regulation (Ts cells)

Table 3 (contd)

Microenvironment	Cells present	Function
Primary follicles, follicular mantle of secondary follicles	Dendritic cells (subtype of follicular dendritic cells), dendritic macrophages, B cells, small number of T cells	Storage of virgin and memory B cells, recirculating B cells (surface $IgM^{+}IgD^{+}$)
Germinal centre	Follicular dendritic cells, dendritic macrophages (starry-sky macrophages), B cells (centrocytes, centroblasts), Th cells	T Cell-dependent B-lymphocyte differentiation, antigen presentation in the form of immune complexes (with/without complement C3)
Medulla (lymph node), red pulp (spleen)	Plasma cells, T effector cells, reticular cells, polymorphonuclear granulocytes	Termination of antigen-specific reaction: antibody synthesis and immune complex-mediated clearance, Tdth and Tc cell response
Marginal zone (spleen)	Marginal zone macrophages, marginal metallophilic cells, marginal zone B cells	T Cell-independent B-lymphocyte proliferation and differentiation, e.g. to bacterial polysaccharides, B-cell memory (surface $IgM^{+}IgD^{-}$ cells)
Mucosa-associated lymphoid tissue		
Epithelium covering lymphoid tissue (e.g. Peyer's patches)	M (microfold) cells	Transport (uptake) of exogenous substances

Table 3 (contd)

Microenvironment	Cells present	Function
Follicles and interfollicular areas	See 'lymph node and spleen'	For antibody synthesis: precursors of IgA plasma cells
Mucosal epithelium	Epithelial cells, Tc cells, natural killer cells, γ–δ T cells	First line of defence, synthesis of secretory component, transport of IgA (IgM) to lumen
Lamina propria	Plasma cells, macrophages	Synthesis of IgA antibody, phagocytosis and killing

MHC, major histocompatibility complex; Th, T helper; Ts, T suppressor; Ig, immunoglobulin; Tdth, delayed-type hypersensitivity T cells; Tc, T cytotoxic

Thus, there is no unequivocal relationship between CD4 or CD8 expression and cell function, because these cell-surface molecules are expressed in relation to the way in which antigen is recognized: T cells recognize antigen in the context of MHC molecules—MHC class II molecules for $CD4^+$ cells and MHC class I antigens for $CD8^+$ cells (Engleman et al., 1981; Meuer et al., 1983). This phenomenon is further discussed in sections 1.2.2.3 and 1.2.2.4.

Monoclonal antibodies have been developed to most subtypes of leukocytes, including NK cells. The specificity of antibody 3.2.3, anti-NKR-P1, for identifying these cells was described by Chambers et al. (1989). Immunological phenotyping of these cells in rat spleen and lung is illustrated in Figure 14d (p. 65).

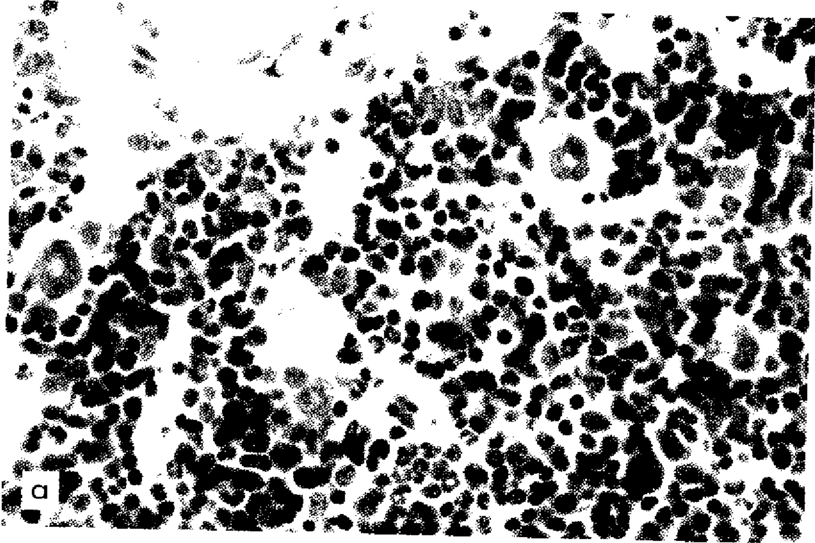
NK cells and their activities are an example of the differences between cell function, cytology, and immunological phenotype. NK cells are characterized by their killer function against selected targets *in vitro*. Cells with cytological features that include cytoplasmic granules, called large-granular lymphocytes, presumably have a natural killer function, as do cells with an immunophenotype defined by certain antibodies. The cells identified in these ways are by no means identical and belong to different, overlapping subpopulations.

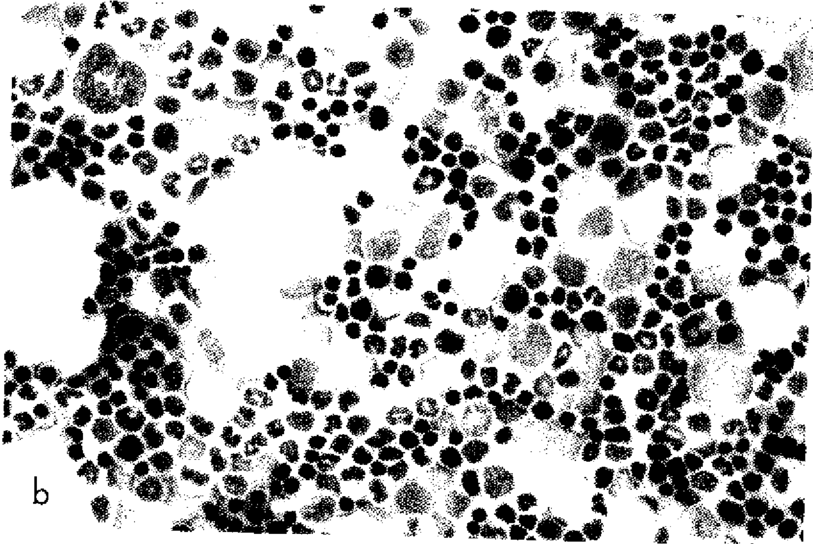
1.2.2.2 *Bone marrow*

The microenvironment of the bone marrow is found in all of the hollow bones of the body and occupies the medullary space of the skeleton (Figure 7). Blood enters the marrow from nutrient arteries at the place where they bifurcate to form the central artery of the medullary canal. Branches of the central artery terminate in capillaries within the medullary space or penetrate the endosteum, where arterial blood from the nutrient artery mixes with blood from muscular arteries in the periosteal capillaries. The blood returns via the vascular sinuses to the central sinus and vein. Blood cells in various stages of development (Figure 1) intermingle with their microenvironment, which is composed of reticular cells (adventitial and fibroblastic), adipocytes, endothelial cells, and extracellular matrix. These, together with passenger accessory cells (macrophages, monocytes, and lymphocytes, especially T cells), support the haematopoietic process (Lichtman, 1981; Weiss & Sakai, 1984; Chanarin, 1985; Weiss & Geduldig, 1991; Mayani et al., 1992).

In many mammals during ontogenesis, haematopoiesis is first located in the yolk sac and fetal liver, until the bone marrow develops and becomes functional. In rodents, haematopoiesis also occurs in splenic red pulp later in life. This effect is less pronounced in other species, but haematopoiesis can occur at other sites in the body, e.g. the

Figure 7. Bone marrow: (a) section; (b) cytocentrifugal preparation





Haematoxylin and eosin stain

thymus. In lower vertebrates, a bone marrow containing haematopoietic stem cells occurs firstly in amphibians. It contains only granulopoietic and lymphoid cells; erythropoiesis and thrombopoiesis usually occur in the blood sinusoids of the splenic red pulp. In fish and more primitive vertebrates, lymphoid tissue is found at many ectopic locations, such as intestine, brain, heart, gonads, pro- and mesonephrons, and liver and is functionally and structurally similar to the bone marrow of higher vertebrates. In birds, the thymus is the main site of erythropoiesis during certain periods of life (Kendall & Ward, 1974; Kendall & Frazier, 1979).

Haematopoiesis: The pluripotent haematopoietic stem cells in the bone marrow proliferate and differentiate to progenitors of myeloid, erythroid, and lymphoid cells. Some further differentiation occurs in the marrow, but the final maturation mainly occurs elsewhere (Figure 1). The common lymphoid progenitor cell differentiates into a T- and a B-lymphoid progenitor. Progenitor T cells then move to the thymus for further maturation. B-Lymphoid progenitors mature via the pre-B cell stage into virgin B cells, which then leave the microenvironment of the bone marrow and lodge in the periphery. In birds, this function in B-cell maturation is performed by the bursa of Fabricius, an epithelial organ located adjacent to the termination of the gastrointestinal tract (B stands for bursa-dependent) (Cooper, 1982; Du Pasquier, 1989; Zapata & Cooper, 1990). Although the bursa is considered essential for the production of B lymphocytes, chickens bursectomized early in embryonic life have B cells in their peripheral lymphoid organs and can produce antibodies, even if their repertoire is very limited. Thus, the bursa seems to be involved in the generation of immunoglobulin diversity rather than just in B lymphatopoiesis.

The development of stem cells into more mature cells requires the presence of a microenvironment, as described above, and is regulated by haematopoietic cytokines (also called haematopoietins) (Fletcher & Williams, 1992; Kincade, 1992; Quesniaux, 1992; Saito, 1992; Williams & Quesenberry, 1992; Wright & Pragnell, 1992; see also Figure 2). Unmyelinated nerve fibres may terminate in the haematopoietic spaces (Lichtman, 1981), and neuropeptides may play a role in the regulation of haematopoiesis. A microgeographical distribution of the various cell lineages in microenvironments has been suggested, such as the preferential associations between fibroblasts and granulocytes, megakaryocytes, and sinus endothelial and adventitial cells and between macrophages and erythroid cells.

The classical bioassay for haematopoietic stem cell activity is measurement of colony-forming units in the spleen of irradiated mice after injection of bone-marrow cells. More differentiated progenitors

can be cultured *in vitro* to provide information on the specific lineage of the colony-forming cell, to evaluate the progenitor activity of macrophages, granulocytes, megakaryocytes, eosinophilic granulocytes, and mast cells, as well as that of several cell lineages, such as macrophages and granulocytes. Progenitors of erythrocytes are assayed as early progenitors or as erythroid burst-forming units. Studies of the mechanisms involved have revealed a number of factors that promote the differentiation of progenitor cells, like granulocyte–macrophage colony-stimulating factor, macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and c-kit ligand. These mediators are produced by various cell types, mainly after activation, and promote haematopoiesis *in vivo* in conditions of e.g. infection and inflammation. Other soluble mediators produced by T cells and by other cells involved in the antigen-driven immune response also promote haematopoiesis; these include IL-1, IL-3, IL-6, IL-11, interferon and tumour necrosis factor. Haematopoiesis in the bone marrow is thus not independent of antigenic stimulation and exposure but is not typically antigen-driven. For instance, in the case of acute infection, the bone marrow produces a large proportion of polymorphonuclear granulocytes within a short time, manifested in blood as leukocytosis and a shift in the differential count to band-type immature granulocytes. This shift may be related to migration of T lymphocytes into the bone marrow microenvironment, where they activate haematopoiesis. Antigen-induced lymphoid cell differentiation also occurs in the bone marrow; for instance, bone marrow cells include the memory lymphocyte pool and the major plasma cell population, which contribute to the intravascular pool of immunoglobulins.

Development and aging: The bone marrow is the primary site of haematopoiesis throughout life, generating over 95% of the haematopoietic activity in adult mammals (Mayani et al., 1992). Essentially all of the medullary space in the bones is occupied by haematopoietic tissue in mice and rats; in contrast, in adult humans, dogs, and rabbits, haematopoietic tissue is restricted to the proximal epiphyses of the long bones, the central skeleton, and the skull; most bone marrow is gradually replaced by fat cells. The normal mean proportions of bone marrow cells and changes with age in rats were reported by Valli et al. (1990).

1.2.2.3 Thymus

The thymus is a two-lobed organ located in the mediastinum, anterior to the major vessels of the heart (Kendall, 1991; Von Gaudecker, 1991; Schuurman et al., 1993), although it is located in the neck region

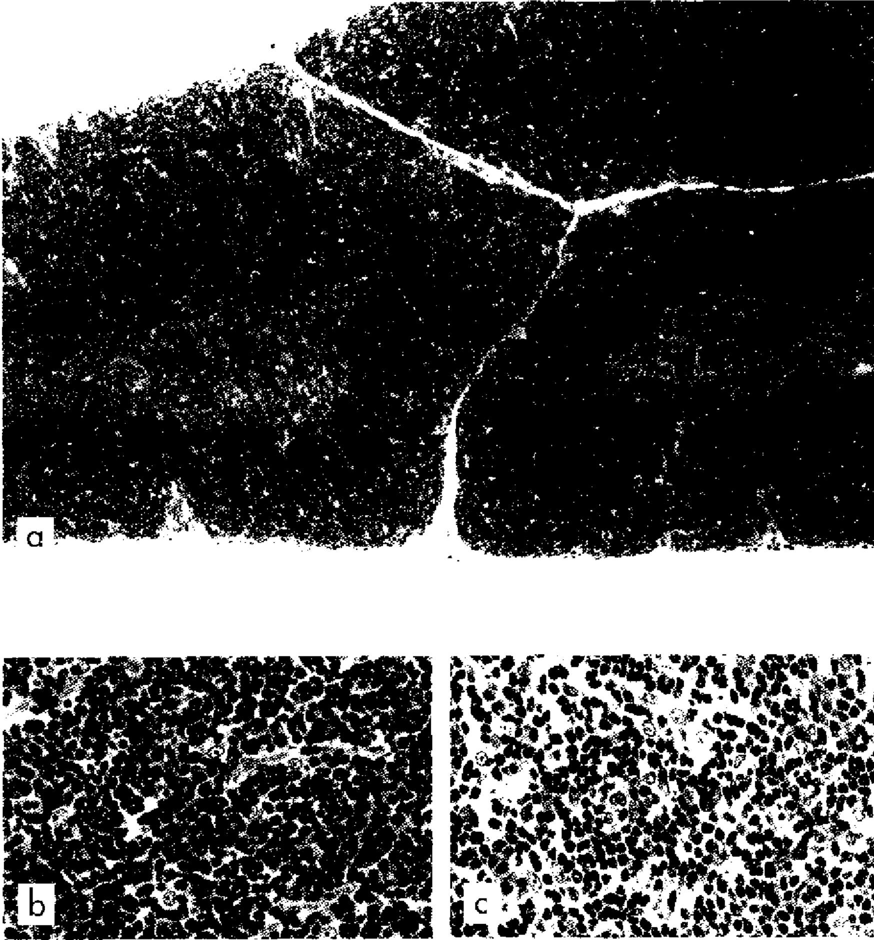
in the guinea-pig. Its anatomical location complicates complete thymectomy *in vivo*. The two independent lobes, attached to each other only by connective tissue, consist of smaller lobules, which have basically the same architecture, with a subcapsular and outer cortical area, a cortex, and a medulla (Figure 8). With conventional histological stains, the cortex is strongly stained, owing to a dense population of small lymphocytes, and the outer cortex and medulla show a paler colouring.

Blood vessels enter the lobules at the cortico-medullary junction and extend radially into the cortex. Nerves course along the blood vasculature. Fenestrated capillaries are very infrequent in the cortex. The thymus is unique among the lymphoid organs in that its microenvironment consists of a reticular epithelium (in birds, the bursa of Fabricius also has an epithelial framework). Macrophages derived from the bone marrow are found in the cortex and medulla as a transient population. Dendritic cells in the medulla, resembling interdigitating dendritic cells in lymph nodes, have a major function in antigen presentation and strongly express MHC class II antigen.

The thymus appears for the first time in vertebrate phylogeny in cartilaginous fish (sharks and rays). More primitive vertebrates lack a thymus, although they can manifest cellular immune responses. Except in bony fish, the thymus is histologically similar in all vertebrates, although it is derived from distinct pharyngeal pouches in different species. The classical cortex–medulla demarcation is not present in fish thymus, and most thymocytes seem to occupy the central part of the organ. In all species, epithelial cells organize a supporting network in both cortex and medulla. Hassall's corpuscles, which are epithelial aggregates with centrally located cell debris, occur in the medulla of the human thymus but are scarce or absent in the rodent thymus and in the thymus of ectothermic vertebrates. In the latter, epithelial cysts are frequent. The thymus of lower vertebrates, in contrast to that of mammals, contains numerous myoid cells; in avian thymus, significant erythropoiesis has been documented (Kendall & Ward, 1974; Kendall & Frazier, 1979), which may also occur in mammals (Kendall, 1980; Kendall & Singh, 1980).

T-Cell maturation: selection in the thymus: T Cells reside in the thymus during their maturation from progenitor cells to immunocompetent T cells. This gland has a privileged function in promoting the maturation process (Brekelmans & Van Ewijk, 1990; Shortman et al., 1990; Van Ewijk, 1991; Boyd et al., 1992). In congenitally athymic, 'nude' animals (Schuurman et al., 1992b,c) and in thymic aplasia in children with complete Di George's syndrome, the absence of a functionally

Figure 8. Thymus of Wistar rat



(a) Overview, showing three lobules; C, cortex; M, medulla; (b) detail of cortex; (c) detail of medulla; haematoxylin and eosin stain

active T-cell system is causally related to the aplasia of the organ. The process of T-cell maturation includes a number of steps located in different microenvironments (Schuurman et al., 1993): The least mature cells, which enter the lobules from the bloodstream at the cortico-medullary junction, first move to the outer subcapsular cortex, where they appear as large lymphoblasts. They then pass through the cortex, where they become small lymphocytes with a scanty cytoplasm. Finally, they move to the medulla, where they appear as medium-sized lymphocytes. These translocational stages in development are monitored on the basis of the immunological phenotype. For the CD4–CD8 phenotype, the cells change from CD^-CD8^- (so-called double-negative) at a very immature stage, then change to a CD4 $CD8^+$ stage and into a $CD4^+CD8^+$ (double-positive) phenotype, which is found on almost all lymphocytes in the cortex. In the medulla, T cells have the phenotype of mature cells, with distinct $CD4^+CD8^-$ (about two-thirds) and $CD4^-$ $CD8^+$ (about one-third) populations.

This phenotypic change is accompanied by a crucial aspect of intrathymic T-cell maturation: genesis of the TCR, which consists of the α–β heterodimer. Initially, the DNA genomic organization that encodes these chains is in germline configuration, with a variety of gene segments encoding the variable part of the receptor molecule. Before transcription and translation into TCR becomes possible, combinations have to be made of the gene segments that encode the variable and constant parts of the TCR. This process of gene rearrangement requires the thymic microenvironment, and only after it is completed can the cell synthesize the receptor. The receptor is then expressed on the cell membrane together with the CD3 molecule, which may act as the transmembrane signal transducing molecule after TCR stimulation; the CD3 molecule is already present in the cytoplasm of the cell even before the TCR has been synthesized. T Cells at this stage of maturation can be recognized by cytoplasmic staining with CD3 reagents.

The TCR gene rearrangement is similar to the rearrangement of genes that encode immunoglobulin heavy and light chains, which takes place in the bone-marrow microenvironment. Once TCR has been expressed at the surface, however, the cell undergoes a process unique to T cells, namely, specific selection on the basis of recognition specificity (Blackmann et al., 1990; Sprent et al., 1990; Von Boehmer, 1990). First, the cell is examined for its capacity to recognize an antigen in the context of its own MHC (self-restriction); then it is allowed to expand (positive selection). Second, the cell is examined for its capacity to recognize a self-antigen (autoreactivity). If it recognizes a self-antigen, it is blocked from further differentiation (negative selection). In this way, the random pool of antigen recognition specificities of T cells is

adapted to the host's situation; the total repertoire of the α–β T-cell population (estimated at 10^{12} different epitopes) changes into the potentially available repertoire (estimated at recognition of about 10^{6} epitopes). Current theories of negative selection state that this step is not feasible for all putative autoantigens in the body. Rather, it applies to a selection of potentially harmful specificities (in particular MHC antigens). If a cell is not selected during positive or negative selection, it dies, possibly by suicide or apoptosis (McDonald & Lees, 1990). A hallmark of apoptosis is endonuclease-induced chromosomal fragmentation into 200 base-pair fragments (McConkey et al., 1990). Histologically, apoptosis is recognized by the presence of condensed, sometimes fragmented nuclei, which can be found in phagocytic macrophages ('tingible body' or 'starry-sky' macrophages) (Kendall, 1991).

Function of the microenvironment: It is generally accepted that the epithelial microenvironment of the thymic cortex plays a major role in positive selection. This microenvironment expresses MHC class I and class II products and shows close interactions with lymphocytes morphologically (at the electron microscopic level). This close interaction is reflected in the complete inclusion of lymphocytes inside the epithelial cytoplasm ('thymic nurse cells') (Van Ewijk, 1988). Negative selection has been ascribed to either the epithelial compartment or the medullary dendritic cells. The different processes occurring in early (cortical) and late (medullary) maturation are associated with differences in the microenvironment. Epithelial cells in the cortex and medulla differ in antigen expression, ultrastructural characteristics, and their capacity to synthesize thymic hormones such as thymulin, thymic humoral factor, thymosin, and thymopoietin. These hormones have a major function late in intrathymic T-cell maturation, and the major site of thymic hormone synthesis is the medullary epithelium (Dabrowski & Dabrowski-Bernstein, 1990).

The cortex can be considered a primary lymphoid organ because it is an antigen-free microenvironment with a blood–thymus barrier. In contrast, antigens can move relatively freely into the medulla and encounter antigen-presenting dendritic cells as well as antigen-reactive T cells. Thus the medulla has the properties of a secondary lymphoid organ (Van Ewijk, 1988; Kendall, 1991).

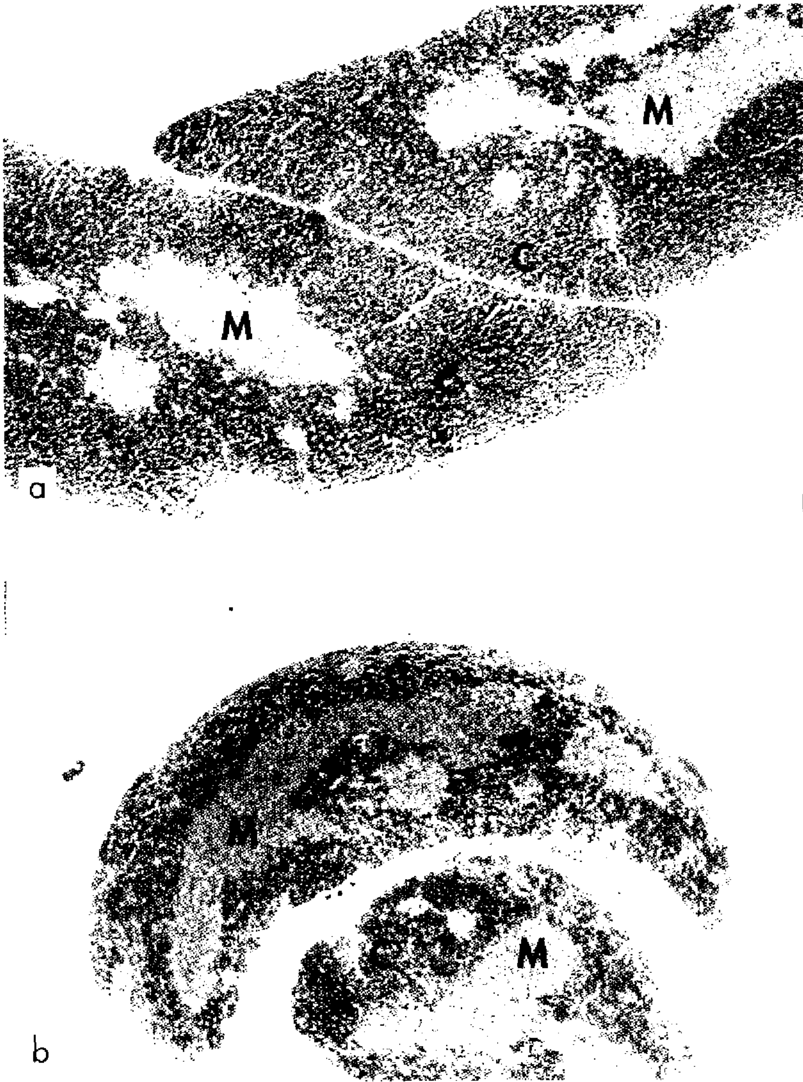
Ontogeny, growth, and involution: The thymus in rodents reaches full development at around day 17 of gestation, that is about five days before birth. In humans, a fully developed thymus is first seen at the 16th to 17th week of gestation (Von Gaudecker, 1986), which is relatively earlier in the gestation period than in rodents, since the human immune

system is more mature at birth than that of rodents. Nevertheless, a thymus that appears histologically to be fully developed may be functionally somewhat immature. For instance, fetal thymus from humans (McCune et al., 1988; Namikawa et al., 1990) or rats (De Heer et al., 1993) can be transplanted into mice with the severe combined immunodeficiency (*scid*) mutation and grow for longer than tissue obtained postnatally (see also section 4.5.3). After birth, when the individual first comes into contact with exogenous antigens, the thymus is called upon to provide large numbers of T cells to the periphery, and the organ grows in a relatively short time—in humans within three to four weeks, from about 15 to 50 g. In rats, the organ reaches it largest relative size about one week after birth; the absolute weight is greatest at about six to eight weeks of age. After adulthood is reached, the thymus starts to involute (Steinmann, 1986; Kuper et al., 1990a; Schuurman et al., 1991a; Kuper et al., 1992a), a process that may be related to changes in the hormonal status of the individual; circulating thymic hormone is reduced to very low levels in adults. The underlying mechanisms are not fully understood. The consequences of age-associated involution are obvious: emigration of lymphocytes from the thymus decreases dramatically, from, for instance, 1.6×10^6/day in one-month-old mice to 4×10^4/day in one-year-old mice (Stutman, 1986; Shortman et al., 1990). Apparently, the persistent generation of a new antigen-recognition repertoire in the T-cell population of adults is not needed. Instead, the body can defend itself using the established repertoire and extrathymic self-renewal of the T cells. Similar processes may occur after artificial involution of the thymus caused by toxic compounds or acute stress (including acute disease); this aspect is further discussed below.

It should be emphasized that the basic architecture of the thymus is not a fixed histological entity; its features depend on the age and the stress hormone status of the individual. A 'normal' architecture can be expected only between the late gestational period and young adulthood, before the start of age-associated involution. This phenomenon has important implications for the selection of rodents according to age for studies of immune function and in the interpretation of studies of immunotoxicity.

In mice, severe but reversible changes occur in the thymus during pregnancy (Clarke, 1984; Clarke & Kendall, 1989; see also Figure 9). The weight of the thymus shows an initial small rise in early pregnancy, from about 35 to 40 mg, and dramatically decreases to 15 mg or less at day 17 of pregnancy (Clarke, 1984). This involution is associated with severe lymphodepletion of the cortex. Cell death is seen by the presence of apoptotic figures and phagocytosis in macrophages and epithelial

Figure 9. Thymus from female CBA mouse during pregnancy



From Clarke & Kendall (1989)
C, cortex; M, medulla; (a) control thymus; (b) thymus on day 17 of pregnancy, showing extensive involution; haematoxylin and eosin stain.

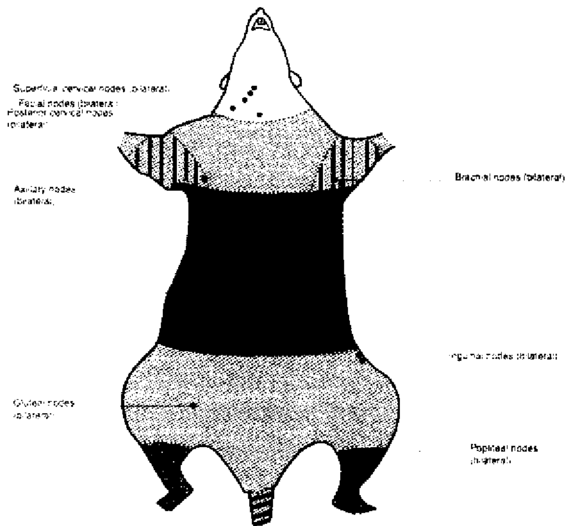
cells. Remarkably, the large lymphoblasts in the outer cortex remain relatively unattached, and the same applies for thymocytes in thymic nurse cells. In birds, changes with breeding sessions have been found (Kendall & Ward, 1974; Ward & Kendall, 1975). In a study of a wild population of adult red-billed queleas, cyclical enlargement and regression of the thymus were documented; at the time of mating and laying, most birds, irrespective of sex, showed an involuted thymus; on subsequent egg incubation the thymus size increased, with a decline in the latter half of the rearing period.

1.2.2.4 Lymph nodes

A finely branched lymph vessel system (lymphatics) is involved in the return of interstitial fluid in tissue to the blood circulation, with lymph nodes spaced at regular intervals (Dunn, 1954; Tilney, 1971). The major sites of lymph nodes (or groups of lymph nodes) are shown in Figure 3 (p. 32); a scheme of the areas drained by distinct lymph nodes or lymph node groups is given in Figure 10, a schematic presentation of individual lymph node architecture is presented in Figure 11, and the histology of a lymph node is shown in Figure 12. The main functions of lymph nodes are to filter pathogens from the afferent lymph and then to initialize immune reactions. The afferent lymphatics penetrate the lymph node capsule and connect with the subcapsular sinus, which in turn connects with the cortical and medullary sinuses. On the basis of the lymph flow through the node, basic units can be recognized, each of which is supplied by its own afferent lymph vessel and which comprise part of the paracortex (Bélisle & Sainte-Marie, 1981; Sainte-Marie et al., 1990). Afferent and efferent blood vessels are connected to the organ at the hilus, where the lymph leaves the node via the efferent lymphatic(s). The efferent lymphatics drain into other lymph nodes or directly into the thoracic duct, which enters the bloodstream, in the rat via the left subclavian vein.

Lymph vessels and lymph nodes occur only in mammals. In some birds and monotremes (primitive mammals), primitive lymph nodes directly interposed in the lymph circulation have been described. Ectothermic vertebrates do not have lymph nodes. Some small lymphoid organs such as lymph glands and jugular bodies have been described in some species of frogs but not in others. These organs presumably filter antigens from both blood and lymph and have been claimed to be phylogenetic precursors of mammalian lymph nodes. Likewise, small lymphoid aggregates associated with the cardinal veins occur in some reptiles.

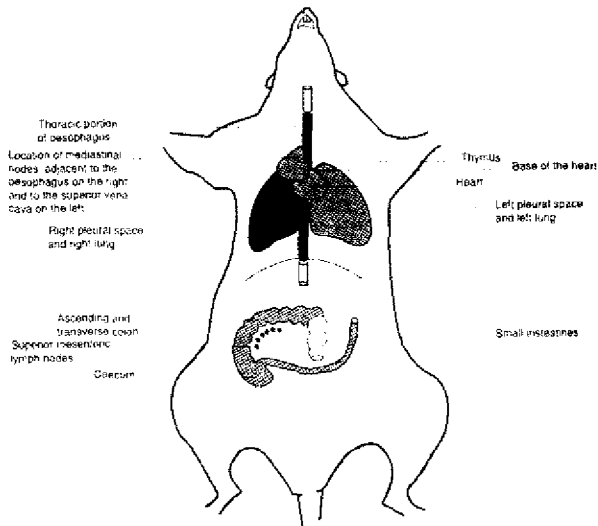
Figure 10. Areas drained by distinct lymph nodes or lymph node groups



a

From Tilney (1971)
(a) Cutaneous areas drained by cervical lymph nodes (head and neck; white), brachial lymph nodes (forefoot, front leg, shoulders, neck, and upper chest; pink), axillary nodes (entire trunk and foreleg; green), inguinal lymph nodes, and gluteal lymph nodes

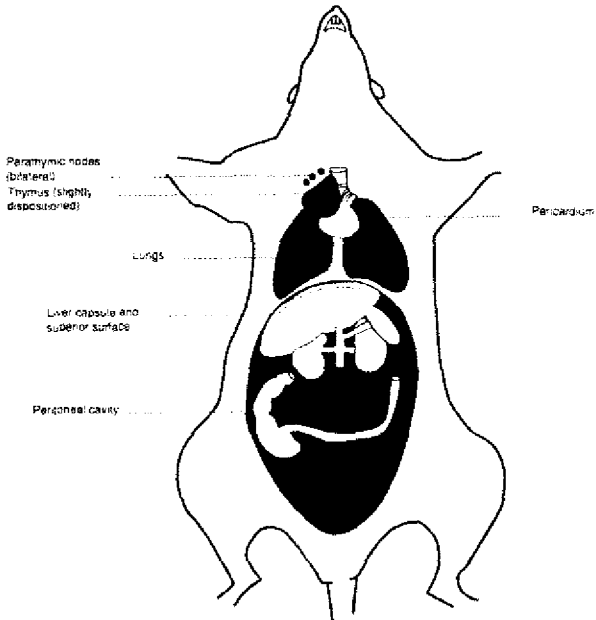
Figure 10 (contd)



b

(b) Organs and areas drained by the superior mesenteric nodes: small intestine, caecum, ascending and descending colon; by the right posterior mediastinal node: right pleural space, right lung, base of the heart, thoracic portion of the oesophagus; and by the left posterior mediastinal node: left pleural space, thoracic viscera, thymus

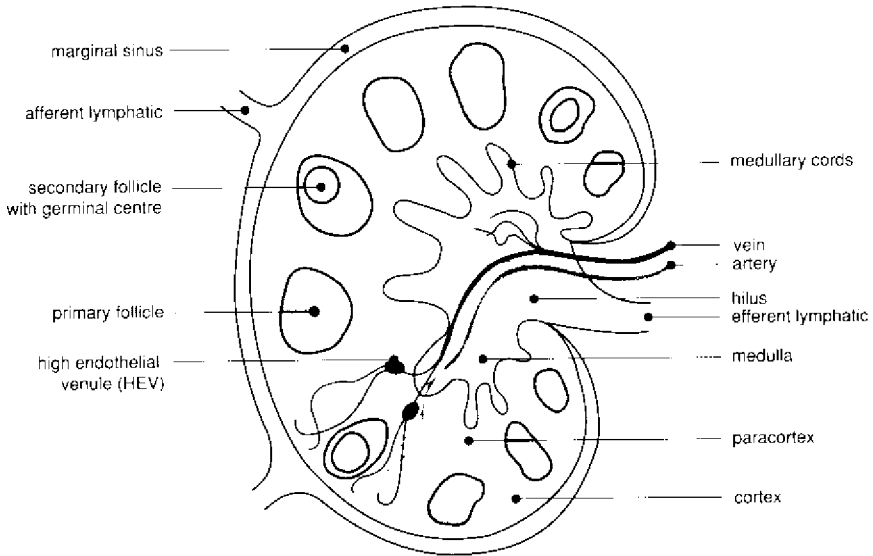
Figure 10 (contd)



C

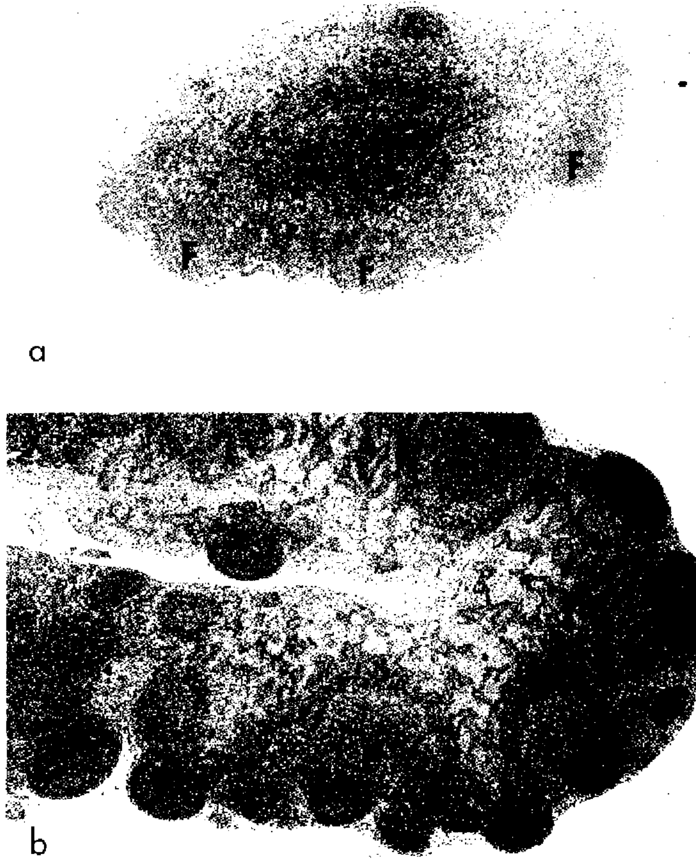
(c) Organs and areas drained by the parathymic lymph nodes. peritoneal cavity, superior surface of liver and liver capsule, thymus, pericardium, lungs

Figure 11. Schematic diagram of the normal lymph node



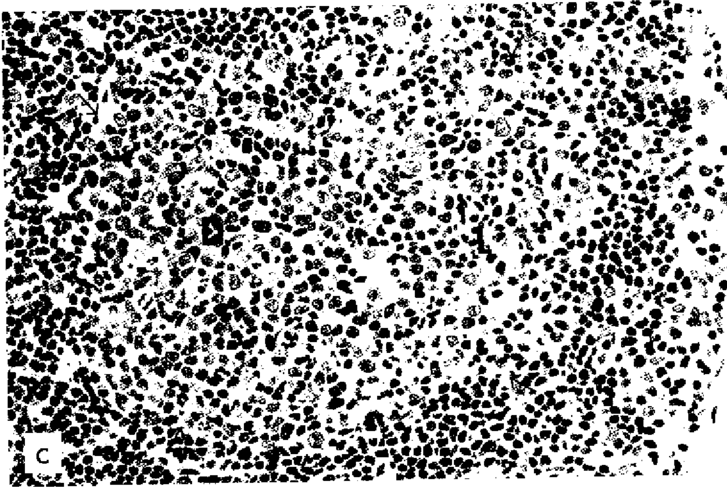
The basic structure comprises the outer cortex, with primary and secondary follicles (B-lymphocyte areas), an interfollicular area that is continuous with the paracortex in the inner cortex (T-lymphocyte area), and the medulla, with medullary cords. Afferent lymphatics enter the lymph node at the capsule and drain through the cortex around the follicles into the medullary sinusoids. The main artery enters at the hilus and divides into capillaries; veins follow the trabeculae and exit at the hilus. The high endothelial (postcapillary) venules, where lymphocytes pass the endothelial barrier and enter the tissue parenchyma, are indicated.

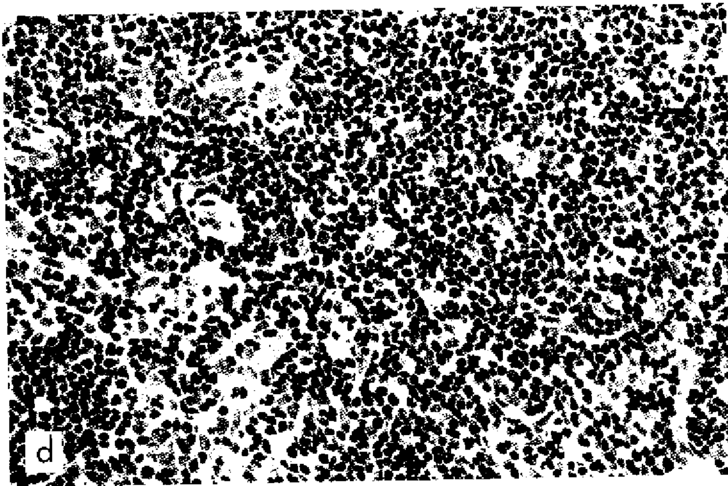
Figure 12. Lymph node of Wistar rat



F, follicles; P, paracortex; I, interfollicular areas; arrows, medullary cords
(a) Overview of the popliteal lymph node, a lymph node that is not normally exposed to antigens and thus has a 'quiescent' or 'resting' appearance, with primary follicles, a relatively small paracortex and interfollicular area, and only small proportions of blastoid cells
(b) Overview of mesenteric lymph nodes which are continuously stimulated by antigens from the gastrointestinal tract; they generally have high intra- and inter-node variability

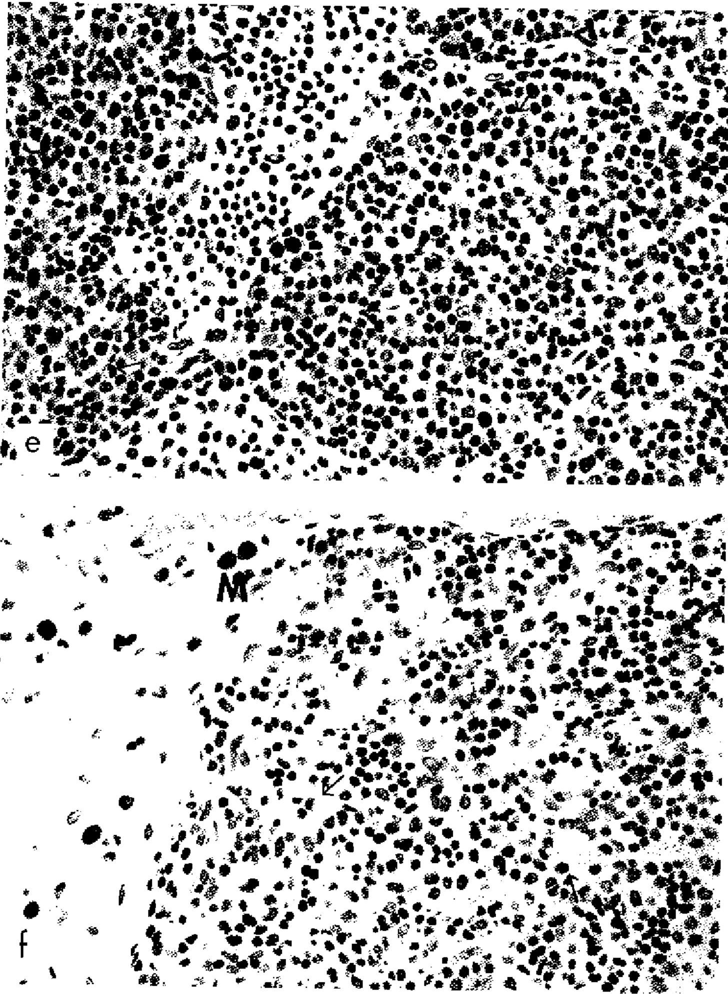
Figure 12 (contd)





(c) Superficial cervical lymph node: germinal centre (arrows indicate border) with light (L) and dark (D) areas. These nodes are continuously stimulated by antigens via nasal and oral uptake. They often manifest prominent follicles with large germinal centres, representing pronounced stimulation of B lymphocytes.
(d) Superficial cervical lymph node: paracortex with high endothelial venules (arrows)

Figure 12 (contd)



(e) Superficial cervical lymph node: plasma cells in medullary cords. Arrows indicate plasma cells with a distinct, optically light zone. A large number of plasma cells give the medullary cords a basophilic appearance in sections stained with haematoxylin and eosin.

(f) Inguinal lymph node showing sinus histiocytosis (arrows), cytoplasm-rich cells; M, mast cells; haematoxylin and eosin stain

Lymph nodes are surrounded by a connective tissue capsule. The nodes comprise various compartments or microenvironments: (i) the outer cortex, with follicles and interfollicular areas; (ii) the inner cortex or paracortex; and (iii) the medulla, with medullary cords and medullary sinuses. These compartments are easily differentiated into sections after conventional histological staining. In the cortex, interfollicular areas and the paracortex (T-lymphocyte area) are differentiated from follicles by the presence of blood vessels lined by high endothelium (high endothelial postcapillary venules, discussed below). Follicles (B lymphocyte area) are rounded structures, located mainly immediately underneath the capsule; they present as accumulations of small lymphocytes (primary follicle) or a pale-stained centre with large lymphoid cells (centrocytes, centroblasts) and tingible-body macrophages surrounded by a mantle with small lymphocytes (secondary follicles). The interfollicular areas are continuous with the paracortex, and the latter is continuous with the medullary cords.

The arterial blood supply enters the node at the medulla and ends in the paracortex as arteriolar capillaries, with branches in the follicles. The capillaries feed venules that are lined with high endothelial cells. The high endothelial venules run from the paracortex into the medullary cords and then leave the node via the vein in the hilus. Lymphocytes migrate through the high endothelial venules after adhering to the endothelium by specific receptor–ligand interactions (Picker & Butcher, 1992). The adhesion molecules on lymphocytes and endothelium that are involved in this binding and subsequent passage thought the endothelial layer are the addressins, reflecting the difference in receptor–ligand interactions that exists between lymph nodes of the internal and external lymphoid systems. Subsequently, lymphocytes can specifically reach the various nodes, using the same route (the blood). After the lymphocytes have migrated into the parenchyma, they move into their microenvironment or compartment.

Antigen encounter and immune reactivity: The main route of access for antigens and pathogens is the afferent lymph flow; antigens can also come into contact with the lymph node tissues via the blood. Antigens in the lymph, either free or processed by veiled macrophages, enter the node through the subcapsular sinus, which is rich in macrophages that can phagocytose free antigen. From there, antigens move to the paracortex, where they are presented to $CD4^+$ Th cells by the antigen-presenting cells for initiation of the immune response. The main antigen-presenting cells in the lymph node are the interdigitating dendritic cells, the tissue equivalents of veiled macrophages, which can arise from Langerhans cells in the skin (for e.g. lymph nodes draining the

skin). The antigen-presenting cells express MHC class II antigens in high density, enabling the α–β TCR of the Th cells to recognize the antigenic determinant complexed with the polymorphic ('self') MHC class II molecule. The $CD4^+$ molecule on the Th cell surface binds to a non-polymorphic determinant of MHC class II molecules and strengthens the binding between Th and antigen-presenting cells (Janeway, 1992). The cellular interaction triggers the synthesis of cytokines like IL-1 and IL-2. This process is down-regulated by Ts cells in a way that is not yet completely understood.

Follicles are involved in antigen-driven B-cell activation, somatic mutation, positive and negative selection, and memory and plasma cell development (Szakal et al., 1989; Kroese et al., 1990; Liu et al., 1992) and are known as primary follicles in the resting state. They contain small, IgM^+IgD^+ virgin B cells in a framework of follicular dendritic cells. During stimulation by antigens, the follicles change into secondary follicles consisting of a germinal centre surrounded by a mantle. Antigen may be transported into the follicle by immune (T) cells, but this route is not yet fully established. Antigen is presented to B cells in immune complexes, with complement split products like C3b, which are trapped in cytoplasmic extensions of the follicular dendritic cells. The interaction between complement and complement receptors on these cells has a pivotal role in the adherence of antigen to them. Fc receptors also play a role, but only in rodents. The complement split products in the trapped immune complexes have an accessory function in antigen presentation.

Local $CD4^+$ Th cells assist in B-cell activation. Antigen-driven B-cell activation and proliferation in the germinal centre are accompanied by an isotype switch of the immunoglobulin class synthesized by the B cell. In addition, the affinity of the antibody increases as a result of somatic mutation (Kocks & Rajewski, 1989). A kind of selection mechanism has been proposed in this antigen-driven process, in which cells that produce antibody of higher affinity are selected preferentially, and cells that produce antibody of lower affinity are not selected and subsequently die, perhaps by apoptosis (programmed cell death) (Liu et al., 1989). This selection resembles that of developing T lymphocytes in the thymus; it differs from T-cell selection by the absence of negative selection and the occurrence of somatic mutation. Antigen may remair in the follicular compartment for quite some time, thereby causin persistent activation of B cells, related to the state of immunological memory within the B-cell population. After the antigen disappears, the immunological memory in the B cells is short-lived and disappears. B-Cell activation in germinal centres leads to activated cells with a specific morphology, the so-called centrocytes and centroblasts. Finally,

B-cell activation leads to the formation of plasma cells, both in the periphery of germinal centres but more predominantly in the medullary cords of the lymph nodes.

The main site of effector immune reactions is the medulla. The medullary cords house macrophages, granulocytes, activated effector T cells, and plasma cells. The effector T cells include $CD4^+$ delayed-type hypersensitivity T cells (mediator-producing cells) and $CD8^+$ Tc cells. The Tc cells bear an α-β TCR that recognizes antigen in the context of the polymorphic determinant of MHC class I molecules. The CD8 molecule has an accessory function in this process, since it binds a non-polymorphic class I determinant (Janeway, 1992). Antigen recognition by Tc cells is thus different from that by Th cells. The reaction products of the effector cells, such as lymphokines, and effector cells like plasma cell precursors leave the lymph nodes via the efferent lymph or blood circulation to go to other sites of the body.

Development and aging: Lymph node morphology is dynamic: its appearance throughout life is directly related to the type and amount of antigenic stimulation. After antigenic contact, the organ increases in size within a relatively short time, with high proliferative activity of lymphocytes and germinal centre formation, depending on the type of reaction and the choice of immunological reaction. In the case of B-lymphocyte reactions, hyperplasia of follicles is seen (e.g. after bacterial infection); in the case of T-cell reactions, the interfollicular areas or paracortex become enlarged (e.g. in viral infection). After the reaction is terminated or is transferred to the next draining lymph node, it regains its normal small size. Germinal centres with an interfollicular microenvironment can develop extranodally, especially at sites of chronic inflammation.

The dynamics of the lymph node can be illustrated by several examples. After immunization of the footpad with antigen mixed with an adjuvant, such as Freund's complete adjuvant, containing killed mycobacteria, the draining popliteal lymph node becomes enlarged (in rats, from about 5 mg to more than 100 mg), and granulomatous reactions (epitheloid-cell granuloma) can be seen histologically. The swelling of lymph nodes is used to assess reactivity towards chemicals and in the evaluation of immunomodulatory drugs (Gleichmann et al., 1989). In the popliteal lymph node assay, a test substance is injected subcutaneously into one footpad and the contralateral side is left untreated or injected with vehicle only. The effect of the substance is subsequently estimated from the difference in weight between the popliteal lymph nodes. Further evidence for immunostimulatory activity *in vivo* is obtained by histological appearance, often manifested as follicular hyperplasia.

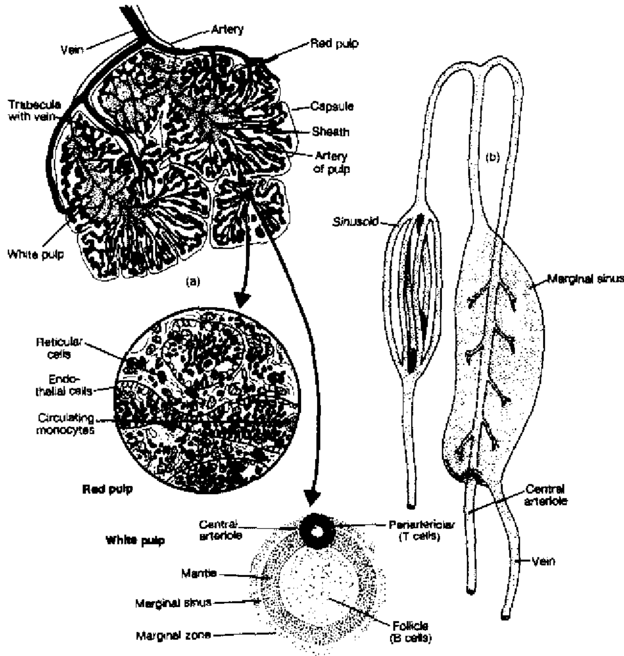
Lymph nodes develop relatively late in fetal life: At birth, the anlage of unstimulated lymph nodes is present, containing few lymph cells. The lymph nodes develop quickly after exposure to many new (exogenous) antigens. In adults, they may become relatively quiescent and small, with virgin T and B cells and primary follicles. The lymph nodes in aged rats are capable of the same degree of activity as those in young individuals upon antigenic stimuli. The state and type of activation in the various nodes of adult and aged animals differ under normal housing conditions and are a reflection of the absence or existence of continuous local stimulation with antigens or disease processes, like inflammation and the presence of a tumour in the drained area (Ward, 1990). The central nodes of the mandibular and superficial cervical group, which are continuously exposed to (aero)antigens via the oronasopharynx, may contain a considerable number of plasma cells and precursors in the medullary cords and relatively well-developed germinal centres. Sinal histiocytosis (that is, considerable numbers of macrophages in the sinuses) and accumulations of pigmented macrophages are often present in the mesenteric lymph nodes, which are continuously exposed to antigens via the digestive tract. An extensive review of lymph node development and aging is given by Losco & Harleman (1992).

1.2.2.5 *Spleen*

The spleen consists of two main compartments: the red and white pulp (Van Rooijen et al., 1989; Dijkstra & Sminia, 1990; Laman et al., 1992; Van den Eertwegh et al., 1992). A schematic drawing of the spleen is presented in Figure 13 and histological views in Figure 14. The red pulp consists of blood-filled sinusoids and Bilroth's cords containing macrophages, lymphocytes, plasma cells, and NK cells. Macrophages perform major functions in clearing blood cells (for instance, old red blood cells) and in phagocytosis, especially of non-opsonized particles. This high-volume filter function is made possible by two factors: the direct contact, unobstructed by blood-vessel walls, between phagocytic cells and blood-borne particles; and the large blood supply, estimated at about 5% of the total blood volume per minute. There are no lymphatics in the spleen.

The phagocytic function is especially important in the case of intravascular pathogenic microorganisms, before antibody formation and subsequent opsonization occur (early bacterial septicaemia). The mononuclear phagocyte system of the liver (Kupffer cells) plays a major role in the removal of opsonized particles. Together with the hepatic phagocytic system, splenic macrophages synthesize complement components, although this is done mainly by hepatocytes. In rats and

Figure 13. Schematic diagram of spleen

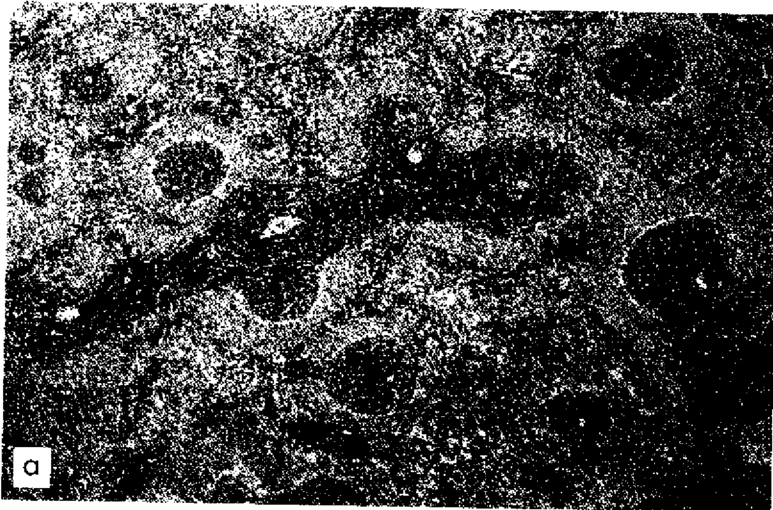


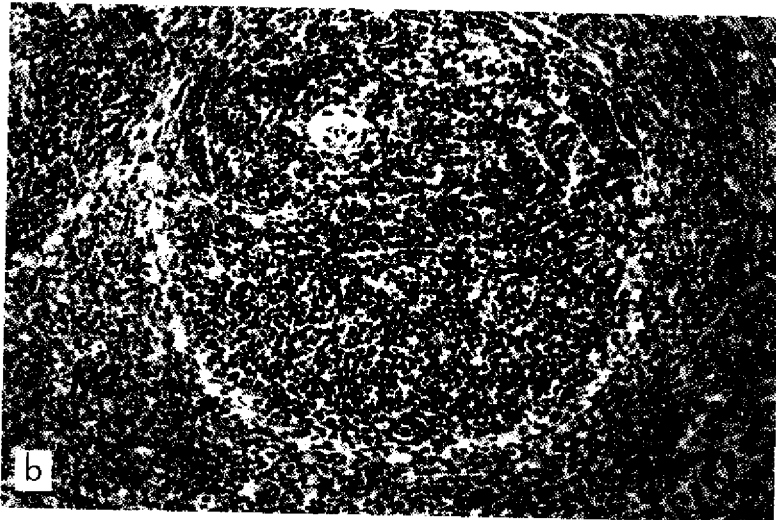
Reproduced with permission from Sell (1987)

(a) The red pulp comprises sinusoidal channels filled mainly with red blood cells. Its main function is phagocytosis and destruction of old red blood cells. In rodents, haematopoiesis also occurs at this location.

(b) In the white pulp, a T-lymphocyte area is found directly around the central arteriole (the periarteriolar lymphocyte sheath), and B-lymphocyte areas are seen in follicles and the marginal zone. Blood entering by the central arteriole supplies either the white pulp or the sinusoids in the red pulp; both branches terminate in the splenic vein.

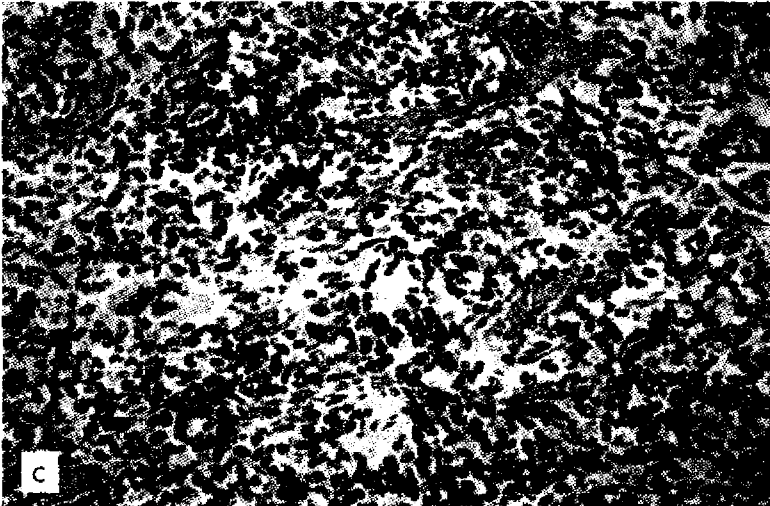
Figure 14. Spleen of Wistar rat

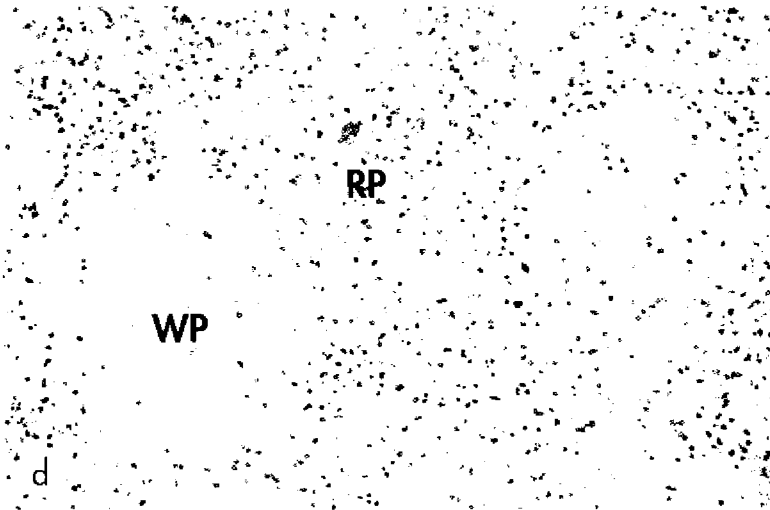




(a) Overview, showing white pulp (within demarcation) with periarteriolar lymphocyte sheath (pals; arrows, central arteriole), follicles (F), a marginal zone (MZ), and red pulp (outside the demarcation)
(b) Detail of white pulp

Figure 14 (contd)





(c) Detail of red pulp
(d) Immunophenotyping for natural killer cells using antibody 3.2.3, showing a relatively high density of cells in the red pulp (RP) and a lower density in the white pulp (WP)
(a–c), haematoxylin and eosin stain; (d), immunoperoxidase technique

mice, the red pulp contains nests of (extramedullary) haematopoiesis, characterized histologically by megakaryocytes and normoblasts. In the case of systemic septicaemia, when pathogenic microorganisms have reached the blood either directly or after inadequate filtering through lymph nodes, the red pulp increases and contains large proportions of (immature) granulocytes. Differentiation between septicaemia and extramedullary haematopoiesis is not always easy; the decreased or absent white pulp in septicaemia can be helpful in making this differentiation.

Phylogenetically, cartilaginous fish are the first species that have a spleen, which consists of lymphoid follicles and a red pulp that generally houses developing erythroid cells (Zapata & Cooper, 1990). The lympho-haematopoietic masses of the intestine that are seen in some lower vertebrates (e.g. lampreys) are not primitive spleens but rather primitive lymphohaematopoietic organs equivalent to mammalian bone marrow. In most bony fishes, the white pulp is poorly developed, probably reflecting the existence of other peripheral lymphoid organs, e.g. the kidney, which participate actively in the immune response. After antigenic stimulation, the amount of splenic lymphoid tissue increases considerably in all lower vertebrates, although germinal centres do not occur. At the cellular level, however, antigen-presenting cells and cells retaining immune complexes on their surface have been described in the spleen of some bony fishes, anurous species, and reptiles.

White pulp: The spleen contains about one-quarter of the body's total lymphocyte population; during lymphocyte recirculation, more cells pass through the spleen than through all the lymph nodes. Lymphocytes in the spleen reside in the white pulp, which consists of a central arteriole surrounded by the periarteriolar lymphocyte sheath, a T-lymphocyte area. The outer sheath contains B lymphocytes and, after antigenic stimulation, plasma cells. Adjacent follicles contain B cells. Around the periarteriolar lymphocyte sheath and follicles is a corona containing B cells, called the marginal zone; this region is easily distinguished, especially in rats. The periarteriolar lymphocyte sheath has a microenvironment and a passenger leukocyte content similar to that of the lymph node paracortex. Some sources claim that the spleen is a rich source of Ts cell activity, exceeding that of lymph nodes. The follicles are not essentially different in structure and function from those of lymph nodes. The spleen performs a major function in humoral immunity by synthesizing IgM class antibodies, especially to blood-borne antigens.

The microenvironment of the marginal zone is unique to the spleen. Histologically, the B cells at this site are of medium size; on histological staining, they are larger and paler than B cells in primary

follicles and in the follicular mantle of secondary follicles (Figure 14). In addition, they do not show the morphology of the centrocytes or centroblasts found in germinal centres, and the phenotypic expression (surface $IgM^{+}IgD^{-}$) indicates that the B cells in the marginal zone are a separate population. Special macrophage types are present, which are known as marginal zone macrophages and marginal metallophilic macrophages. The latter are located at the periphery of the white pulp, along the inner border of the marginal sinus, and can be stained by silver impregnation.

The physiological function of the marginal zone has been characterized recently. First, the site retains B-lymphocyte memory; second, it mediates humoral responses that do not directly involve T cells. These T-independent responses are elicited by polysaccharide antigens of encapsulated bacteria, which are present in repeating units on the microorganism and are presented to the B cells by marginal zone macrophages. The response may not be completely T cell-independent in all cases, as T cell-derived factors enhance the response to some of these antigens. The antibodies generated are mainly of the IgM class, as T-cell help is required for an isotype switch.

In conclusion, the main immunological function of the spleen is to defend the body's vascular compartment by generating T cell-independent IgM antibody responses to bacterial polysaccharides and by exerting an enormous phagocytic power. This function is lost after splenectomy, when reduced nonspecific phagocytosis of non-opsonized particles, lowered serum IgM levels, and increased susceptibility to infections by encapsulated bacteria have been described.

1.2.2.6 *Mucosa-associated lymphoid tissue*

The secretory epithelial surfaces of the body form a major route of entry for potentially pathogenic substances. These surfaces include the epithelia of the gastrointestinal, upper and lower respiratory, and urogenital tracts (Miller & Nicklin, 1987; Sminia et al., 1989). The host response at these locations ranges from nonspecific constituents, such as a physical or mechanical component (epithelial barrier, motility of the gastrointestinal tract, and the mucociliary escalator in the respiratory tract), to a chemical component (low gastric pH, mucus, lysosomal and digestive enzymes), and antigen-specific components of the immune system.

Nonspecific killer cells are found in significant numbers in the lungs and along the epithelium of the gastrointestinal tract, where lymphocyte-like cells have been found to kill pathogens, presumably without prior sensitization (Hanglow et al., 1990). In mice, these cells have been characterized as T cells with a γ–δ TCR, which, in contrast to

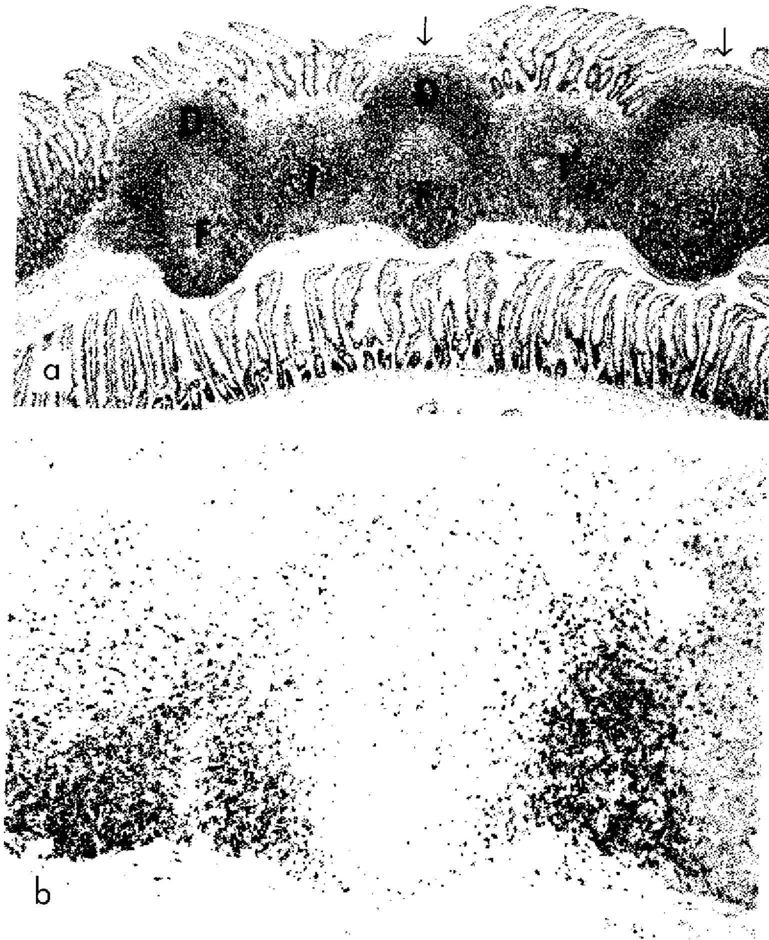
Tc cells that express α–β TCR, kill targets in an MHC-nonrestricted manner (Raulet, 1989). The cells have antigen specificity that is encoded at the DNA level by variable gene segments, but the repertoire appears to be smaller than that which encodes TCR α or β chains. These γ–δ T killer cells are not generated under the strict influence of the thymus, as are their α–β T-cell counterparts (Bell, 1989; Haas et al., 1993). Apart from their killer activity, these cells may serve as inducing elements for the response mediated by α–β TCR-expressing T subsets. In this initiating activity, γ–δ TCR molecules shed from the lymphocyte surface may act as antigen-specific factors (De Weger et al., 1989).

Lymphoid tissue: Lymphoid tissue occurs just underneath the secretory epithelium, in the duodenum and jejunum as Peyer's patches (Figure 15), in the appendix of the large intestine, along the bronchi (Sminia et al., 1989), and in the oro- and nasopharyngeal regions (Kuper et al., 1992b). These mucosal lymphoid tissues share structural and functional characteristics and are strongly interrelated. The common designation 'mucosa-associated lymphoid tissue' (MALT) is therefore used to refer to bronchus-associated, gut-associated, and nasal-associated (nasal cavity and nasopharynx) lymphoid tissue. Nasal-associated lymphoid tissue has been identified in horses, monkeys, and rats (Figure 16) (Kuper et al., 1990b). In humans and domestic animals, the larger lymphoid nodules in the pharyngeal region are called tonsils. Together with the intermediate lymphoid tissue, the tonsils form Waldeyer's tonsillar ring.

The organization of MALT is similar to that of lymph nodes, with B cell-containing follicles and T cell-containing interfollicular areas. Afferent lymph vessels are lacking, because pathogens can enter the tissue through the covering epithelial layer. The epithelial cells at this location (the 'M' or microfold cells) are often thinner than those at other secretory sites, in order to enable efficient passage of antigens. Stimulated gut-associated lymphoid tissue and human tonsils often have prominent follicles with germinal centres. In contrast, germinal centres are scarce in stimulated bronchus-associated and nasal-associated lymphoid tissue in rodents, due to the fact that immunological reactions occur mainly in the draining cervical lymph node. Th medulla-like areas seen in lymph nodes are absent in MALT. Lymphocytes and NK cells are found in the lymphoid tissue, in interstitial tissue in the lung, and in the lamina propria along the gastrointestinal tract.

The homing specificity of lymphocytes into lymphoid tissue by migration through high endothelial venules is described above. The specificity of the homing phenomenon to MALT has the advantage that the same circulation pathway (i.e. the blood) is used by the secretory and internal immune system (with the intrinsic possibility of mutual

Figure 15. Swiss roll of small intestine of rat



(a) Peyer's patches with follicles (F), interfollicular area (I), and dome (D), showing the direct association between the covering epithelium (arrows) and the lymphoid area; hamatoxylin and eosin stain

(b) Frozen section showing the interfollicular area, immunolabelled with antibody specific for T cells (cells with a dark rim); immunoperoxidase technique

Figure 16. Paired nasal-associated lymphoid tissue (arrows) at the entrance of the nasopharyngeal duct

Cross-section through rat nasal passages; haematoxylin and eosin stain

contact). In addition, the antigen message received at one secretory site is followed by effects at all secretory surfaces. Thus, after antigen presentation in the gastrointestinal tract, effector cells (e.g. IgA antibody-synthesizing plasma cells, see below) are found at the site of stimulation and at other secretory sites (e.g. the respiratory tract). Thus, the major function of MALT is to initiate immune responses, which are then passed on to draining lymph nodes, such as mesenteric lymph nodes in the gastrointestinal tract.

The secretory IgA antibody response: The immune response in MALT differs from that at other sites of the body in that it is devoted to the generation of an IgA antibody response. Thus, MALT contains precursors of IgA antibody plasma cells and populations of T cells capable of promoting a B-cell immunoglobulin class switch into IgA-producing B cells or plasma cells. B-Cell differentiation into IgA-producing plasma cells after local antigen presentation is accompanied by lymphocyte migration and specific homing. Precursors move through draining lymph nodes into the blood and from there to the secretory surface, where they lodge as IgA plasma cells in the lamina propria.

Specific homing mechanisms exist by which these cells are able to select the secretory surface of their final mucosal destination.

In contrast to IgA produced by the bone marrow and circulating in blood, IgA synthesized by plasma cells of MALT consists of a dimeric immunoglobulin subunit. The two monomers are linked by a polypeptide called the J chain (about 15 kDa). These IgA antibodies have their main effect outside the body itself, for example in salivary and gastrointestinal secretions. The transport from the site of synthesis across the epithelial barrier is specifically adapted for dimeric IgA, and to a lesser extent for pentameric IgM. Epithelial cells express a receptor for these immunoglobulins, called secretory component (a polypeptide of about 70 kDa). After binding to this receptor, the molecule is transported through the epithelium, possibly through its cytoplasm, and excreted on the luminal surface. During this process, secretory component attaches to the immunoglobulin molecule (coiled around the Fc fragments); the composite molecule, comprising dimeric IgA, the J chain, and secretory component, is called secretory IgA. In rodents, a similar secretory component-mediated transport occurs in the liver (Brown & Kloppel, 1989). Here, a secretory component on the hepatocyte surface mediates the passage of dimeric IgA from the sinusoids to the bile canaliculi. In this way, dimeric IgA entering the liver by the portal vein efficiently recirculates to the bile and from there into the gastrointestinal lumen. Secretory IgA is more resistant to luminal conditions (especially proteolytic enzymes) than dimeric IgA and is thus better able to function there.

IgA lacks the effector reactivity of IgM and IgG in complement activation by the classical cascade, opsonization and phagocytosis, or antibody-mediated cellular cytotoxicity. This lack appears to be related to the absence of effector systems (complement, phagocytes) in secretory fluid. The main function of IgA is to prevent the entry of potentially pathogenic substances into the body, by a specific antigen exclusion function in which the epithelium is coated with 'antiseptic paint'.

Induction of immunological tolerance: A final feature of MALT is its capacity to generate immunological tolerance. After antigenic priming at secretory surfaces, subsequent systemic antigenic challenge often results in nonresponsiveness (Strobel & Ferguson, 1984; Challacombe, 1987; Holt & Sedgwick, 1987; Mowat, 1987). Suppressor cells have been found in the spleen and suppressor factors in the circulation after local immunization. The induction of tolerance pertains primarily to dead microorganisms and inactivated proteins which come into contact with the MALT. The mechanism of tolerance induction and different responses to live and dead microorganisms is not completely defined

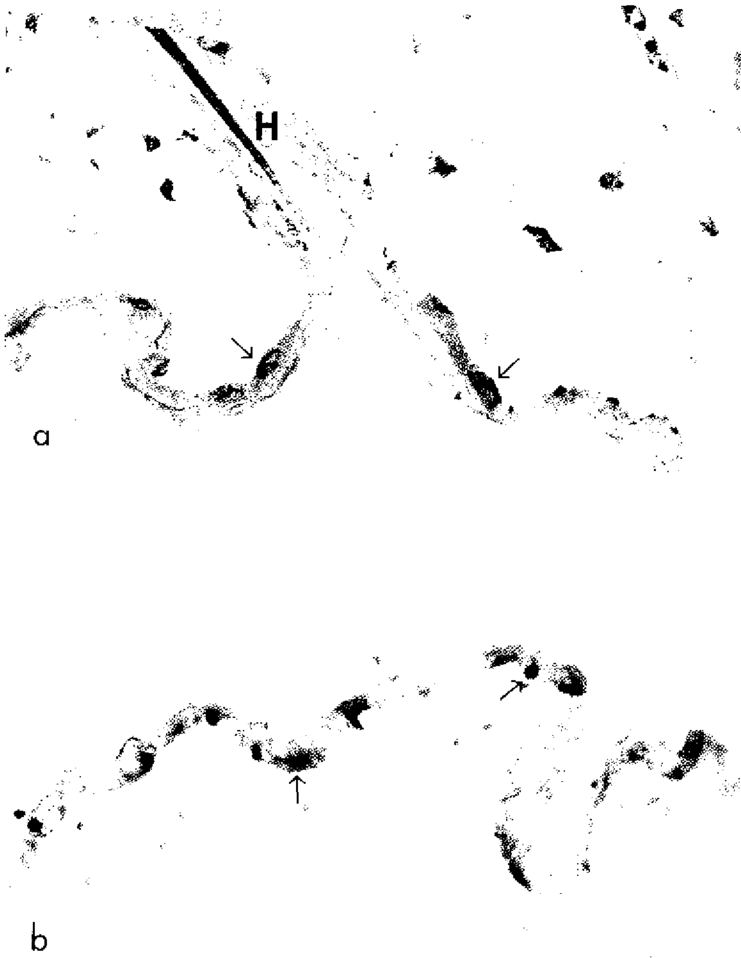
but is important in tolerance to food antigens and the development of food allergies.

In summary, the host's defence in MALT is different from the response of the internal immune system. The responses at this first line of defence range from NK cell activity in the epithelium to specific IgA antibody-mediated exclusion in the secretory fluid. Immune responses to antigens entering the body at secretory sites are initiated by lymphocytes in the epithelium, in lymphoid tissue immediately underneath the epithelium, and in the lymph nodes that drain the site (e.g. the mesenteric node). The liver may also contribute to the response, as antigens passing directly into the portal vein are efficiently removed and processed by the hepatic mononuclear phagocyte system (Kupffer cells). Because MALT can function independently of the internal immune system, blood analysis alone may not provide complete information on MALT. Instead, analysis of secretory fluids, such as saliva (for IgA antibody), bronchoalveolar lavage fluid, and jejunal fluid, or direct investigation of the tissue itself, are more appropriate approaches.

1.2.2.7 *Skin immune system or skin-associated lymphoid tissue*

As the skin is the largest organ of the body, its principal physical function is to act as a barrier to water-soluble compounds, to mechanical trauma, and to trauma caused by potentially pathogenic microorganisms and the photons of sunlight. The physicochemical characteristics of the outermost layer, the corneal or horny substance of the epidermis, underlie the resistance to exogenous pathogenic substances. The skin also has a host defence function that can be designated as immunological. Some studies have suggested that the skin might function as a primary organ (Fichtelius et al., 1970; Bos & Kapsenberg, 1986), but most of the relevant immune reactions in the skin appear to be antigen driven. The components of the skin immune system, or skin-associated lymphoid tissue, are the following (Streilein, 1983, 1990) (Figure 17): (i) Langerhans cells in the epidermis, which are adapted for processing antigen and transporting it to the draining lymph node, where they are called interdigitating cells and present the antigen to lymphocytes; (ii) epidermotrophic recirculating T lymphocyte subpopulations (homing T lymphocytes); (iii) keratinocytes, which can synthesize cytokines after activation, thereby influencing T-cell differentiation and haematopoiesis; they can have an antigen-presenting function, especially after activation resulting in MHC class II expression; (iv) Thy-1^{+} dendritic epidermal cells, described in rodent skin epidermis: a special T cell that bears the γ–δ TCR and has an antigen-presenting function; and (v) skin-draining lymph nodes comprising high endothelial venules through which lymphocytes enter from the blood circulation.

Figure 17. Components of the skin immune system



(a) Frozen skin section with hair follicle (H), showing Langerhans cells (arrows) in epidermis (immunolabelling by MHC class II antibody)
(b) Frozen skin section (C57Bl mouse) showing dendritic epidermal cells (arrows; immunolabelling by an antibody to the T-cell receptor Vg3)
Kindly provided by R.A. de Weger, Department of Pathology, University Hospital, Utrecht, Netherlands (immunoperoxidase technique)

Immune components exist not only in the epidermis but also in the dermis. At this location, T cells and macrophages have preferential distributions, especially in the papillary region. T Cells, macrophages, mast cells, endothelial cells, and dendritic cells are found in the connective tissue of the dermis, as in connective tissue at other locations in the body. The reactivity of these cells in the dermis may differ, however, from those at other locations. For instance, skin mast cells (Van Loveren et al., 1990a) behave differently from mast cells at other places. These immunological components cannot always be recognized in conventional histological preparations of skin. For instance, Langerhans cells and dendritic epidermal cells require special immuno-histological staining (Figure 17).

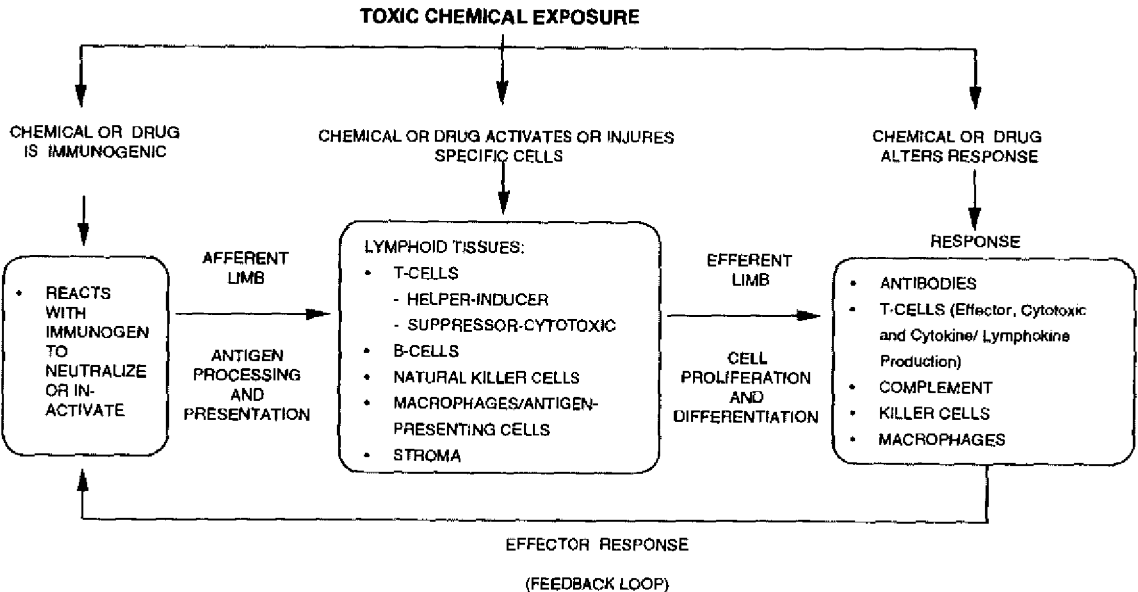
Various inflammatory and immune mediators are also present in skin. These include antimicrobial peptides, complement components, immunoglobulins, cytokines, fibrinolysins, eicosanoids, and neuro-peptides. They are partly derived from the blood circulation and are partly of local origin (Bos, 1990). Streilein (1990) described the function of the skin-associated lymphoid tissue as follows: induction of primary immune responses to new cutaneous antigens, expression of immunity to previously encountered antigens, and avoidance of deleterious immune responses to non-threatening cutaneous antigens.

1.3 Pathophysiology

1.3.1 Susceptibility to toxic action

The dynamic nature of the immune system renders it especially vulnerable to toxic influences. The major target sites of the immune system for toxicity are presented in Figure 18. The reactions of lymphoid cells are associated with gene amplification, transcription, and translation, and compounds that affect these processes of cell proliferation and differentiation are especially immunotoxic, particularly to the rapidly dividing thymocytes and haematopoietic cells of the bone marrow. Thus, the disappearance of lymphoid cells from bone marrow, blood, and tissue, and thymus weight may be the first and most obvious signs of toxicity, as was seen in application of the tiered approach to assessing the immunotoxicity of pesticides, mentioned previously (Vos & Krajnc, 1983; Vos et al., 1983a). Effects on the constituents of the framework (stationary stroma), which support and steer the activation, proliferation, and differentiation of lymphoid cells, are observed less often (Krajnc-Franken et al., 1990; Schuurman et al., 1991b). Such effects result mostly in degeneration, ending in atrophy and fibrosis. Alternatively, framework cells and passenger leukocytes may persist but are rendered unable to function by the toxic insult, and the delicate

Figure 18. Interrelationships of the major components of the immune system and sites of potential toxic effects



For completeness, the figure includes toxicity resulting from an immune response to therapeutic chemicals, although this aspect is not discussed in the text.

interactions between these cells may be affected. This result of toxicity is not always observed by conventional histology; it may be visualized by changes in immunolabelling for markers that have functional significance. Otherwise, functional assessment *in vivo* or *in vitro* is required. As shown in Figure 18, the effects on specific cells in lymphoid tissues are generally reflected in altered histology of lymphoid organs, but this is not always the case for effects on responses.

The skin, respiratory tract, and gastrointestinal tract together form an enormous surface that is in close contact with the outside world and is potentially exposed to a vast magnitude of microbial agents and potential toxicants. The toxic effects on these components of the immune system can differ in (histo)pathological manifestation from those on internal lymphoid organs. In the human respiratory tract, these effects include asthma, fibrosis, and pulmonary infections. Examples of inhaled pollutants that may induce these effects are oxidant gases and particulates such as silica, asbestos, and coal dust. The cellular and biochemical profiles of bronchoalveolar lavage constituents after exposure of experimental animals and humans by inhalation (Koren et al., 1989) are valuable for screening immune-mediated lung injury. The products of pulmonary epithelial cells and alveolar macrophages appear to be key factors. A number of studies have indicated that lung disease progresses with postactivational release of cytokines, such as IL-1, tumour necrosis factor, platelet-derived growth factor, and transforming growth factors. Alveolar macrophages secrete not only cytokines but also a variety of short-lived products that may contribute to altered resistance to pulmonary infections and inflammation; these include reactive oxygen species, such as superoxide, nitric oxide, and hydrogen peroxide, and arachidonic acid metabolites. The overall suppression of these humoral systems, in combination with effects on e.g. NK cells, may predispose individuals to infectious agents or tumour development or may alter the inflammatory and degenerative response (Van Loveren et al., 1990b; Khan & Gupta, 1991; Denis, 1992; Denis et al., 1993).

The skin is an important target in immunotoxicology, for instance for chemical allergens (Kimber & Cumberbatch, 1992) and ultraviolet B (UVB) radiation (Goettsch et al., 1993). The skin can respond to many xenobiotics by a specific immune response (contact hypersensitivity) or by a nonspecific inflammatory response (contact irritancy); both responses are associated with the induction of pro-inflammatory cytokines. The cells of the immune system are readily recruited from the circulation to the skin in response to dermal stimulation by xenobiotics. In addition, various resident immune cells can be activated, for instance Langerhans cells during the induction of the contact hypersensitivity response. Soluble mediators can be produced locally, and antigen–antibody complexes can be formed at the site of

inflammation. Exogenous factors such as ultraviolet light (Applegate et al., 1989) and 7,12-dimethylbenz[*a*]anthracene (DMBA) (Halliday et al., 1988) can cause the disappearance of Langerhans cells from the skin (or the loss of their function), with consequent disturbance or dysregulation of the skin's immune function. Keratinocytes, which comprise the vast majority of cells in the epidermis, have an important role in immune and inflammatory responses, serving as a significant source of cytokines, which contribute either quantitatively or qualitatively to the nature of the response of the skin to exogenous stimulation.

Reactions to drugs illustrate the skin's susceptibility to toxic influences. Cytostatic drugs used in cancer therapy often induce bone-marrow depression as a major side-effect, resulting in an increased risk for infections, so that blood leukocyte counts, which reflect bone-marrow depression, must be monitored during administration. Conversely, a number of cytotostatic drugs are immunosuppressive. A well-known example is azathioprine (see section 2.2.1.1). A number of new immunosuppressive drugs inhibit DNA synthesis, including Mizoribine (Bredinin, an imidazole nucleoside), Brequinar sodium (a quinoline carboxylic acid derivative), and RS61443 (morpholino-ethylester of mycophenolic acid) (Thomson, 1992). Their specificity to cells of the immune system may be related to the distinct pathways in purine and pyrimidine metabolism that are preferentially used by lymphocytes, such as guanine nucleotide synthesis promoted by inosine monophosphate dehydrogenase in the case of Mizoribine and RS61443. Another example of the particular sensitivity of the immune system to toxic damage is its response to irradiation. Ionizing radiation is commonly used in cancer therapy. Of the body's constituents, the haematopoietic system is particularly sensitive to irradiation; when pluripotent stem cells are affected, regenerative activity is lost. Other systems destroyed by this treatment, like the intestinal epithelium, have an intrinsic self-renewal capacity and do not need replacement therapy. The lymphoid constituents of the immune system differ in radiosensitivity. The dose of radiation that causes a reduction in the cell population by a factor of 1/e (or 0.37), the D_0, has been estimated at about 0.9 Gy for bone-marrow lymphoid stem cells, 0.4–0.9 Gy for pre-B cells, and 0.7 Gy for peripheral lymphocytes (Anderson & Williams, 1977; Anderson et al., 1977). The thymus is very sensitive to irradiation (Sharp & Watkins, 1981; Anderson et al., 1986; Adkins et al., 1988). Cortical lymphocytes manifest a D_0 value of about 0.6 Gy. The intrathymic T-cell precursor has a D_0 value of about 1.4 Gy. A refractory population (about 20–30%) is present within the medulla, but most cells are radiosensitive (D_0, about 0.7 Gy). The capacity of thymic stroma to support precursor T-cell processing is radioresistant (Huiskamp et al.,

1988). In addition to cell depletion, peripheral lymphoid tissues manifest decreased lymphocyte recirculation at a dose of only 0.5 Gy (Anderson et al., 1977), suggesting that high endothelial venules, or cell surface molecules involved in lymphocyte migration through these venules, are radiosensitive. The sensitivity of lymphocytes to ionizing radiation cannot be ascribed solely to their susceptibility to death during proliferation. Cells in the resting state disappear after irradiation, at a time that does not correspond to their physiological half-life. Apparently, the death of lymphocytes occurs at phases between cell division. This phenomenon, known as interphase death, appears to be a distinct characteristic of lymphocytes and sensitizes the immune system to radiation. Thus, only peripheral blood lymphocytes need be assessed as dosimeters after accidental irradiation (such as at Chernobyl in 1986).

Toxicity to the thymus: Of the lymphoid cells of the body, the lymphocytes of the thymus (thymocytes) are especially susceptible to the action of toxic compounds (Schuurman et al., 1992d). Table 4 presents data on the susceptibility of various thymic components to toxic damage, illustrating the particular vulnerability of the passenger lymphocyte population. The microenvironment appears to be more resistant, mainly on the basis of histopathological assessment. Thymocyte depletion, suggestive of toxicity towards this population, may actually be an indirect effect, in that the microenvironment is damaged and unable to support thymocyte growth. This situation may exist in the thymus after exposure to 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD), which preferentially attacks the thymic reticular epithelium, resulting in lymphocyte depletion histologically (see section 2.2.2.1). Similarly, the reduction in the cellularity of the medulla of the thymus after treatment with cyclosporin A is due to the absence of the medullary lymphocyte population, but the basis of the reduction is the disappearance of dendritic cells (as seen by immunohistochemistry; see section 2.2.1.2).

The susceptibility of thymocytes to toxicity is also related to their fragile composition, especially cortical thymocytes, and to the delicate interactions between them and their microenvironment. For instance, they are programmed to enter apoptosis when activated during the physiological process of selection. A decrease in size or involution of the organ may thus be the first manifestation of toxicity. It should be noted that stress itself can induce thymic involution; furthermore, thymic status is dependent on nutritional status and age. The main function of the thymus is to generate the T-cell repertoire during fetal and early postnatal life. Its susceptibility to toxic compounds and the subsequent effects on the cell-mediated immune system are most evident during this period of life.

Table 4. Sensitivity of cell populations in the thymus to toxic chemicals

Cells	Location	Compound
Lymphocytes		
Immature lymphoblasts	Outer cortex	Some organotin compounds, 2,3,7,8-tetrachlorodibenzo-*para*-dioxin
Small cells	Cortex	Glucocorticosteroids, cytostatic drugs (e.g. azathioprine)
Intermediate-sized cells	Medulla	Ammonia caramel (THI)
Epithelial cells	Cortex	2,3,7,8-Tetrachlorodibenzo-*para*-dioxin
	Medulla	Cyclosporin, FK-506
Dendritic cells	Medulla	Cyclosporin, FK-506
Macrophages	Cortex	Ammonia caramel (THI)

THI, 2-acetyl-4(5)-tetrahydroxybutylimidazole; caramel colour III

An ever-increasing number of toxic compounds has been shown to affect the immune system. Induced and spontaneous immuno-pathology in rodents have been described in reviews and textbooks (Dean et al., 1982, 1985; Irons, 1985; Descotes, 1986; Lebish et al., 1986; Gopinath et al., 1987; Luster et al., 1989; Vos & Luster, 1989; Jones et al., 1990; Krajnc-Franken et al., 1990; Vos & Krajnc-Franken, 1990; Schuurman et al., 1991b; Dean et al., 1994; Frith et al., in press). The weight and histology of lymphoid organs are the main parameters in evaluating toxicity. The examples given in section 2.2 illustrate the various ways in which the immune system is injured.

1.3.2 *Regeneration*

The dynamic nature of the immune system provides it with a strong regenerative capacity. In principle, the leukocyte population is generated by a single pluripotent progenitor cell in the bone marrow. In the thymus, single thymocyte precursors have an enormous capacity for expansion (Ezine et al., 1984). Thus, after exposure to xenobiotic compounds that destroy the immune system, regeneration can occur within a relatively short time: The original architecture is restored within three to four weeks after involution caused by radiation or

treatment with glucocorticosteroid or organotin compounds. The restoration process can occur in waves, depending on the sensitivity of the precursor T cells in the thymus and bone marrow (Huiskamp et al., 1983; Penit & Ezine, 1989). Regeneration does not occur after destruction of the white blood cell system, e.g. by sublethal irradiation, when the stem cells in the bone marrow are affected. In such cases, bone-marrow transplantation is required, and the anlage of the lymphoid organs then supports generation of the newly built immune system. After bone-marrow transplantation, polymorphonuclear granulocytes are the first cells to appear in the circulation, within two to three weeks; lymphocytes appear after three to four weeks; however, establishment of a fully developed T-cell system takes about six months (De Gast et al., 1985). These examples illustrate the vulnerability of the bone marrow-derived component to toxic action; regeneration requires substitution of this component and not the relatively more resistant stromal component in lymphoid tissue.

1.3.3 *Changes in lymphoid organs*

The weight and gross morphology of lymphoid organs are the first parameters studied in assessing toxicity, as the response to injury is often expressed as changes in tissue or organ weight, size, colour, and gross appearance. These observations are combined with leukocyte counts, studies of differentiation, and the results of histopathological evaluations of lymphoid organs and tissues. Conventional histopathology allows evaluation of the effects of xenobiotics on the main cell subsets on the basis of their distinct morphology, tissue location, and density. In this way, the effects on lymphocytes, lymphoblasts, and stromal cells can be evaluated (Schuurman et al., 1994; Kuper et al., 1995). The disappearance of lymphocytes from blood and tissues and the decrease in size and weight are often first seen in the thymus, as this organ is very sensitive to toxicity (see above). Evaluations of effects on distinct subpopulations must be confirmed by immunohistochemical characterization (Schuurman et al., 1992a). The sensitivity of histopathology can be increased by quantitative microscopy and morphometry and organ cell counts.

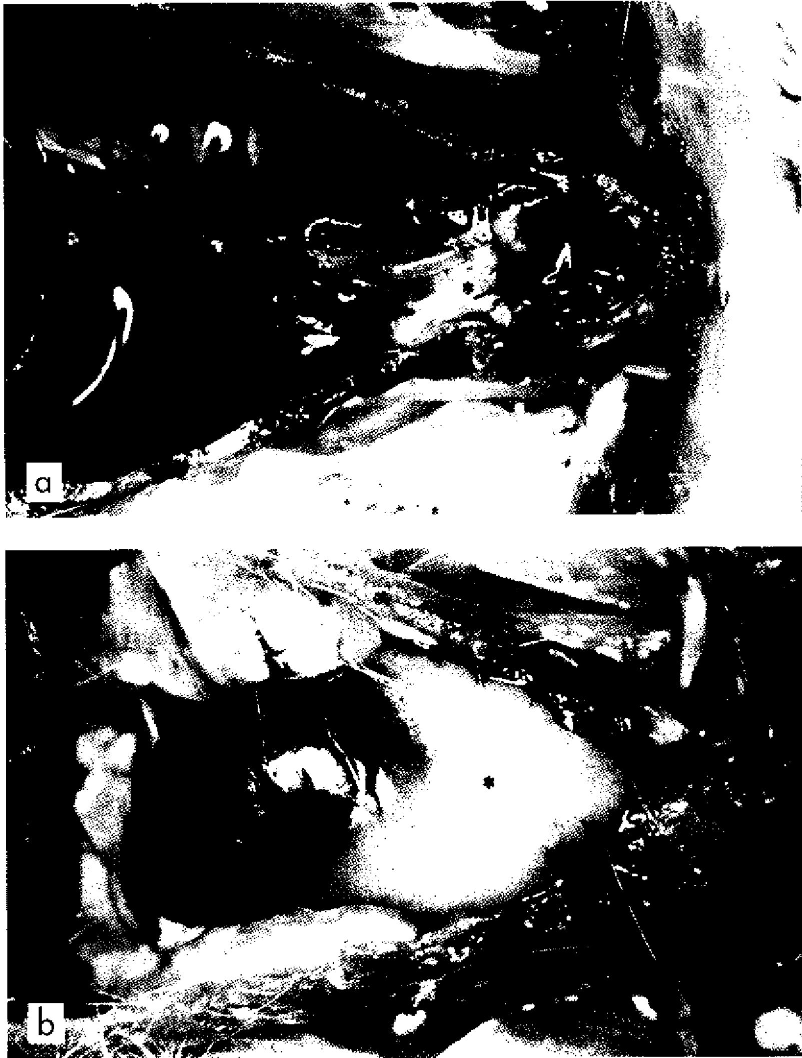
During microscopic examination, important aspects to be considered are the cell density and the size of the various compartments of the lymphoid organs, as well as qualitative changes like germinal centre development; however, microscopic examination of lymphoid organs reveals these highly dynamic and complex processes only at a static stage. Changes in the number of cells are best reported by descriptive terms like 'increases' or 'reductions' in cellularity rather than by interpretive terms like 'involution', 'atrophy', or 'hyperplasia'.

The pathology working group of the IPCS–European Union international collaborative immunotoxicity study has started to develop such a descriptive approach. A window of control values and gross and microscopic appearance must be established for recognition of deviations from normal; however, normal and control values and histological features are influenced by various endogenous and exogenous factors, including the age and hormonal (especially sex hormone) status of the animal (Kammüller et al., 1989; Goonewardene & Murasko, 1992; Kuper et al., 1992a; Losco, 1992; Losco & Harleman, 1992). The influence of sex hormonal status on the histology and histophysiology of lymphoid organs is illustrated by the following examples. Considerable changes are seen in the lymphoid organs of mice during pregnancy (Clarke, 1984), in birds during hatching (see Section 2), and in ectothermic vertebrates during different seasons of the year (see section 1.2.1.5). Castration results in an increase in thymic size in old animals (Kendall et al., 1990), as has been observed in old rats with an involuted thymus after treatment with an analogue of luteinizing hormone-releasing hormone (Greenstein et al., 1987). Figure 19 shows the wet thymic weight of groups of male rats in order to illustrate these phenomena: the mean values were about 500 mg in young male rats at 2.5 months of age, about 125 mg in rats at 12–15 months of age, about 250 mg in aged rats after surgical castration, and about 300 mg in aged rats after treatment with the hormone (Kendall et al., 1990).

The neuroendocrine system and the sex steroid hormone balance may also underlie the changes in lymphoid organs of ectothermic vertebrates with seasonal changes like temperature and photoperiod (mentioned above). The thymic status is also dependent on nutritional status (Van Logten et al., 1981; Corman, 1985; Mittal et al., 1988; Good & Lorenz, 1992), and stressful conditions influence the appearance of lymphoid organs, especially the thymus. Finally, housing conditions, diet, and microbiological status are important. For instance, the presence of γ–δ T cells in the rodent intestinal epithelium is dependent on the presence or absence of microbiological stimulation (that is, whether the animals are bred and maintained under specified pathogen-free conditions) (Bandeira et al., 1990; Dobber et al., 1992; Umesaki et al., 1993). The role of genetic factors is reflected in strain differences in lymphoid organ histology, e.g. in rats (Losco & Harleman, 1992; Schuurman et al., 1992e), seen on examination of data on concurrent and historical controls.

Genetic factors may also contribute to the individual variability in response to a given compound. This variability can be relatively high, even among random-bred and inbred rats, as illustrated in the following example (Figure 20), derived from a study of the toxicity of the

Figure 19. Thymus from male rats, 12–15 months of age

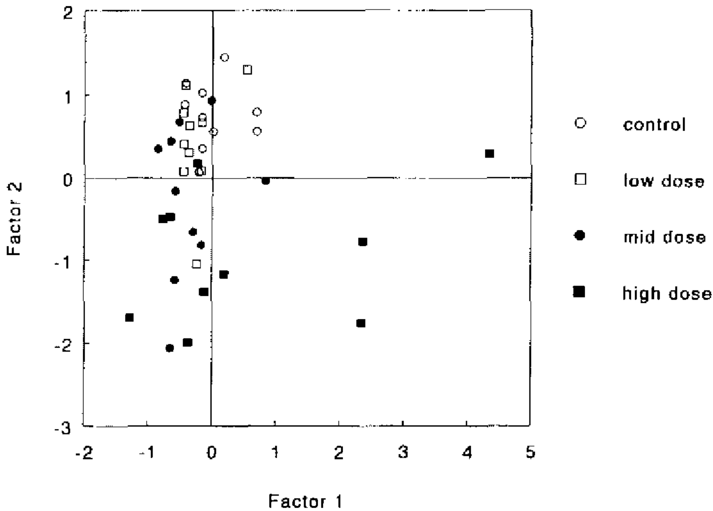


Reproduced from Kendall et al. (1990)

(a) Control thymus (asterisk)

(b) Thymus (asterisk) after reversible chemical castration with luteinizing hormone releasing hormone, showing enlargement of the organ

Figure 20. An example of individual sensitivity to a xenobiotic: effects of the immunosuppressive drug azathioprine on haematological and histopathological parameters in random-bred Wistar rats



Data for animals treated with azathioprine at 0.5 (low dose), 12.5 (mid dose), or 25 (high dose) mg/kg body weight per day are plotted with respect to the two most important clusters of parameters ('factors'). The ordinates represent the computed individual scores for each factor. Factor 1 includes the parameters 'bone-marrow cellularity', assessed from bone-marrow sections, 'spleen cellularity', from splenic sections, 'thymic cellularity, from thymic sections, and 'spleen weight'; factor 2 comprises 'bone-marrow cell counts', 'body weight', and 'thymic weight'. The values for untreated control animals and animals that did not respond to the drug (mainly animals in the low-dose group) appear in the upper left-hand corner. The data for three of 10 animals in the high-dose group, on the right, indicate that they were high responders to azathioprine, especially with respect to factor 1; one animal in this group (arrowhead) was untreated and was considered to be a nonresponder; all of the other six animals in this group were considered to be low to medium responders.

immunosuppressive drug azathioprine in random-bred Wistar rats. The data on haematological and histopathological parameters were subjected to factor analysis in order to facilitate examination of individual and group responses to the drug. Factor analysis involves clustering of parameters into composite groups, or 'factors', by arithmetic manipulation of the data, so that the parameters within a cluster are

related mathematically. It is up to the investigator to decide whether the clustering is meaningful biologically. Figure 20 shows that not all of the 10 animals that received the highest dose of azathioprine had the same response: three were considered to be high responders, six low or medium responders, and one a non-responder. The high individual variability in response illustrates the significance of outliers in studies with a limited number of animals.

The relevance of changes observed in a study of exposure *in vivo* must be based on knowledge of the nature of the response. Current understanding of the relationship between the structure and function of the lymphoid organs and their components often allows only a provisional hypothesis to be made about the mechanisms of toxicity. Moreover, different mechanisms of tissue injury can yield similar histopathological features. Therefore, in-depth, specific histological examination of tissue response and immune function are indispensable for interpreting changes and for risk assessment. In interpreting quantitative changes, like changes in cellularity, it should be noted that particular components of the immune system may be decreased in number or size (suppressed or involuted) or increased (stimulated or expanded), but this does not necessarily reflect the overall effect on the immune system or lymphoid organs.

The presence of tissue damage, protein complex deposits, and inflammatory cell infiltrates may indicate the induction of autoimmunity or the presence of allergy or hypersensitivity. The site at which such responses are seen is often not a lymphoid organ, but blood vessels, renal glomeruli, synovial membranes, thyroid, skin, liver, or lung, which are well-known sites of autoimmunity and hypersensitivity. Non-lymphoid organs should also be examined in order to determine whether the effects on the lymphoid system are secondary, for instance mediated by acute stress. This aspect is not considered in detail in this monograph, which concerns mainly the direct toxic action of xenobiotics on the immune system.

2. HEALTH IMPACT OF SELECTED IMMUNOTOXIC AGENTS

2.1 Description of consequences on human health

2.1.1 Consequences of immunosuppression

Since immunosuppressive drugs were introduced clinically to prevent allograft rejection more than 25 years ago, the human consequences of immunosuppression are well known. Infections and cancers are the main consequences of immunosuppressive therapy, as exemplified by a number of isolated case reports and epidemiological studies (IARC, 1987; Descotes, 1990; IARC, 1990). The cancers are often lymphomas and carcinomas, which are likely to be of viral origin, especially in immunosuppressed patients. Recurrent respiratory viral infections should also be considered as sentinel conditions for immunotoxicity, both in individuals and in community-based epidemics, including, but not confined to, opportunistic infections. Additional evidence that immunosuppression can enhance the risk of cancer is the increased incidence of an atypical form of Kaposi's sarcoma and of lymphomas frequently located in the brains of patients with AIDS. It is important to distinguish between profound immunodepression (mainly seen clinically, e.g. after renal transplantation or cytotoxic therapy for neoplasia) and the less severe suppression of immune function that is more likely to be associated with exposure to an environmental immunotoxic agent.

2.1.1.1 Cancer

Evidence from three sources, namely cancer patients on chemotherapy, organ transplant patients, and patients with autoimmune disorders undergoing long-term immunosuppressive therapy, demonstrates that immunosuppressed patients are at a higher risk than others of developing malignancies (Boyle et al., 1984; Penn, 1988; IARC, 1990; Barrett et al., 1993; Bouwes Bavinck et al., 1993; Penn, 1993a,b; Descotes & Vial, 1994), although not all immunosuppressive drugs have been shown to be carcinogenic, e.g. prednisone and methotrexate (IARC, 1987). Immunotoxic effects might result in tumour formation through reduced immune surveillance, i.e. tumours might escape the guard of the immune system. Reduced immune surveillance can thus be regarded as tumour promotion.

The risk for second malignancies after prolonged cancer chemotherapy has been shown in numerous case reports and epidemiological studies (Henne & Schmähl, 1985; Boivin, 1990; Blatt et

al., 1992). Acute leukaemia is the most frequently reported second cancer (Kyle, 1984); overall, iatrogenic leukaemias account for 10% of all leukaemias, with an incidence 5–100 times higher than in the general population. Non-Hodgkin's lymphoma develops in 0.5–4.5% of patients within 10 years after cytotoxic therapy. The risk for solid tumours, e.g. carcinomas of the lung, skin, breast, colon, and pancreas, is also increased after cytotoxic therapy but with a different trend: the increase in risk is more prolonged and slower (Swerdlow et al., 1992).

Although most cytotoxic drugs are genotoxic, their immunosuppressive effects may also account for the increased risk of second cancers, as indicated by results obtained in organ transplant recipients. The Cincinnati Transplant Tumour Registry collected data on more than 3600 cases of cancer in transplant patients up to June 1988 (Penn, 1988). Large numbers of cancers were also included in the Australian and New Zealand Combined Dialysis and Transplant Registry (Sheil et al., 1991). Overall, 1–15% of organ transplant patients developed cancer within the first five years after transplantation; whatever the therapeutic regimen, the incidence of cancer was at least three times that of the general population and increased logarithmically with the length of follow-up to reach more than 50% after 20 years in some series. Cancers of the skin and lips were reported in 18% of patients after 10 years of immunosuppressive therapy. Squamous-cell carcinoma was the most frequent skin cancer and was about 250 times more frequent in transplant patients than in the general population (Hartevelt et al., 1990). Lymphomas accounted for 14–18% of neoplasms in transplant patients, and high-grade non-Hodgkin's lymphomas accounted for 95% of these lymphomas. The incidence of Kaposi's sarcoma was 400–500 times more frequent in transplant patients than in the general population.

A similar pattern of neoplasias was observed in patients on immunosuppressive therapy for autoimmune diseases (Sela & Shoenfeld, 1988). Non-Hodgkin's lymphomas were 11 times more frequent than in the general population, and other cancers, namely leukaemias, primary liver cancers, and squamous-cell carcinomas, were also found to be more frequent (Penn, 1988; Descotes & Vial, 1994). The respective roles of immunosuppressive therapy and of the underlying disease remain to be established, however.

Cancers have been reported to occur after immunosuppressive therapy with both cytotoxic and noncytotoxic drugs. Even though the respective roles of genotoxicity and immunosuppression are difficult to ascertain, cancers have been described in patients on azathioprine after organ transplantation (Wessel et al., 1988; Singh et al., 1989) or on low-dose methotrexate for autoimmune disorders (Ellman et al., 1991; Shiroky et al., 1991; Kingsmore et al., 1992; Kamel et al., 1993);

immunosuppressive drugs increased the risk of malignancies (especially lymphomas) in the treated patients. Interestingly, all of the noncytotoxic immunosuppressive drugs were reportedly associated with a variety of malignancies presumably related to immunosuppression. Whatever the type of tumour, the time to tumour development after treatment with cyclosporin A was shorter (26 months on average; 14 months for lymphomas) than after conventional immunosuppressive therapy (60 months on average) (Penn, 1988). The murine monoclonal antibody OKT3 was also shown to increase the risk for lymphoproliferative disorders (Swinnen et al., 1990): Lymphomas developed 1–18 months after starting OKT3, and a correlation was found between the dose and time to neoplasm development. Lymphoproliferative disorders were also shown to occur following treatment with FK 506 (Reyes et al., 1991). There are insufficient data to conclude a direct causal relationship between immunosuppression induced by environmental chemicals and the development of cancer; however, there is epidemiological evidence that exposure to various potentially immunotoxic chemicals (e.g. pesticides, benzene) is associated with increased risks for cancers that also occur in immunosuppressed patients (e.g. non-Hodgkin's lymphoma and leukaemia).

No marked difference was found in the relative risks for lymphoproliferative disorders associated with the various immunosuppressive drugs currently used, suggesting that immunosuppression is the causative factor, particularly when account is taken of the different carcinogenic potentials of azathioprine and cyclosporin A, both clinical reference immunosuppressive agents (Ryffel, 1992). Reactivation of latent viruses, e.g. Epstein-Barr virus, due to immunosuppression was suggested to be involved. Indeed, most lymphoproliferative disorders induced with cyclosporin A or methotrexate were B-cell malignant lymphomas associated with this viral infection (Starzl et al., 1984; Kamel et al., 1993). Uninhibited proliferation of Epstein-Barr virus in B lymphocytes caused by the efficient immunosuppression of one or more kinds of controls by T lymphocytes is the commonly accepted mechanism. Interestingly, Epstein-Barr virus-associated lymphomas in patients on immunosuppressive therapy are usually reversible upon cessation of treatment (Starzl et al., 1984).

2.1.1.2 *Infectious diseases*

Whatever the primary cause of the immune deficiency, patients develop more frequent, more severe, recurring, and often atypical infections, depending on the type and severity of the deficiency. The complications associated with severe immunosuppression include

bacterial, viral, fungal, and parasitic infections (Waldman, 1988; Barr et al., 1989; Mandell, 1990; Tieben et al., 1994). The pathogens most frequently encountered in immunodeficient patients include the bacterial agents *Staphylococcus aureus, Streptococci, Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes, Mycobacterium tuberculosis,* and atypical mycobacteria. Herpes virus, cytomegalovirus, Epstein-Barr virus, and human papillomavirus are the leading causes of viral infections in immunosuppressed patients. Fungal opportunistic infections include those induced by *Candida, Aspergillus,* and *Cryptococcus* species. The immunotoxicity induced by environmental chemicals often results in subtle changes in the immune system, which have been suggested to result in increased incidences of common infections like influenza and the common cold (see section 2.4).

The respiratory tract is a primary target for infectious pathogens, especially in immunosuppressed patients. Pulmonary infections and infections of the upper respiratory tract are the most common (Frattini & Trulock, 1993). Cytomegalovirus infections, often asymptomatic, are particularly frequent in renal transplant patients (Rubin, 1990). In addition, *Pneumocystis carinii* can cause a particular form of pneumonia in immunosuppressed patients. Even though respiratory diseases usually predominate, gastrointestinal infectious diseases may constitute the leading consequences of immunosuppression (Bodey et al., 1986). Infections of the central nervous system and isolated fever are also extremely frequent. Interestingly, the type of infection that develops in immunosuppressed patients is largely dependent on the type of immune defect, as illustrated in Table 5.

Table 5. Pathogens frequently associated with immune defects

Humoral immunity	Cellular immunity	Neutrophil functions	Complement system
Campylobacter	*Candida*	*Aspergillus*	*Neisseria*
Echovirus	*Coccidioides*	Bacteroides	Staphylococci
Gardia	Cryptococci	*Escherichia coli*	Streptococci
Haemophilus	Cytomegalovirus	*Klebsiella*	
Pneumococci	Herpes virus	*Pseudomonas*	
	Salmonella		
	Legionella		
	Mycobacteria		
	Staphylococci		
	Histoplasma		
	Toxoplasma		
	Pneumocystis		
	Human papillomavirus		

Infectious complications have been commonly described in patients treated with various cytotoxic drugs for cancer and with immunosuppressants, such as cyclosporin A, for the prevention of allograft rejection or the treatment of autoimmune disorders (Kim, 1989; Descotes & Vial, 1994).

2.1.2 *Consequences of immunostimulation*

The health consequences of immunostimulation are less well established than those associated with immunosuppression. A number of adverse effects have, however, been reported after treatment with immunostimulating drugs, including influenza-like reactions, facilitation or exacerbation of underlying diseases, and inhibition of hepatic drug metabolism (Descotes, 1992).

Patients with influenza-like reactions present with mild to moderate fever associated with chills, malaise, and hypotension. The reaction usually develops within hours after taking an immunostimulating drug, and the patient recovers uneventfully within a few hours. Such reactions are relatively uncommon with most immunomodulating agents but have been shown to limit treatment with several recombinant cytokines, e.g. IL-1 and tumour necrosis factor.

Facilitation and/or exacerbation of underlying diseases have been ascribed to most immunostimulating drugs, but the incidence of this adverse event differs markedly from one drug to another. Exacerbation of chronic infections, psoriasis, and Crohn's disease have been reported. More interestingly, several autoimmune diseases are more frequent in patients treated with various (recombinant) cytokines, e.g. autoimmunity treated with IFNγ (Jacob et al., 1987), thyroiditis with IL-2 (Vial & Descotes, 1993), and lupus erythematosus with IFNα (Vial & Descotes, 1993). Exacerbation or facilitation of allergic reactions to unrelated allergens has also been reported: starting an immunostimulating treatment has been associated with exacerbation of underlying eczema, asthma, or rhinitis. Allergic reactions to radiological contrast media have been shown to be more frequent in IL-2-treated patients than controls (Vial & Descotes, 1992).

Oxidative drug metabolism by the hepatic cytochrome P450 system has been shown to be inhibited by immunostimulating drugs (Descotes, 1985) and by administration of bacillus Calmette-Guérin (BCG) vaccine or interferon (Vial & Descotes, 1993). Although the mechanism of this inhibition is still unknown, activation of macrophages resulting in the release of IL-1 and IL-6 has been suggested to be involved. Likewise, vaccination has been shown to compromise drug metabolism to a sufficient extent that normally therapeutic doses of theophylline caused acute toxicity in humans (Renton et al., 1980). Stimulation of the

immune system has also been shown to alter drug metabolism in humans (Renton, 1986). A similar effect of infection has been reported in laboratory animals (Selgrade et al., 1984); infection with mouse cytomegalovirus before exposure to the insecticide parathion reduced the total P450 concentration and dramatically increased the toxicity of parathion.

So far, only a few environmental chemicals have been shown to exert immunostimulating properties, e.g. hexachlorobenzene and selenium. There have been no reports of clinical reactions to such chemicals that are similar to the adverse effects seen with immunostimulating drugs.

2.2 Direct immunotoxicity in laboratory animals

The following are some illustrative examples of immunotoxic chemicals.

2.2.1 *Azathioprine and cyclosporin A*

The immunosuppressive effects of azathioprine and cyclosporin A are considered because they can shed light on the direct immunotoxicity of environmental chemicals.

2.2.1.1 *Azathioprine*

Azathioprine is a thiopurine that is used as cytostatic drug in the treatment of leukaemias and as an immunosuppressant in patients who have received allogeneic organ transplants or who have autoimmune diseases. When used as an immunosuppressant, its main side-effect is bone-marrow depression, reflected in blood leukocytopenia; its administration must therefore be monitored through blood leukocyte counts. Another side-effect, especially after long-term administration, is tumour formation (IARC, 1987).

In rats, azathioprine is cytotoxic for all cell lineages in the bone marrow, and strong cellular depletion is observed histologically. It decreases the cellularity in thymus, blood, and peripheral lymphoid organs, but it is mainly in the thymus that the immature lymphocyte population of the cortex is affected. This effect is a general feature of most cytostatic drugs. A similar effect is seen in the thymus after treatment with glucocorticosteroids, but the molecular mechanism resulting in lymphocyte depletion is obviously different: interference with DNA synthesis resulting in lymphocyte proliferation in contrast to binding to glucocorticosteroid receptors and cell down-modulation. Azathioprine affects a number of indicators of immune function, like macrophage cytotoxicity (Spreafico et al., 1987), lymphocyte prolifera-

tion *in vitro* after mitogen stimulation (Weissgarten et al., 1989) and in the mixed leukocyte reaction (Mellert et al., 1989), and cytotoxicity by NK cells (Pedersen & Beyer, 1986; Spreafico et al., 1987; Versluis et al., 1989). Both stimulation and suppression of these functions have been found in experimental animals, depending on the dosage and the time of testing after exposure. These findings are in accordance with those in azathioprine-treated patients, who showed no change in primary antibody response, a decrease in secondary antibody response, and some or no effect on lymphocyte proliferation *in vitro* after mitogen stimulation. The time of testing after the start of exposure to azathioprine was a crucial factor in the detection of effects. Azathioprine was tested in the IPCS–European Union international collaborative immunotoxicity study (see section 1.1) and showed a significant strain-dependent sensitivity.

2.2.1.2 *Cyclosporin A*

Cyclosporin A is one of the most powerful immunosuppressive drugs (Kahan, 1989). It is a neutral lipophilic cyclic peptide consisting of 11 amino acids (relative molecular mass, 1203 Da) isolated from the fungus *Tolypocladium inflatum*. Its main use is in bone-marrow transplantation to prevent transplant rejection and graft-versus-host reactions. It is also used in the therapy of various autoimmune diseases.

A complication of cyclosporin A treatment is nephrotoxicity. Another side-effect, especially after long-term administration, is tumour formation (IARC, 1987). In its immunosuppressive action, cyclosporin A does not affect resting lymphocytes but blocks the events occurring after stimulation, particularly the synthesis of lymphokines, including IL-1 and IL-2, and IL-2 receptors. The synthesis of IL-1 by antigen-presenting cells and of IL-2 by Th cells is inhibited, and the synthesis of IFNγ and tumour necrosis factor is blocked. These events occur inside the cell at the transcriptional level. Cyclosporin A binds to an intracellular receptor, cyclophilin, forming a complex with calcineurin; this complex in turn interferes with the activation of genes, resulting in inhibition of lymphokine gene transcription (Baumann et al., 1992; Sigal & Dumont, 1992).

An interesting feature of cyclosporin A is its specific action on the thymus and the induction of autoimmune phenomena. Rats treated with total body irradiation and syngeneic or autologous bone-marrow transplantation, followed by treatment with cyclosporin A at a dose of about 10 mg/kg body weight per day subcutaneously for four weeks, developed signs of acute graft-versus-host reactions, with lymphocytic infiltration at multiple epithelial sites (Glazier et al., 1983). A similar pseudo-graft-versus-host reaction has also been evoked in mice. It is

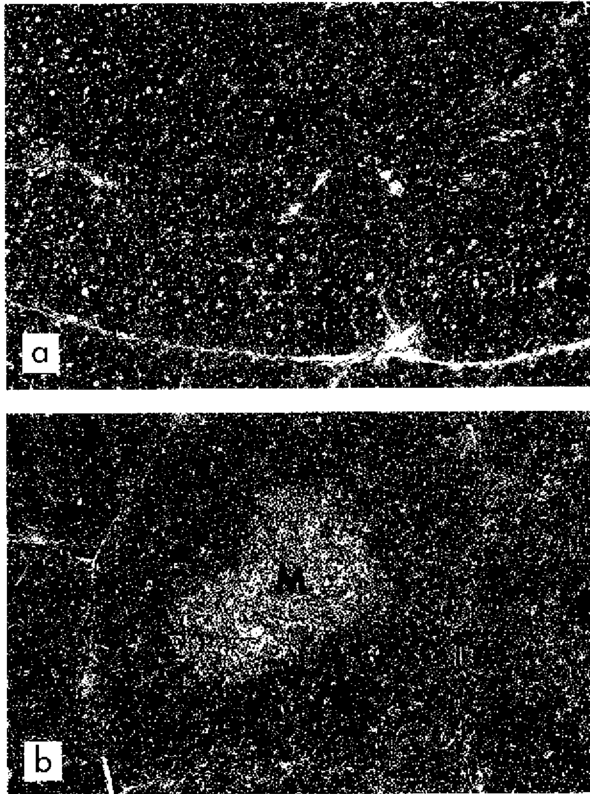
associated with thymic changes, because it can be transferred in whole thymus or thymocytes (Sakaguchi & Sakaguchi, 1988). Histologically, the medullary area is diminished (Beschorner et al., 1987a; Schuurman et al., 1990; see also Figure 21). The medullary stroma shows a decrease in MHC class II expression, indicating a loss of dendritic cells, which has been confirmed by electron microscopy (De Waal et al., 1992a). As these cells normally contribute to the negative selection process, their depletion (or reduced MHC class II expression) may be related to an absence of negative selection. The autoreactive T cells may even attack the medullary epithelium.

The effect of cyclosporin A on thymic functions, i.e. the induction of 'leakiness', with export of T cells that have not been negatively selected, has not yet been studied for other drugs, but may not be specific to cyclosporin A. It represents a distinct mechanism of autoimmunity induced by the action of toxic compounds on the immune system, mediated via thymic selection. Although the medullary area is reduced in young rats after treatment with cyclosporin A, this is not the case in one-year-old rats, which presumably have a lesser output of mature T cells because of thymic involution (Beschorner et al., 1987b).

The effect of cyclosporin A in inducing syngeneic graft-versus-host disease in rodents has an application in clinical medicine: Patients treated for cancer with high-dose chemotherapy and/or total body irradiation, followed by autologous bone-marrow transplantation, develop a recurrence of the original tumour at a higher incidence than patients who receive an allogeneic bone-marrow transplant. This difference has been ascribed to the addition of a graft-versus-tumour effect to the graft-versus-host reaction. Trials have now been initiated to induce a graft-versus-host reaction with cyclosporin A treatment after autologous bone-marrow transplantation, in order to reduce tumour recurrence. The initial results are promising (Hess et al., 1992; Yeager et al., 1993; Kennedy et al., 1994).

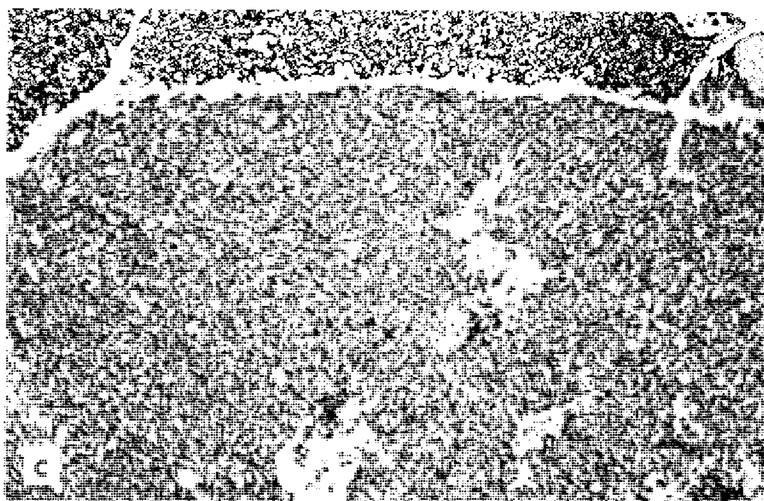
Interestingly, two other immunosuppressive drugs, FK-506 and rapamycin, which also interfere with gene activation in T lymphocytes, do not bind to cyclophilin but to another intracellular receptor, the FK-binding protein. The effect of FK-506 on the thymus is similar to that of cyclosporin A, i.e. a decrease in the medulla (Pugh-Humphreys et al., 1990), and rapamycin causes severe acute involution with disappearance of lymphocytes from the cortex (Zheng et al., 1991). These findings indicate that the two compounds have different molecular mechanisms of action on the thymus from those of cyclosporin A, which have not yet been elucidated.

Figure 21. Effects of cyclosporin A on the histological appearance and immunohistochemistry of rat thymus



Histological appearance (a and b), haematoxylin and eosin stain; immunohistochemistry (c and d), antibody for RT1 class II that labels epithelium in the cortex (C) in a dendritic pattern and interdigitating cells in the medulla (M) in a more confluent pattern, whereas the epithelium in the medulla is not stained
Rat thymus 14 days after daily administration of cyclosporin A (a and c) and after a subsequent recovery period of four weeks (b and d)
(a) The medullary area has disappeared, as judged by the uniform presence of the small lymphocytes that normally occur in the cortex. The original boundary of the cortex and medulla can still be discerned (demarcation line) on the basis of the structure of the vasculature.
(b) The thymus has recovered its normal histological appearance, with cortex–medulla demarcation.

Figure 21 (contd)



(c) Most of the section is filled with cortical-type epithelium in an adendritic staining pattern, due to the collapse of the medullary microenvironment. A few RT1 class II-negative areas are seen, with some RT1 class II-positive interdigitating cells (arrows).
(d) Recovery, with a clear cortex–medulla demarcation

2.2.2 *Halogenated hydrocarbons*

2.2.2.1 *2,3,7,8-Tetrachlorodibenzo*-para-*dioxin*

The halogenated hydrocarbon most closely studied for its immunotoxic effects is TCDD. It has a variety of toxic effects, with a remarkable interspecies variation; however, it causes atrophy of the thymus and immunotoxicity in all species investigated (Vos & Luster, 1989; Holsapple et al., 1991; Neubert, 1992; Kerkvliet & Burleson, 1994). Atrophy of the thymus is reflected histologically by lymphocyte depletion of the cortex (Figure 22). Functionally, cell-mediated immunity appears to be suppressed in a dose-dependent fashion, as manifested in delayed-type hypersensitivity responses, rejection of allogeneic skin transplants, graft-versus-host reactivity, and lymphocyte proliferation *in vitro* after mitogen stimulation. This immune suppression is age-related: more severe immunotoxic effects are observed after perinatal administration than after administration in adulthood (Vos & Moore, 1974; Thomas & Hinsdill, 1979). TCDD can also impair antibody-mediated immunity after primary or secondary immunization. A sensitive parameter of the immunotoxicity of TCDD and TCDD congeners in mice is suppression of the T cell-dependent antibody response to sheep red blood cells in mice (Vecchi et al., 1980; Davis & Safe, 1988; Kerkvliet et al., 1990). No effects have been observed on classical macrophage functions.

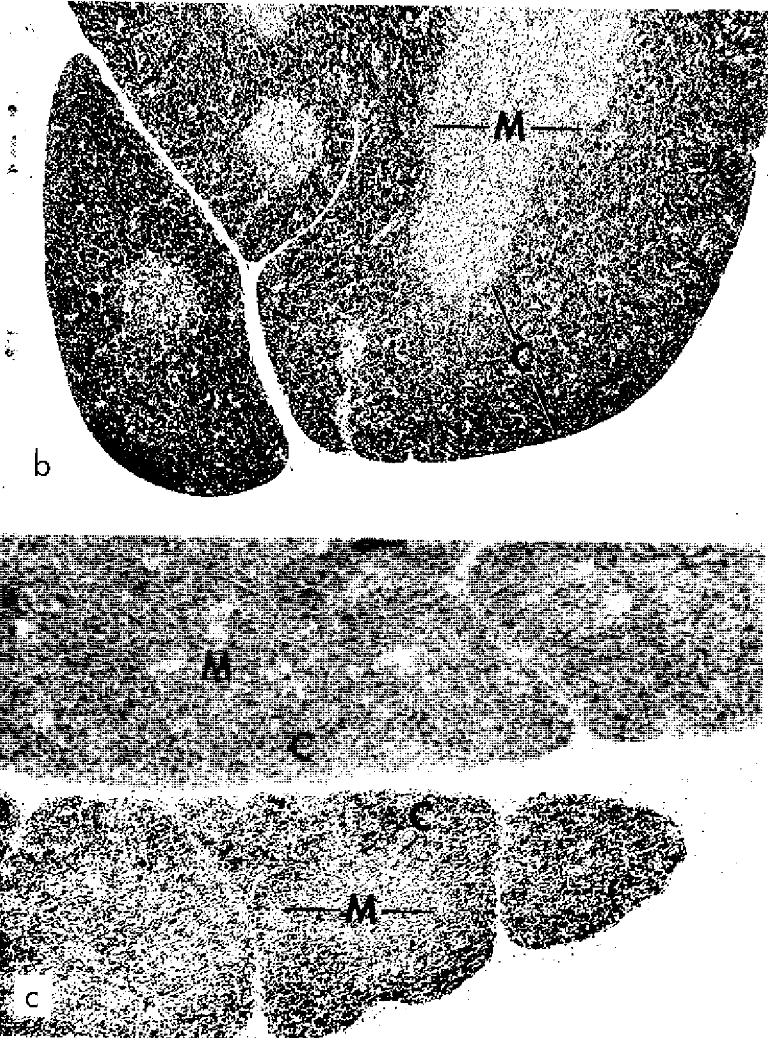
In mice, susceptibility to TCDD is genetically determined and is segregated at the locus that encodes a cytosolic protein which mediates aryl hydrocarbon hydroxylase activity (Poland & Knutson, 1982). This Ah (aromatic hydrocarbon) receptor has a high affinity for TCDD and is strongly active in mouse and rat thymus (Gasiewicz & Rucci, 1984), particularly in epithelial cells (Greenlee et al., 1985; Cook et al., 1987). Ah receptor-dependent immunotoxicity has been demonstrated in mice for thymic atrophy and the antibody response to sheep red blood cells (Tucker et al., 1986; Kerkvliet & Burleson, 1994); however, the importance of Ah receptor-mediated events in chronic, low-level TCDD immunotoxicity is controversial (Morris et al., 1992).

Immunosuppression in adult mice manifests almost exclusively as suppressed antibody responses and does not appear to be related to thymic atrophy in experiments in thymectomized (Tucker et al., 1986) and nude (Kerkvliet & Brauner, 1987) mice. Both T and B lymphocytes involved in antibody responses can, however, be affected by TCDD. For example, exposure to TCDD *in vivo* alters regulatory lymphocyte function (Kerkvliet & Brauner, 1987) and antigen-specific T lymphocyte activation (Lundberg et al., 1992). TCDD also inhibits T-independent antigen responses (Vecchi et al., 1983) and T-dependent responses

Figure 22. Thymus atrophy in the rat after exposure to 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD)

(a) Treated animal (left) showing atrophy of the thymus (asterisk) as compared with a control animal (right)

Figure 22 (contd)



(b) Thymus of control rat; haematoxylin and eosin stain
(c) Thymus of TCDD-treated rat, showing severe atrophy of the cortex (C); the cellularity of the remaining cortex is less dense, making a distinction between the cortex and medulla (M) difficult; haematoxylin and eosin stain.

when only B cells are treated (Dooley & Holsapple, 1988). Studies of the effects of TCDD on enriched B-cell populations *in vitro* have shown that it selectively inhibits late stages of the cell cycle and the development of B cells into plasma cells after antigen-specific activation (Luster et al., 1988). The molecular events responsible for TCDD immunosuppression have not been examined in detail. While early events in B-cell maturation, such as inositol phosphate accumulation, are not affected (Luster et al. 1988), activation of protein kinase (Kramer et al., 1987) and tyrosine kinase (Clark et al., 1991) have been observed.

A consequence of TCDD-induced immunosuppression is impaired resistance to infection by bacterial, viral, and protozoan microorganisms (Vos et al., 1991). In various mouse strains with different treatment schedules, TCDD suppressed resistance to models of infectious diseases with *Salmonella bern*, *S. typhimurium*, *Streptococcus pneumoniae*, herpes II, *Plasmodium yoelli* and influenza viruses. Various effects have been reported on resistance to *L. monocytogenes*. TCDD had no effect on the mortality of mice infected with *Herpes suis* (pseudorabies), whereas the mortality of mice infected with influenza virus was enhanced by a single oral dose of TCDD as low as 10 ng/kg body weight (Burleson et al., in press).

Many studies have been performed to investigate the mechanisms of TCDD-induced thymic atrophy, and a number have presented evidence that the effect may occur through an action on epithelial cells:

1. The enhanced lymphoproliferation of thymocytes after coculture with cultured mouse and human epithelial cells was reduced when the epithelial cells were pretreated with TCDD (Greenlee et al., 1985; Cook et al., 1987).
2. In mouse radiation chimaeras, TCDD-induced suppression of Tc lymphocytes is determined by the host (epithelium) and not the donor (bone marrow, subsequently thymocytes) (Nagarkatti et al., 1984).
3. Histological and electron microscopy studies of TCDD-exposed rats reveal formation of epithelial aggregates and a more differentiated state of cortical epithelium, indicating that TCDD acts on the thymic epithelium (De Waal et al., 1992b, 1993).

A direct action of TCDD on rat thymocytes has also been documented *in vitro* as cell death due to apoptosis (McConkey et al., 1988), but this effect requires higher concentrations than those that affect epithelial cell function *in vitro*. In bone marrow, TCDD affects myelopoiesis (Luster et al., 1985a) but may be more selective for prothymocytes (Fine et al., 1989, 1990; Holladay et al., 1991; Blaylock et al., 1992), thus indirectly affecting thymic function.

2.2.2.2 *Polychlorinated biphenyls*

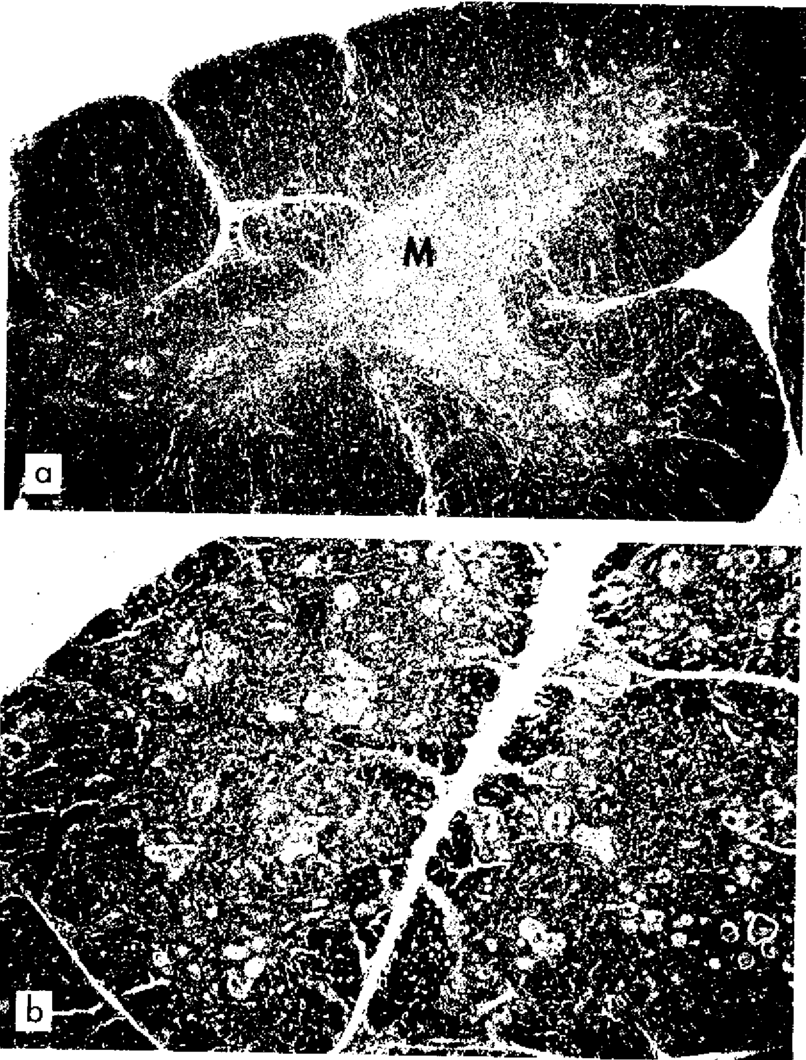
Polychlorinated biphenyls (PCBs) are important environmental chemicals shown in numerous studies to have immunotoxic properties. PCB mixtures alter several morphological and functional aspects of the immune system in rodents, guinea-pigs, rabbits, and chickens (Vos & Luster, 1989). The first suggestion that PCBs might affect the immune system came from observations on the weight and histology of lymphoid organs. Oral exposure of chickens to PCBs resulted in small spleens (Flick et al., 1965) and atrophy of lymphoid tissue (Vos & Koeman, 1970). Similar effects were noted in rabbits and guinea-pigs (Figure 23). Dermal application of PCBs to rabbits caused lymphopenia, atrophy of the thymic cortex, and a reduced number of germinal centres in spleen and lymph nodes (Vos & Beems, 1971). Oral exposure of guinea-pigs significantly reduced the number of circulating lymphocytes and the relative thymus weight (Vos & Van Driel-Grootenhuis, 1972).

Functional tests have been focused on humoral immune responses. Exposure of guinea-pigs, rabbits, mice, and rats to PCBs at different regimens reduced antibody production to foreign antigens, including tetanus toxoid, pseudorabies virus, sheep red blood cells, and keyhole limpet haemocyanin (Vos & Van Driel-Grootenhuis, 1972; Koller & Thigpen, 1973; Loose et al., 1977; Wierda et al., 1981; Exon, 1985; Kunita et al., 1985). These data are in line with the observations of Loose et al. (1977) and Thomas & Hinsdill (1978) that exposure to PCBs lowered circulating immunoglobulin levels in mice. No reduction was reported in antibody responses to bovine serum albumin (Talcott & Koller, 1983).

The response to sheep red blood cells in the plaque-forming cell assay has been used to establish dose–response relationships for several potentially immunotoxic Aroclors in mice given a single intraperitoneal injection of PCB mixtures (Davis & Safe, 1989). These studies indicate that the higher chlorinated PCB mixtures are more immunotoxic than the lower chlorinated Aroclors (Allen & Abrahamson, 1973; Loose et al., 1978; Tryphonas, in press). Data on the effects of PCBs on total serum immunoglobulin levels have not been reported in non-immunized animals.

While the suppressive effects of PCBs on humoral immunity are well documented, the effects on cell-mediated immune parameters are less clear. The delayed-type hypersensitivity reaction to tuberculin was suppressed in guinea-pigs (Vos & Van Driel-Grootenhuis, 1972) but not in rabbits treated with PCBs (Street & Sharma, 1975). Decreased delayed-type hypersensitivity reactions were reported in mice by Smith et al. (1978) but not by others (Talcott & Koller, 1983). Kerkvliet & Baecher-Steppan (1988) reported that 3,4,5,3',4',5'-hexachlorobiphenyl reduced

Figure 23. Thymus of guinea-pigs



(a) Control

(b) Animal treated with a polychlorinated biphenyl (PCB) mixture, showing severe atrophy of the cortex (C) and the presence of large cystic Hassall's bodies (arrows) in the medulla (M); haematoxylin and eosin stain

Tc lymphocyte activity in the spleens of mice. In contrast, the graft-versus-host reaction was increased following PCB treatment (Carter & Clancy, 1980). Studies on the mitogen-induced responses of splenic mononuclear leukocytes from PCB-treated mice *in vitro* resulted in either enhanced or unaltered responses (Bonnyns & Bastomsky, 1976; Wierda et al., 1981; Davis & Safe, 1989; Smialowicz et al., 1989).

Functional impairment of the non-specific resistance of macrophages has been reported, including reduced phagocytic activity and clearance of pathogenic bacteria by the spleens and livers of PCB-exposed animals (Smith et al., 1978) and decreased NK cell activity (Talcott et al., 1985; Smialowicz et al., 1989). Exposure of mice to PCBs also enhanced their sensitivity to endotoxin shock (Loose et al., 1978; Thomas & Hinsdill, 1978).

PCB treatment was shown to protect mice and rats against Ehrlich's tumour (Keck, 1981) and Walker 256 tumours (Kerkvliet & Kimeldorf, 1977), shown as reduced tumour growth and metastasis after transplantation; in other studies, however, no influence of PCB on tumour-cell implants was reported (Koller, 1977; Loose et al., 1981). PCBs also affect the resistance of animals to infectious diseases. Thus, ducklings exposed to low levels of PCBs were more susceptible to challenge with duck hepatitis virus (Friend & Trainer, 1970), and mice were more susceptible to challenge with Moloney leukaemia virus (Koller, 1977), *Plasmodium berghei* (Loose et al., 1978), *S. typhimurium* (Loose et al., 1978), *L. monocytogenes* (Thomas & Hinsdill, 1978), and *Herpes simplex* and *Ectromelia* viruses (Imanishi et al., 1980).

The immunotoxic effects of PCBs have also been investigated in a number of studies with non-human primates. Decreased titres of anti-sheep red blood cells have been observed in PCB-exposed rhesus (Thomas & Hinsdill, 1978) and cynomolgus monkeys (Hori et al., 1982; Truelove et al., 1982; Kunita et al., 1985). Immunotoxic effects were also reported in adult female rhesus monkeys and their infants (exposed *in utero* and through lactation) after low-level exposure (Tryphonas et al., 1989, 1991a,b). In this study, five groups of female rhesus monkeys were administered PCB (Aroclor 1254) at 0, 5.0, 20.0, 40.0, or 80.0 μg/kg body weight per day orally. Immunological effects were reported after both 23 and 55 months of exposure and comprised significantly decreased IgM and IgG responses to sheep red blood cells at the lowest dose. Alterations in T-cell subsets were reported in the group receiving the high dose in comparison with the controls, which were characterized by an increase in Ts/Tc (CD8) cells and a reduction in the relative numbers of Th/inducer cells (CD4) and in the CD4:CD8 ratio. No effects were seen on total lymphocytes or on B cells or on total serum IgG, IgM, and IgA levels. A further study indicated that Aroclor

1254 had no effect on B lymphocytes, since antibody responses to T-independent pneumoccocal antigen were not significantly affected. A trend for reduced incorporation of ^{3}H-thymidine by mitogen-induced lymphocyte proliferation was noted only for the T mitogens phytohaemagglutinin and concanavalin A and not for the B pokeweed mitogen. A significant augmentation of NK cell activity was noted at the highest dose. Total serum complement activity (CH50) was also increased. The serum levels of corticosteroids (hydrocortisone), which were measured throughout the study, were not affected by treatment (Loo et al., 1989), clearly indicating that the changes in several of the immune parameters were direct effects of Aroclor 1254 on the immune system.

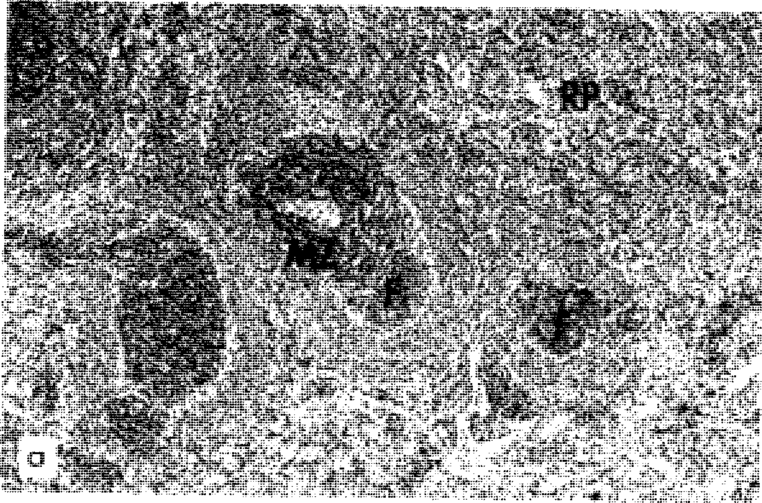
2.2.2.3 *Hexachlorobenzene*

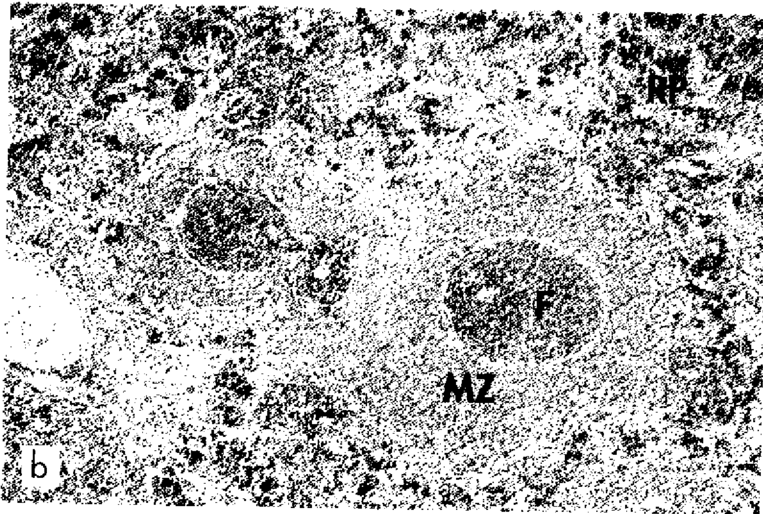
Hexachlorobenzene (HCB) is a highly persistent chemical which was used in the past as a fungicide. Emissions to the environment now occur owing to its use as a chemical intermediate and its presence as a by-product in several chemical processes. It is an immunotoxic compound (Vos, 1986), with different effects in rats and mice. In rats, the main changes seen after subacute exposure are increased weights of spleen and lymph nodes; the serum levels of IgM are also increased. Histologically, the spleen shows hyperplasia of follicles and the marginal zone (Figure 24); the lymph nodes have more follicles with germinal centres and greater proportions of high endothelial venules, indicating activation (Figures 25 and 26). High endothelial-type venules are also induced in the lung (Figure 27), and macrophages accumulate in lung alveoli (Kitchin et al., 1982; Vos, 1986; see also Figure 28).

Functional assessment showed an increase in cell-mediated immunity (delayed-type hypersensitivity) and an even greater increase in antibody-mediated immunity (primary and secondary antibody response to tetanus toxoid). Macrophage functions were unaltered. Stimulation of immune reactivity occurs at dietary levels as low as 4 mg/kg after combined pre- and postnatal exposure for six weeks, whereas the conventional parameters of hepatotoxicity are not altered at this dose (Vos et al., 1979a; Vos, 1986). The developing immune system of the rat therefore seems to be particularly vulnerable to the immunotoxic action of HCB. Reduced NK cell activity has also been found in the lung after oral exposure to 150–450 mg/kg HCB in the diet (Van Loveren et al., 1990c).

Studies on the mechanism of action of HCB indicate a role for T cells: congenitally athymic *rnu/rnu* rats, which lack T cells, do not manifest the hyperplasia of B lymphocytes in splenic follicles and the marginal zone after administration of the compound; but endothelial

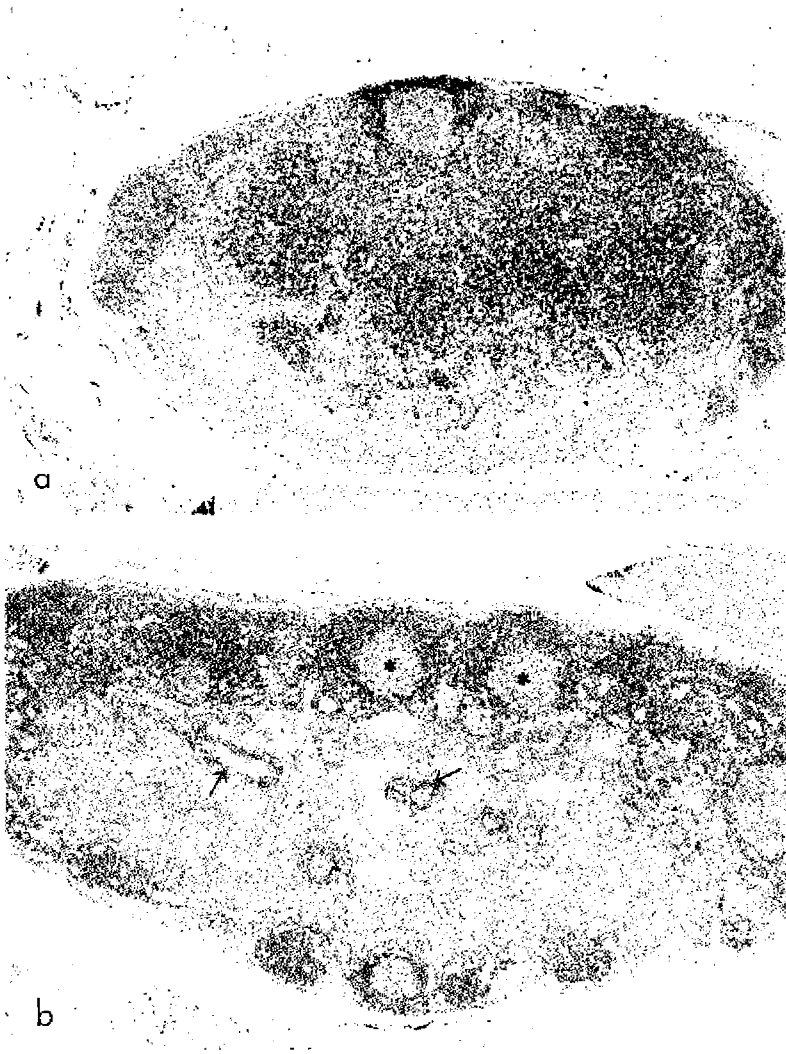
Figure 24. Spleen of control rat (a) and of an animal treated with hexachlorobenzene (b)





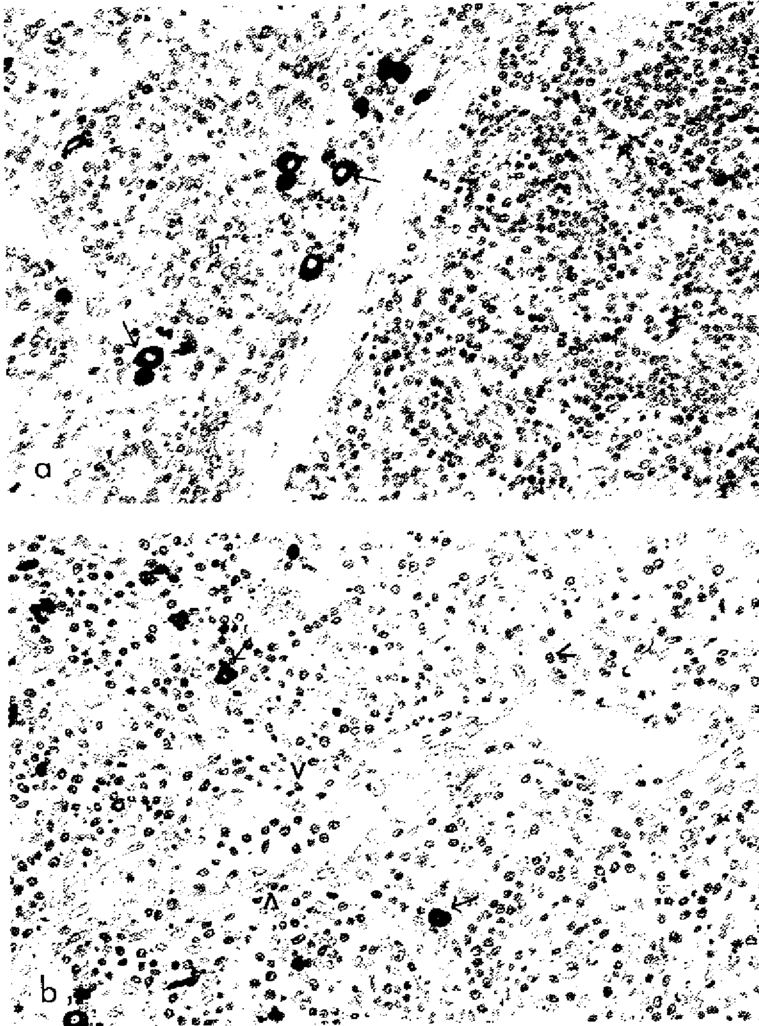
Treatment caused hyperplasia of the white pulp, particularly in the marginal zones (MZ) and follicles (F). Note also the increase in extramedullary haematopoiesis in the red pulp (RP) of the exposed animal; haematoxylin and eosin stain.

Figure 25. Popliteal lymph node of control rat (a) and of an animal treated with hexachlorobenzene (b)



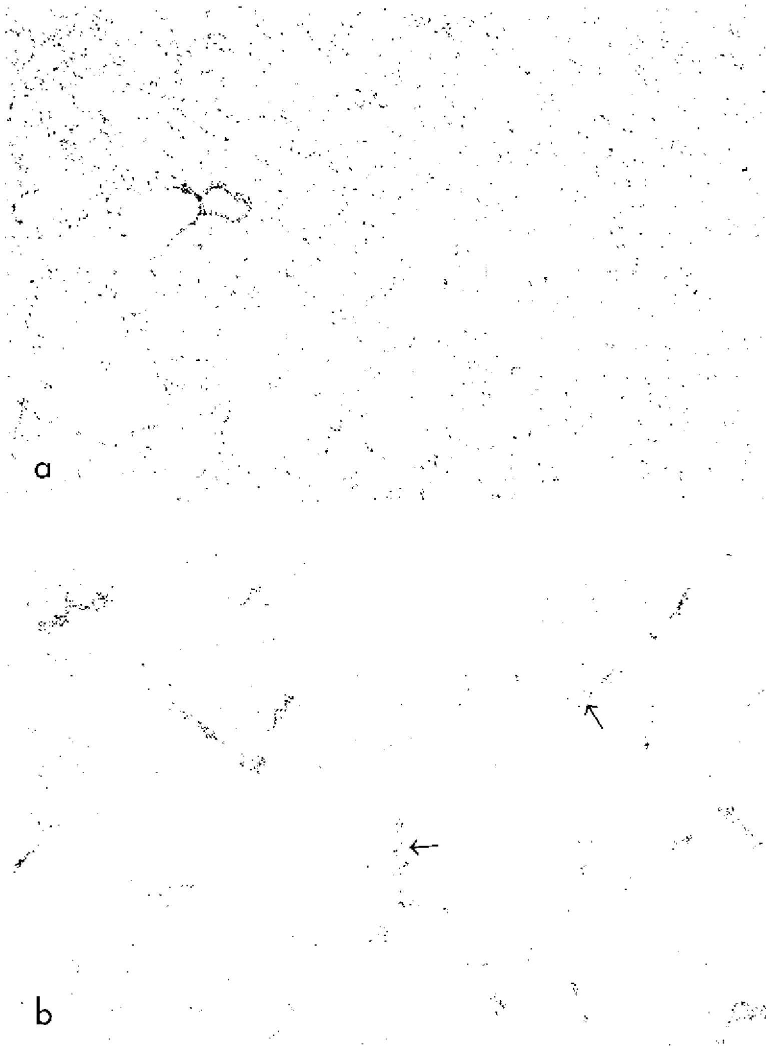
Treatment induced activation, as demonstrated by follicles with germinal centres (asterisks). An increase in high-endothelial venules (arrows) is seen in the paracortex; haematoxylin and eosin stain.

Figure 26. Popliteal lymph node of control rat (a) and activated lymph node of rat exposed to hexachlorobenzene (b)



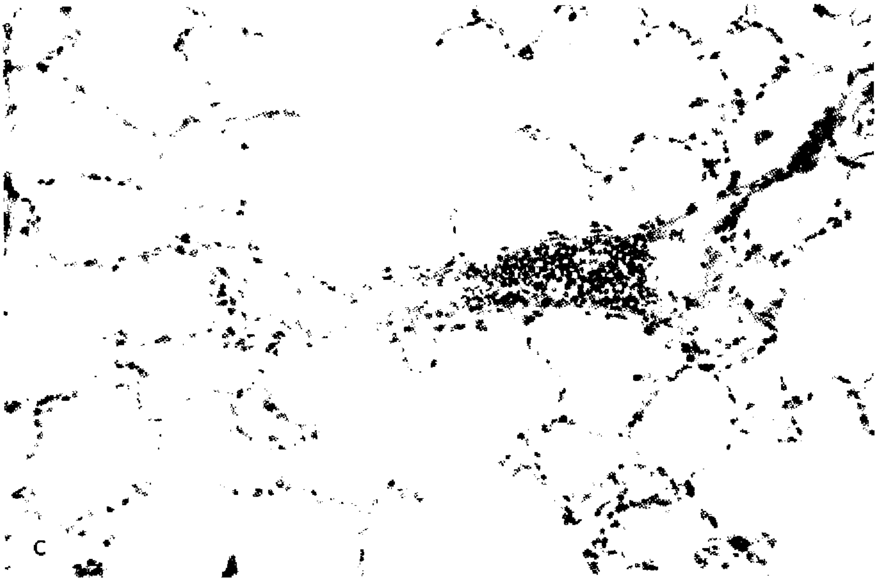
Proliferation of high-endothelial venules is seen, with an increased number of lymphocytes migrating through the endothelium (arrowheads). Note also the relatively large number of mast cells (arrows), glycolmethacrylate embedding, Giemsa stain.

Figure 27. Lung of control rat (a and c) and of an animal treated with hexachlorobenzene (b and d)



Vessels resembling high-endothelial venules (arrows) are seen at low (b) and high (d) magnification. Note the hypertrophy and proliferation of the lining endothelial cells of the venule; haematoxylin and eosin stain.

Figure 27 (contd)



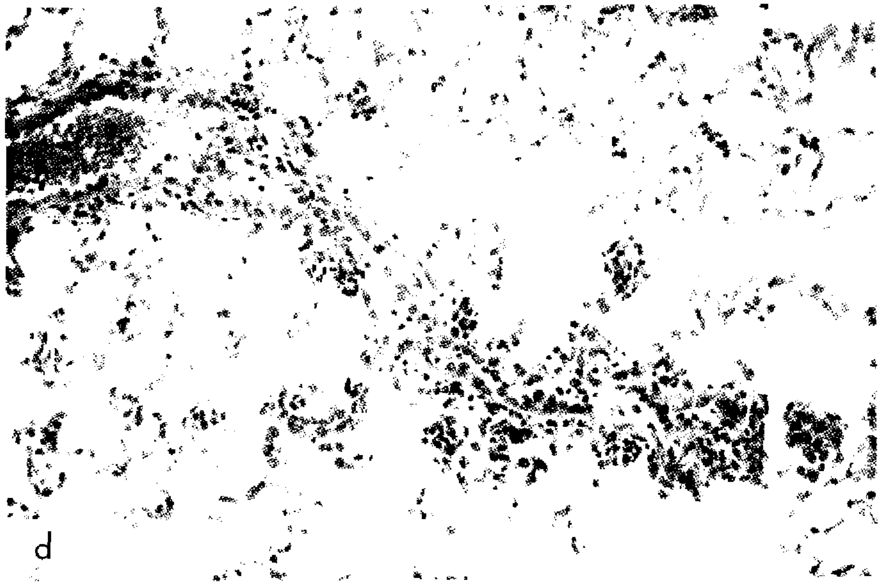


Figure 28. Immunohistochemical staining for lysozyme in lung from rat exposed to hexachlorobenzene

Note the macrophage nature of the accumulated mononuclear cells in the alveolar lumina (arrows); immunoperoxidase technique.

cell proliferation and macrophage accumulation in the lung are apparently T cell-independent, as these effects were seen in athymic animals (Vos et al., 1990b). In contrast to the immunostimulatory effect in rats, HCB suppresses cell-mediated and antibody-mediated immunity in mice, as well as their resistance to protozoan infections (*Leishmania* and *Plasmodium berghei*) and to inoculated tumours (Loose et al., 1977, 1978, 1981). The susceptibility of mice to HCB is also higher after pre- or perinatal administration (Barnett et al., 1987). Recent studies indicate that the immunostimulatory effect of HCB in rats may be related to autoimmunity:

1. Exposure to HCB of Lewis rats, which develop autoimmune disease after sensitization with complete Freund's adjuvant (adjuvant arthritis) or with guinea-pig myelin (experimental allergic encephalomyelitis), had clear effects (Van Loveren et al., 1990c): Whereas the allergic encephalomyelitis response was severely enhanced, the arthritic lesions were strongly suppressed.
2. Wistar rats treated with HCB produce antibodies to autoantigens; thus, IgM, but not IgG, levels against single-stranded DNA, native DNA, rat IgG (representing rheumatoid factor), and bromelain-treated mouse erythrocytes (indicating that phosphatidylcholine

is a major autoantigen) were elevated. It has been suggested that HCB activates a B-cell subset committed to the production of these autoantibodies and associated with various systemic autoimmune diseases (Schielen et al., 1993).

2.2.3 *Pesticides*

A large number of studies have focused on the immunotoxicity of pesticides. Because of the chemical heterogeneity of these compounds as a class, the reported effects vary widely (Barnett & Rodgers, 1994).

2.2.3.1 *Organochlorine pesticides*

The evidence for the immunotoxicity of organochlorine pesticides as a class is inconclusive.

DDT: Wistar rats treated with 40 mg/kg body weight per day DDT orally for 60 days showed increased anti-bovine serum albumin titres (Lukic et al., 1973). Studies by Vos et al. (Vos & Krajnc, 1983, Vos et al., 1983a), however, showed no changes in thymus or spleen weights, leukocyte counts, or total serum IgG and IgM levels at doses up to 800 mg/kg body weight per day.

Chlordane: Prenatal exposure of mice to chlordane was reported to reduce contact and delayed hypersensitivity responses, suggesting an effect on T-cell responses. Attempts to elucidate the mechanism have, however, been unsuccessful. Johnson et al. (1986) observed increased lymphocyte proliferation only at a dose of 8 mg/kg body weight in B6C3F1 mice and concluded that chlordane has no significant immunotoxicity in this model.

Chlordecone: Chlordecone reduced thymus and spleen weights by 40% in Fischer rats at a dose of 10 mg/kg body weight per day but had no significant effect at or below 5 mg/kg body weight per day. T-Lymphocyte proliferation was unaffected at all doses (Smialowicz et al., 1985a).

Lindane: Lindane had various effects on the anti-sheep red blood cell response, depending on the immunization protocol. Specific IgM levels were unchanged by parenteral immunization after four weeks' treatment with 150 mg/kg in the diet, but specific IgG2b levels were raised after intragastric immunization (André et al., 1983); however, the duration of *Giardia muris* infection was significantly prolonged. Five weeks' oral treatment with up to 12 mg/kg of diet decreased the antibody titre to TY3 vaccine in rabbits in a dose-dependent manner (Desi et al., 1978).

Toxaphene: Toxaphene given at 100 or 200 mg/kg of diet decreased anti-bovine serum albumin antibody titres in Swiss mice treated for eight weeks and in the offspring of dams given the same diet. Macrophage activity was also reduced in these offspring, but there were no changes in the delayed-type hypersensitivity response to purified protein derivative (Allen et al., 1983).

Endosulfan: Endosulfan had no immunotoxic effect in Wistar rats (Vos & Krajnc, 1983; Vos et al., 1983a).

2.2.3.2 *Organophosphorus compounds*

Single doses of the insecticides parathion, malathion, and dichlorvos cause significant reductions in anti-sheep red blood cell plaque-forming cell responses (Casale et al., 1983, 1984). The relevance of these findings is questionable, however, as they occurred only if cholinergic or parathion symptoms were also induced. Administration of multiple doses of malathion resulted in conflicting findings: C57Bl6 mice given four doses of 240 mg/kg body weight over eight days had unchanged plaque-forming cell responses to sheep red blood cells. In contrast, rabbits treated with 5–10 mg/kg body weight per day over 5–6 weeks had reduced antibody titres after vaccination with *S. typhimurium*. Parathion also failed to suppress anti-sheep red blood cell plaque-forming cell formation when given as four doses of 4 mg/kg body weight (Casale et al., 1983). Parathion-methyl given to rabbits for four weeks did not affect immune responses (Desi et al., 1978). In mice, however, both cellular and humoral responses were reported to be suppressed by subacute administration of parathion (Wiltrout et al., 1978).

The immunotoxicity of MPT-IP (the industrial compound for the production of Wofatox EC50, containing 60% parathion-methyl) was studied in mice given single oral doses of 8.9 mg/kg body weight or repeated doses of 0.890 or 0.445 mg/kg body weight for four weeks. Depending on the day of administration, the single dose increased the IgM plaque-forming cell content of the spleen and the serum anti-sheep red blood cell antibody titre. In the subacute system, the smaller dose (0.445 mg/kg) increased the splenic plaque-forming cell content and serum antibody titre (Institoris et al., 1992).

Dimethoate was administered by gavage to three generations of Wistar rats at doses of 14.1, 9.39, and 7.04 mg/kg body weight (equivalent to 1/50, 1/75, and 1/100 of the LD_{50}), and parathion-methyl was administered at doses of 0.436, 0.291, a,d 0.218 mg/kg body weight. The highest dose of dimethoate significantly decreased the plaque-forming capacity of spleen cells in the first generation and increased thymic

weight in the second and third generations. All three doses of parathion-methyl decreased the number of red blood cells and the haematocrit value, and the two highest doses decreased the leukocyte count. The nucleated cell content of the bone marrow was increased in the second and third generations, and decreased relative thymic weight was seen at all three doses in the third generation (Institoris et al., 1995). In a similar experiment, dichlorvos was administered at doses of 1.85, 1.24, or 0.972 mg/kg body weight. A significant decrease in leukocyte count, lowered spleen cellularity, and decreased plaque-forming capacity were seen with the highest dose in the second generation. In the third generation, there was a dose-dependent decrease in femoral bone-marrow cellularity (Institoris et al., in press). *In vitro*, 250 μmol/litre of paraoxon, a parathion metabolite, suppressed mitogenic lymphocyte proliferation in spleen cells from Sprague-Dawley rats (Pruett & Chambers, 1988).

Reduction of antibody titre against Ty3 vaccine was observed by the end of six weeks' oral treatment of rabbits with 5–100 mg/kg body weight of malathion or with 1.25 or 2.5 mg/kg body weight of dichlorphos (Desi et al., 1978). In the same system, a dose-dependent decrease was observed in the tuberculin skin reaction after administration of 0.31, 0.62, or 1.25 mg/kg body weight of dichlorphos. The cholinesterase activity of red blood cells was decreased only by the two higher doses.

Convincing evidence for immunotoxicity has been obtained only for *O,O,S*-trimethylphosphorothiate (*O,O,S*-TMP), a contaminant of various commercial organophosphorus formulations, such as malathion, fenitrothion, and acephate. This compound was shown to suppress humoral and cellular immunity in mice exposed to 10 mg/kg body weight orally (Devens et al., 1985). Several organophosphorus derivatives can alter some immune functions *in vitro*, including mitogen-induced lymphocyte proliferation (Pruett & Chambers, 1988), T-lymphocyte cytotoxicity, and production of hydrogen peroxide by macrophages (Pruett, 1992), at concentrations that can theoretically be attained *in vivo*.

Several mechanisms have been proposed to explain organophosphorus-induced immunosuppression (Pruett, 1992). A direct cholinergic mechanism is unlikely to be involved, as the addition of various cholinergic agonists does not suppress immune responses *in vitro*. In addition, *O,O,S*-trimethylphosphorodithioate, a structural analogue of *O,O,S*-TMP, modulates cholinesterase activity but does not alter immune competence. An indirect mechanism involving stress caused by neurotoxicity has also been proposed. Finally, a direct action on cells of the immune system, and particularly macrophages, has been

suggested to be involved. Mice treated with *O,O,S*-TMP, which is not neurotoxic, generate a population of macrophages, contraindicating lymphocyte proliferation. Antigen processing and presentation by these highly activated (inflammatory) macrophages are severely impaired; however, the changes in macrophage function are not correlated with suppression of humoral or cellular immunity. While there is no direct evidence that B and T lymphocytes are the predominant targets of organophosphorus compounds, their mechanisms of action on macrophages are largely unknown.

2.2.3.3 *Pyrethroids*

Dose-dependent decreases in the serum anti-*S. typhimurium* antibody titre and in the tuberculin skin reaction were observed in rabbits fed 25, 12.5, or 6.25 mg/kg body weight of technical-grade cypermethrin (93.5%) for seven weeks (Desi et al., 1985). Single oral doses (23.5, 20.7, or 18.7 mg/kg body weight) of supermethrin, the active substance of the pyrethroid pesticide Neramethrin EC 50, decreased the number of IgM plaque-forming cells in the spleens of mice but had no effect on the delayed-type hypersensitivity reaction. Repeated doses of 2.97, 1.49, and 0.743 mg/kg body weight caused only slight changes in the leukocyte count and in the nucleated cell content of femoral bone marrow (Siroki et al., 1994).

2.2.3.4 *Carbamates*

Carbaryl induced marked increases in serum IgG1 and IgG2, but not IgA, IgG3, or IgM, levels of mice exposed to 150 mg/kg of diet for one month (André et al., 1983). Rabbits given carbaryl at 4–150 mg/kg of diet for four weeks had no changes in anti-sheep red blood cell haemolysin or haemagglutinin titres or in the delayed-type hypersensitivity response to tuberculin, whereas oral treatment with carbofuran at 0.5–20 mg/kg of diet for four weeks induced a 60–75% decrease in the delayed-type hypersensitivity response (Street & Sharma, 1975). Aldicarb induced no changes in a large battery of assays for immune function and host resistance in B6C3F1 mice exposed to 0.1–1000 mg/litre of drinking-water daily for 34 days (Thomas et al., 1987).

2.2.3.5 *Dinocap*

Dinocap is a dinitrophenol compound used as a fungicide. Female C57Bl/6J mice were given doses of 12.5–50 mg/kg body weight per day by gavage for 7 or 12 days. All mice given the highest dose died after

four days. Mice given 25 mg/kg for 12 days had decreased thymus weights and cellularity and increased spleen weights but no changes in body weight, leukocyte count, lymphoproliferative response to B- or T-cell mitogens, mixed lymphocyte reaction, or NK cell activity of spleen cells; lymphoproliferative responses to concanavalin A and phytohaemagglutinin in thymocytes were reduced. In mice exposed for seven days to 25 mg/kg body weight per day, the cytotoxic T lymphocyte response to P815 mastocytoma cells was enhanced, and there was a significant reduction in the IgM and IgG plaque-forming cell response to sheep red blood cells. *In vitro* in murine thymocytes, a concentration of 10 μg/ml dinocap for 72 h suppressed the proliferative response to concanavalin A and phytohaemagglutinin; exposure for as little as 30 min suppressed the mitogen-stimulated response with no direct cytotoxicity (Smialowicz et al., 1992a).

2.2.4 *Polycyclic aromatic hydrocarbons*

A major concern for human health is the carcinogenic potential of most polycyclic aromatic hydrocarbons (PAHs). Interestingly, those which are carcinogenic also have potent immunosuppressive properties, whereas those which are not carcinogenic lack marked immunotoxic effects (Ward et al., 1985; White, 1986). Suppression of humoral immunity has been observed frequently after exposure to a number of PAHs, including benzo[*a*]pyrene, DMBA, and 3-methylcholanthrene (Ward et al., 1985). Structure–activity studies by White et al. (1985), in which the antibody-forming cell response was used to evaluate 10 PAHs in B6C3F1 and DBA/2 mice, demonstrated a wide spectrum of activity: compounds like benzo[*e*]pyrene and perylene were not immunotoxic, whereas dibenz[*a,h*]anthracene and DMBA were potent immunosuppressors of the plaque-forming cell response. Interestingly, the DBA/2 mice were more susceptible to the immunosuppressive effects than the B6C3F1 mice.

PAHs also suppress cell-mediated immunity. T-Lymphocyte cytotoxicity and mixed lymphocyte responsiveness were found to be impaired by most PAHs. Differences between PAHs are seen, however, in that benzo[*a*]pyrene may be less suppressive of cell-mediated immunity than DMBA, accounting for the greater host susceptibility to *L. monocytogenes* and PYB6 sarcoma challenges in DMBA- than in benzo[*a*]pyrene-treated rodents (Ward et al., 1985). Thurmond et al. (1987) evaluated immunosuppression in B6C3F1 (*Ah*-responsive) and DBA/2 (*Ah*-nonresponsive) mice and in *Ah*-congenic C57Bl/6J (responsive B6-Ah^bAh^d and nonresponsive B6-Ah^dAh^d) mice after exposure to DMBA in a battery of immunological assays, including

evaluation of organ weights, plaque-forming cell response, mitogen responses, and mixed lymphocyte responses. The authors concluded that the immunosuppressive action of DMBA was independent of the *Ah* locus and associated induction of cytochrome P_1-450 metabolizing enzymes.

The mechanisms of PAH-mediated immunosuppression remain to be elucidated. PAHs may exert their immunotoxic effects as the parent compound or as metabolites. *In vitro* many of the metabolites of benzo[*a*]pyrene and DMBA are immunosuppressive, the diol metabolites being the most potent (Kawabata & White, 1987; Ladics et al., 1991). Several possible mechanisms of action have been proposed, including altered interleukin levels (Lyte & Bick, 1986; Pallardy et al., 1989), a direct effect on transmembrane signalling (Pallardy et al., 1992), and alterations in intracellular calcium mobilization (Burchiel et al., 1991; Davis & Burchiel, 1992).

Earlier studies suggested that Th cells or faulty antigen recognition by T cells were possible mechanisms of DMBA-induced immunosuppression (House et al., 1987, 1989). Myers et al. (1987) also reported that benzo[*a*]pyrene alters macrophage antigen presentation. Studies by Ladics et al. (1992) demonstrated that the only splenic cell type capable of metabolizing benzo[*a*]pyrene was the macrophage and that the predominant immunosuppressive metabolite formed was the benzo[*a*]pyrene-7,8 diol epoxide, which is also believed to be the ultimate carcinogenic metabolite of benzo[*a*]pyrene.

2.2.5 *Solvents*

2.2.5.1 *Benzene*

Exposure to benzene is associated with myelotoxicity, and a strong correlation was noted between lymphocytopenia and abnormal immunological parameters. The myelotoxicity may be due, in part, to altered differentiation of marrow lymphoid cells, as suggested by the finding that acute exposure of IgM^+ cell-depleted marrow cultures to hydroquinone, an oxidative metabolite of benzene, blocked the final maturation stages of B-cell differentiation (King et al., 1987). In addition, it was shown that the hydroquinone metabolite inhibits lectin-stimulated lymphocyte agglutination and mitogenesis by reacting with intracellular sulfhydryl groups (Pfeifer & Irons, 1981).

Immunosuppression associated with exposure to benzene was found in rabbits to be an impaired antibody response together with an increased susceptibility to tuberculosis and pneumonia. Similarly, C57Bl/6 mice exposed to benzene had a lower antibody response and reduced mitogen-induced lymphocyte proliferation (Wierda et al.,

1981). Chronic inhalation of concentrations as low as 30 ppm impaired resistance to *L. monocytogenes* (Rosenthal & Snyder, 1985). Similarly, increased susceptibility to PYB6 tumour cell challenge was seen at concentrations that also impaired Tc lymphocyte function.

The mechanism of benzene-induced immunosuppression is unclear. Cellular depletion may be the major effect, although B- and T-cell dysfunction may also be involved. The antiproliferative effects of benzene may be related to its ability to alter cytoskeletal development through inhibition of microtubule assembly. Polyhydroxy metabolites of benzene (*para*-benzoquinone and hydroquinone) have been shown to bind to sulfhydryl groups on the proteins necessary for the integrity and polymerization of microtubules. This effect may alter cell membrane fluidity and may explain the sublethal effect of benzene on lymphocyte function.

2.2.5.2 *Other solvents*

Hexanediol (1.2 mg/kg per day for seven days) decreased thymus and spleen weights, antibody production, and delayed-type hypersensitivity in mice (Kannan et al., 1985). Humoral immunity was suppressed to a greater extent in female than in male mice after a four-month exposure to trichloroethylene in the drinking-water at doses of 0.1, 1.0, 2.5, or 5.0 mg/ml; cell-mediated immunity and bone-marrow stem-cell colonization were inhibited only in females (Sanders et al., 1982). The immunotoxicity of glycol ethers and some of their metabolites has been studied in rats by measuring the plaque-forming cell response to trinitrophenyl lipopolysaccaride. The glycol ethers 2-methoxyethanol and 2-methoxyethylacetate were immunosuppressive, as was the principal metabolite of the latter, 2-methoxyacetic acid. The glycol ethers 2-(2-methoxyethoxy)ethanol, bis(2-methoxyethyl) ether, 2-ethoxyethanol and its principal metabolite 2-ethoxyacetic acid, 2-ethoxyethyl acetate, and 2-butoxyethanol were not immunosuppressive (Smialowicz et al., 1991, 1992b, 1993)

Dichloroethylene did not induce immunotoxic changes in mice given up to 2 mg/litre per day for 90 days (Shopp et al., 1985). Similarly negative findings were obtained with trichloroethane (Sanders et al., 1985).

2.2.6 *Metals*

Heavy metals have been shown to alter immune responsiveness in laboratory animals (Koller, 1980). Alterations in B lymphocyte function have been observed most frequently after exposure to lead and cadmium, but T-cell and macrophage changes have also been described. In

addition, exposure to metals is correlated better with impaired resistance to experimental infections than with changes in B- or T-cell functions. Interestingly, immunostimulation has been shown to occur at levels of exposure lower than those associated with immunosuppression. Metals have also been shown to induce immunopotentiation, at lower doses than those that cause immunosuppression.

2.2.6.1 *Cadmium*

Conflicting results have been obtained with regard to the effect of cadmium on humoral immunity in animals (Descotes et al., 1990). Cell-mediated immunity, however, is consistently depressed after both short- and long-term exposure, and phagocytosis and NK cell activity are found to be depressed. Susceptibility to *L. monocytogenes*, *Herpes simplex* 1 and 2, and influenza virus was increased in B6C3F1 mice exposed for long periods (Thomas et al., 1985a).

2.2.6.2 *Lead*

Experimental studies suggest that lead has immunosuppressive effects in rodents (Lawrence, 1985; Descotes et al., 1990; Koller, 1990). Early studies demonstrated that lead can suppress the humoral immune response of mice exposed as adults (Koller & Kovacic, 1974) and of rats exposed pre- and postnatally (Luster et al., 1978). In contrast, no change in humoral immunity was found in mice exposed to 0.08–10 mmol/litre in drinking-water (Lawrence, 1981) or after a 10-week oral treatment with 13, 130, or 1300 mg/kg of diet (as lead acetate) (Koller & Roan, 1980). Delayed-type hypersensitivity was found to be depressed by lead acetate and lead chloride but not in mice treated with lead oxide, nitrate, or carbonate. The most consistent finding in experimental studies of the effects of lead on host resistance, however, is increased susceptibility to infectious agents (McCabe, 1994).

With respect to nonspecific host defence mechanisms, mice treated with lead at doses of 5, 10, or 20 µg/kg body weight given intraperitoneally once or at doses of 25, 50, or 100 µg/kg body weight given orally once showed increased clearance of colloidal carbon (Schlick & Friedberg, 1981). Furthermore, treatment of mice with 130 or 1300 ppm of lead orally for 10 weeks impaired the phagocytosis of sheep red blood cells (Koller & Roan, 1977). Lead also has consistent overall effects on host resistance to infection. Thus, treatment resulted in significantly decreased resistance of mice to *Klebsiella pneumoniae* (Hemphill et al., 1971) and *S. typhimurium*, and decreased resistance of rats to a bacterial endotoxin and to a challenge with *E. coli*, *S. epidermidis*, or *S. enteritidis*. The increased susceptibility of rodents to Gram-negative

bacteria after exposure to lead is likely to be due to hypersensitivity to an endotoxin of bacterial origin (Cook et al., 1974, 1975)

Organolead compounds, such as tetrethyllead, can also be immunotoxic (Luster et al., 1992).

2.2.6.3 *Mercury*

Mercuric salts have been shown repeatedly to depress both humoral and cellular immunity and nonspecific host defences in animals. For instance, B6C3F1 mice given mercuric chloride orally for seven weeks had decreased thymus and spleen weights, an impaired plaque-forming cell response, and inhibition of lymphocyte proliferation at a daily dose of 75 mg/litre of drinking-water (Dieter et al., 1983). Methylmercury was reported to decrease humoral immunity in mice treated orally for three weeks with 0.5, 2, or 10 mg/litre drinking-water (Blackley et al., 1980).

2.2.6.4 *Organotins*

Several organotins have been shown to be markedly immunotoxic and are considered as prototype immunotoxicants (Penninks et al., 1990), even though no human data are available.

Di-*n*-octyltin dichloride at 50 or 150 mg/kg of diet for six weeks induced a dramatic, dose-related decrease in the weight of the thymus in rats, associated with a less severe decrease in spleen and lymph node weights (Seinen & Willems, 1976). The numbers of cells in the thymus and spleen, but not the bone marrow, were decreased. Histologically, lymphocyte depletion was seen in the thymus and in thymus-dependent areas of the spleen. Interestingly, thymic atrophy recovered quickly after cessation of exposure (Seinen et al., 1977). It was later shown to be associated with a 25% decrease in peripheral blood lymphocytes, with a preferential loss of Th lymphocytes. As expected, T-cell functions, such as the delayed-type hypersensitivity response and T-lymphocyte proliferation, were depressed. Inhibition of humoral immunity was also seen, with reduced numbers of plaque-forming cells and decreased circulating antibody titres. NK cell activity was not affected, whereas susceptibility to *L. monocytogenes* infection was markedly increased.

Immune function is not impaired in guinea-pigs or mice fed di-*n*-octyltin dichloride, which correlates with the absence of thymic atrophy (Seinen & Penninks, 1979). Mice treated intravenously or intraperitoneally develop thymic atrophy, however, suggesting interspecies variability in the disposition of dialkyltins after oral intake, although other, poorly understood mechanisms may account for this variability

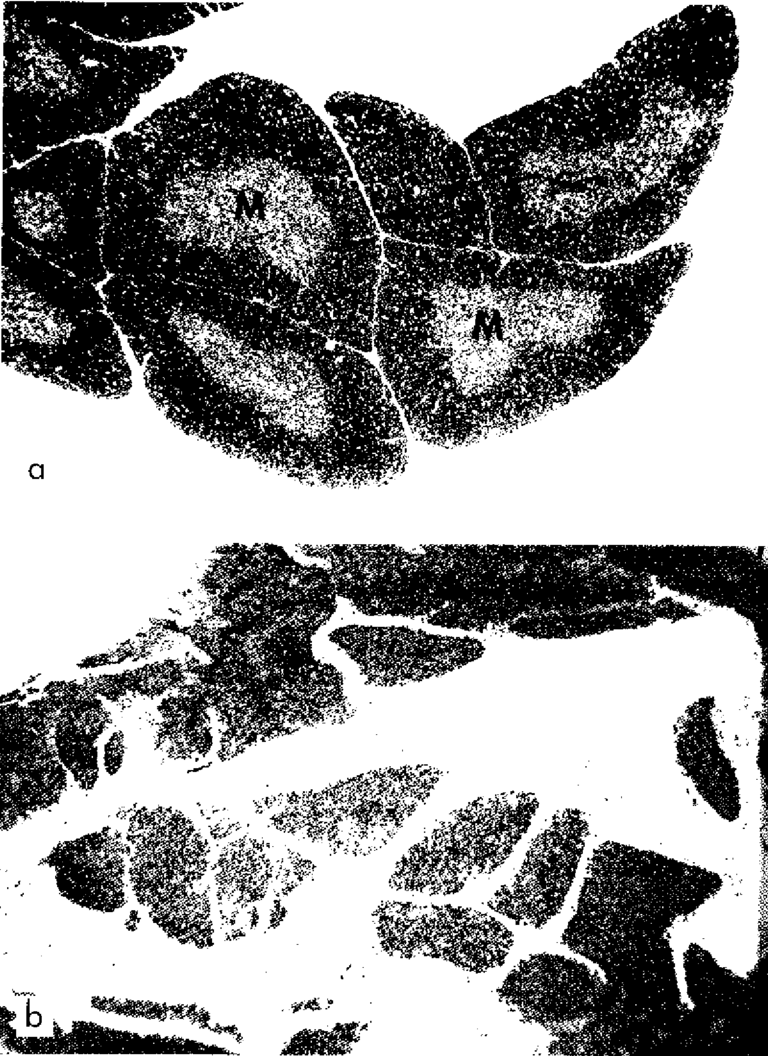
(Penninks et al., 1990). No interspecies differences in lymphocyte functions were noted after exposure *in vitro*.

Generally similar findings were made with the trialkylorganotin, tri-*n*-butyltin oxide (TBTO). As trisubstituted organotins are rapidly metabolized to disubstituted derivatives, the latter are considered to be involved in the reported thymic effects (Snoeij et al., 1988). In a short-term study in rats, pronounced effects were found on the lymphoid organs: thymus (Figure 29), spleen, and lymph nodes. These effects were most pronounced in thymus-dependent areas (Figure 30) (Krajnc et al., 1984). Interestingly, thymus atrophy also occurred in fish, as guppies exposed to organotin compounds showed severe thymic atrophy (Figure 31). In function tests (Vos et al., 1984), rats that were exposed to TBTO for six weeks after weaning had suppressed delayed-type hypersensitivity responses to ovalbumin and tuberculin and suppressed IgG responses to sheep erythrocytes. *In vitro* mitogen responses to concanavalin A in thymus and spleen and NK cell activity in both the spleen and the lungs were decreased (Van Loveren et al., 1990b). Exposure to TBTO at 20 or 80 mg/kg of diet for six weeks led to decreased resistance to infection with *L. monocytogenes* or *Trichinella spiralis*. The latter effect was evidenced by increased numbers of adult worms in the gut as a result of impaired worm expulsion, increased numbers of muscle larvae in the striated tissue, decreased inflammatory responses around these larvae, and decreased antibody responses to *T. spiralis*, especially in the IgE class (Vos et al., 1984). After long-term exposure (15–17 months) to 5 or 50 mg/kg of diet, delayed-type hypersensitivity was not suppressed, but assays for NK cell activity and resistance to infection indicated suppression.

As the immune responsiveness of older animals can be expected to be less strong than that of younger rats, the effects of exposure to immunotoxic chemicals may become evident less easily; however, tests for function still indicated immunotoxicity. In experiments in which exposure to TBTO was begun only at 12 months of age, both infection models showed immunotoxicity to TBTO. Very few studies have focused on the immunotoxic effects of chemicals on the gut immune system, but the studies of TBTO showed both a decreased capacity of the host to expel adult *T. spiralis* worms from the gut and increased production of serum IgA specific for this parasite (Vos et al., 1990a).

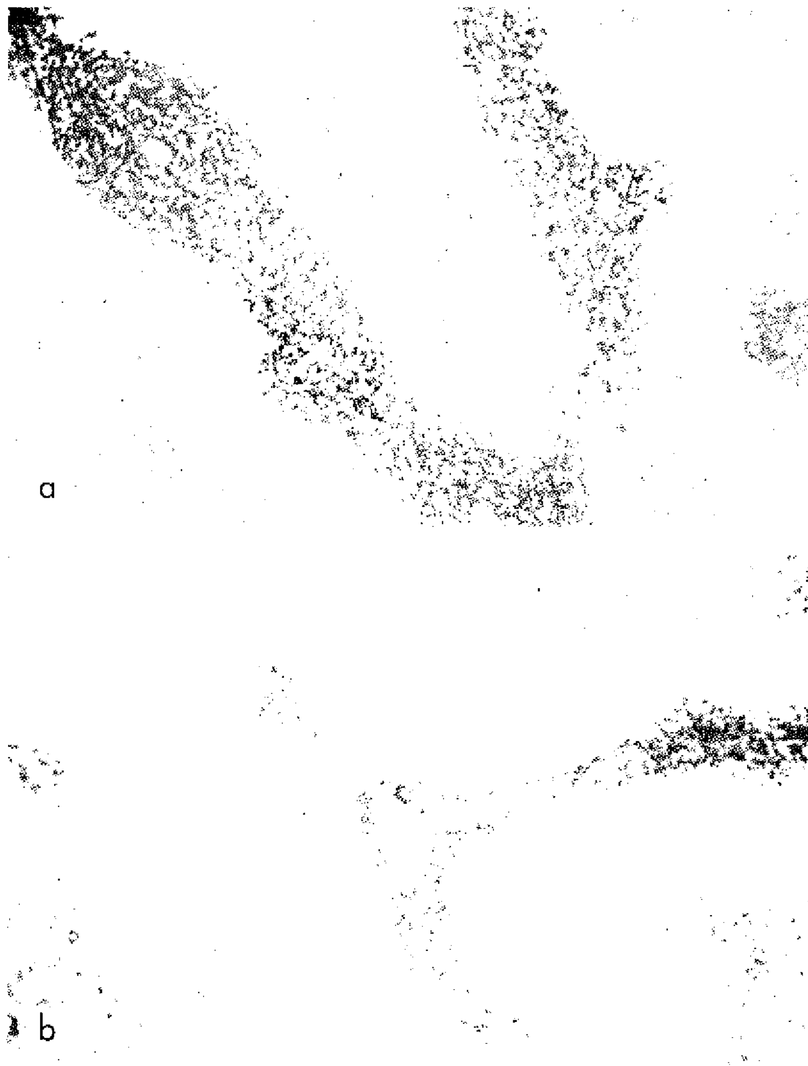
The mechanism of the immunotoxicity of organotin compounds has been investigated extensively (Penninks et al., 1990). A direct influence on the synthesis of thymic hormones is uncertain, as conflicting results have been obtained in different experiments. Interference with the influx of prothymocytes can be ruled out, as thymic atrophy develops too rapidly. Interestingly, organotins reduced the proliferative

Figure 29. Thymus from a control rat (a) and from a rat exposed to tri-n-butyltin oxide (b)



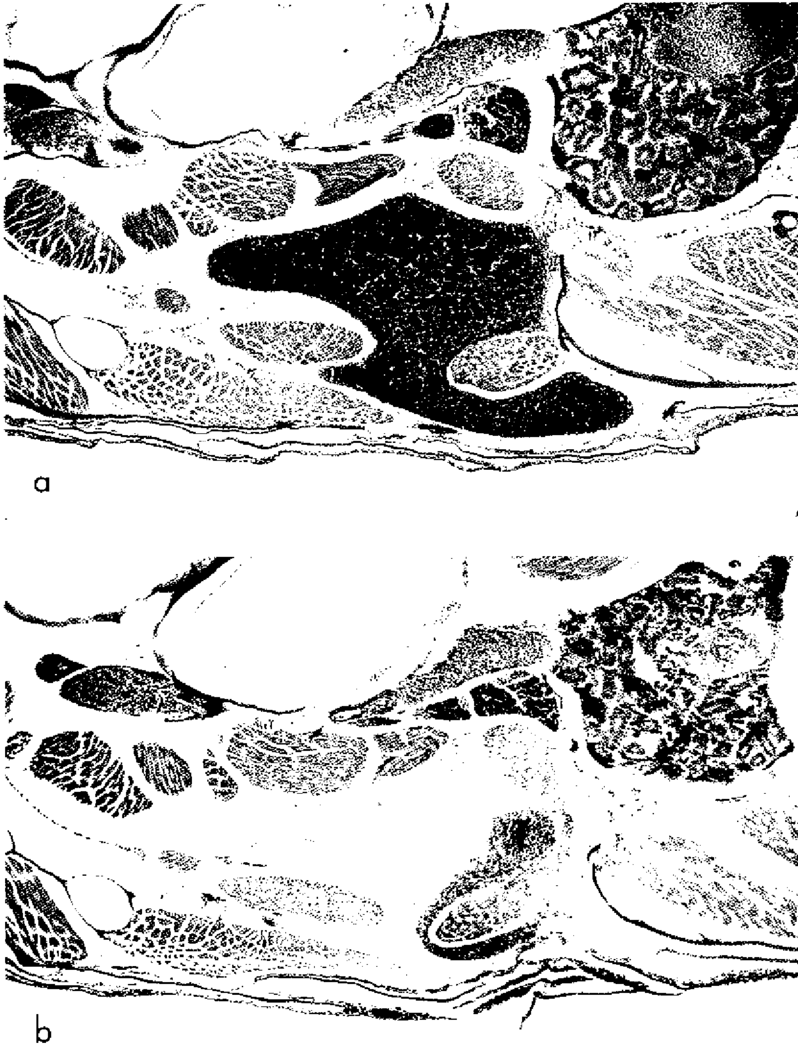
Note the severe lymphocyte depletion and atrophy of the cortex (C) and the absence of distinct corticomedullary junctions. M, medulla; haematoxylin and eosin stain

Figure 30. Immunohistochemical staining for T lymphocytes in the spleen of a control rat (a) and an animal treated with tri-*n*-butyltin oxide (b)



Note the T-lymphocyte depletion in the periarteriolar lymphocyte sheaths; immunoperoxidase technique.

Figure 31. Section of guppy (Poecilia reticulata) showing the thymus (T) in control fish (a) and in fish exposed to tri-*n*-butyltin oxide (b)



Note the strong depletion of the thymus in the treated animal; haematoxylin and eosin stain.

activity of thymocytes and the number of proliferating thymoblasts within 24 h after exposure was begun, at a time when thymic atrophy was not evident. This selective effect on thymoblasts and the physiological destruction of most cortical thymocytes would result in marked depletion and, finally, in thymic atrophy.

2.2.6.5 *Gallium arsenide*

Gallium arsenide is an intermetallic compound used widely in the electronics industry, primarily in the manufacture of transistors and light-emitting diodes. A single intratracheal instillation of 50, 100, or 200 mg/kg body weight into female B6C3F1 mice resulted in a dose-related decrease in the IgM and IgG antibody response to sheep erythrocytes. Similarly, cell-mediated immunity, as evaluated by the delayed-type hypersensitivity reaction to keyhole limpet haemocyanin and the mixed lymphocyte response, was also decreased in a dose-dependent way. Increases were observed in complement C3 levels, mitogen response to lipopolysaccharide, and NK cell activity. No effects were observed on response to T-cell mitogens, total complement CH50 activity, or host resistance to *Plasmodium yoelii* or *Streptococcus pneumoniae*; however, a significant decrease in host resistance was observed to *L. monocytogenes* and B16F10 tumour challenge (Sikorski et al., 1989).

2.2.6.6 *Beryllium*

Beryllium induces a variety of diseases, including granulomatous lung (chronic beryllium disease) and skin conditions. These granulomatous reactions involve a lymphocyte response to beryllium salts. The major lymphocyte population consists of Th cells (CD4). The T-cell response to beryllium is IL2-dependent (Saltini et al., 1989). The antigen has not been identified, but may be a beryllium-protein complex. There appears to be a genetic predisposition, as the majority of patients with beryllium lung disease share a particular HLA-Dp allele (HLA-DpB1) (Richeldi et al., 1992). The development and maintenance of lung and skin granulomas depend on the presence of antigen, antigen-presenting cells, and memory T lymphocytes and the release of proinflammatory cytokines by macrophages and lymphocytes (Boros, 1988; Kunkel et al., 1989).

2.2.7 *Air pollutants*

Pollutants characteristic of occupational and urban environments may cause or aggravate pulmonary diseases. Pulmonary defence

mechanisms to pathogens comprise mechanical defences, nonspecific defences (ingestion by phagocytic cells, lysis of virus-infected cells), and specific immunity. A number of studies in experimental animals have shown that exposure to air pollutants, including ozone, nitrogen dioxide, sulfur dioxide, some volatile organic compounds, and metal particulates, adversely affects pulmonary defences, and primarily nonspecific defences important in clearing certain Gram-positive bacteria from the lung (Graham & Gardner, 1985; Jakab & Hmieleski, 1988; Selgrade & Gilmour, 1994).

In dogs, exposure to ozone at 3 ppm for 2 h per day for three days markedly increased the number of epithelial neutrophils, whereas the number of circulating neutrophils was decreased (O'Byrne et al., 1984). A significant decrease in absolute thymocyte numbers was also observed in mice continuously exposed to 0.7 ppm ozone for three to seven days (Li & Richters, 1991). Decreased spleen and thymus weights were reported in mice exposed to ozone alone or in combination with nitrogen dioxide (Fujimaki, 1989; Goodman et al., 1989). The numbers of neutrophils and alveolar macrophages in bronchoalveolar lavage fluid were found to be increased in rats, and T-lymphocyte infiltrations were seen in ozone-induced lesions of mice. Accumulations of macrophages are located mainly at the bronchoalveolar junction and in alveoli (Figures 32 and 33).

Modulation of nonspecific defence mechanisms by ozone has also been described (Goldstein et al., 1971; Holt & Keast, 1977; Van Loveren et al., 1988a, 1990b). Thus, phagocytic activity in alveolar macrophages is suppressed, but this depends on the concentration and duration of exposure; enhanced phagocytic activity was also observed. Alterations in the macrophage production of arachidonic acid metabolites, resulting in increased prostaglandin 2 production, have been suggested to be involved (Gilmour et al., 1993). NK cell activity is either unaffected or stimulated by low ozone concentrations, whereas high concentrations decreased both the number and the activity of splenic and pulmonary NK cells (Burleson et al., 1989; Van Loveren et al., 1990b). Ozone also affects T cells (Dziedzic & White, 1986; Van Loveren et al., 1988a; Bleavins & Dziedzic, 1990; Dziedzic et al., 1990). Ozone-induced systemic dysfunction has been reported in animals and probably contributes to impaired host defences (Aranyi et al., 1983). Humoral immunity, e.g. circulating antibody titres to a variety of antigens and the plaque-forming cell response to sheep erythrocytes, is depressed after exposure to ozone; cellular immunity is also inhibited. The numbers of all major T lymphocyte subsets, mitogen-induced T lymphocyte proliferation, and delayed-type hypersensitivity responses were all shown to be decreased. Numerous studies with infectivity models show that

Figure 32. Lungs of control rat (a) and animal exposed to ozone (b), showing accumulation of macrophages (arrows) in alveoli and in the centroacinar junction (scanning electron microscopy)

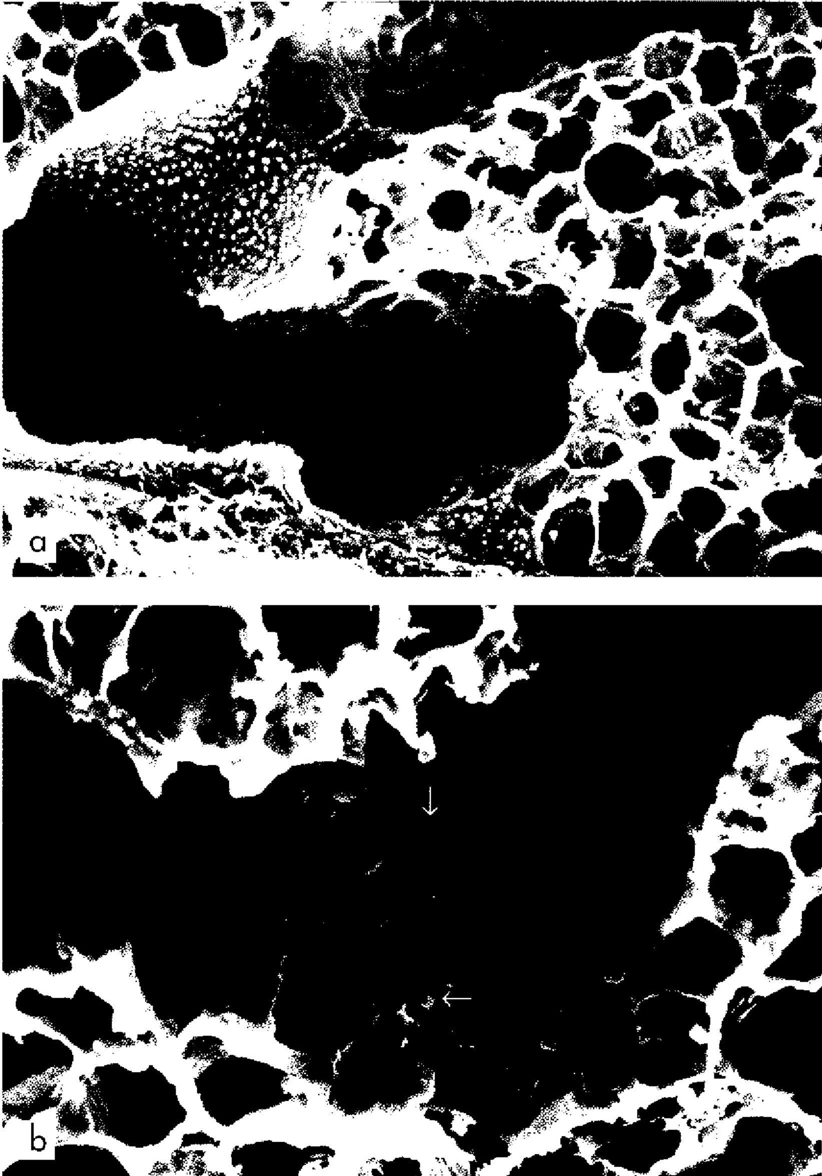
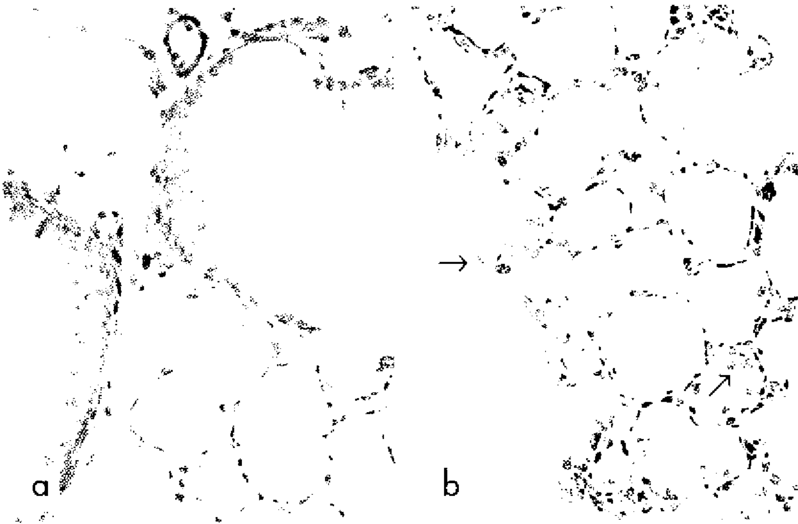


Figure 33. Light microscope images of areas similar to those shown in Figure 32, with accumulation of macrophages (arrows) in the lungs of an ozone-exposed rat (b) as compared with a control rat (a); glycolmethacrylate embedding, toluidine blue stain



exposure to ozone has an adverse influence on the host defences to respiratory infections (Van Loveren et al., 1994), and most of the studies demonstrate that the primary targets are the alveolar macrophages (Selgrade & Gilmour, 1994).

Although the influence of other air pollutants such as nitrogen dioxide and sulfuric acid on host defences has been the subject of fewer studies, the available data suggest that they have similar adverse effects (Graham & Gardner, 1985). In view of the numerous possible targets of air pollutants on respiratory defences and because of the intricate mechanisms involved, infectivity models in animals are particularly relevant for ascertaining the likely consequences of air pollution for exposed human populations.

2.2.8 *Mycotoxins*

Mycotoxins are structurally diverse secondary metabolites of fungi that grow on feed. Mycotoxin-induced immunosuppression may be manifested as depressed T- or B-lymphocyte function, decreased antibody production, or impaired macrophage activity. Immuno-

stimulation may also be observed with the tricothecenes under some experimental conditions. Similar effects have been found on the proliferative responses of human and rodent lymphocytes *in vitro* (Lang et al., 1993). Most of the data have been obtained *in vivo* or *in vitro* in animal systems, and there is only limited evidence that mycotoxins are immunosuppressive in humans (Lea et al., 1989).

Dietary exposure to various mycotoxins resulted in decreased antibody production, T-lymphocyte proliferative response, delayed-type hypersensitivity, and NK cell activity (Pestka & Bondy, 1990). Interestingly, dietary intake was associated with increased susceptibility to experimental infections.

Aflatoxin is markedly immunosuppressive in cattle and poultry (see below). Thymic atrophy, suppression of mitogen-induced T- and B-lymphocyte proliferation, and decreased antibody responses to various microbial antigens and sheep erythrocytes have been observed (Corrier, 1992). Cell-mediated immune responses appear to be affected at lower concentrations than antibody responses. The mechanism of action seems to be related to impaired protein synthesis.

Ochratoxin, a mycotoxin produced by several species of *Aspergillus* and *Penicillium*, causes depletion of lymphoid cells in the spleen and lymph nodes of dogs, swine, and mice (Corrier, 1992). The dose, the route of administration, and the animal species appear to be critical factors, however; for instance, administration of 13 mg of ochratoxin to mice in six intraperitoneal injections did not impair T-lymphocyte proliferation (Luster et al., 1987), whereas intraperitoneal injections of 5 mg/kg body weight for 50 days did (Prior & Sisodia, 1982). Ochratoxin also impairs NK cell activity and increases tumour cell growth in mice (Luster et al., 1987).

The trichothecenes, including T-2 toxin and deoxynivalenol (vomitoxin), are a structurally related group of mycotoxins produced by *Fusarium*. T-2 toxin has been studied extensively and has been shown to induce lymphoid depletion in the thymus, spleen, and lymph nodes of numerous laboratory animals (Pestka & Bondy, 1990; Corrier, 1992). In addition, mitogen-induced T- or B-lymphocyte proliferation, antibody production, and macrophage activation have been found to be depressed after exposure to either T-2 toxin or vomitoxin. The impaired immune responsiveness is associated with increased susceptibility to a variety of experimental infections. As the tricothecenes are currently considered to be the most potent small-molecule inhibitors of protein synthesis in eukaryotic cells, the immunosuppression associated with exposure to these mycotoxins is likely to be directly or indirectly linked to inhibition of protein synthesis.

2.2.9 *Particles*

2.2.9.1 *Asbestos*

Exposure to asbestos is associated with the development of inflammatory, fibrotic, and malignant (i.e. pleural mesothelioma and bronchogenic carcinoma) diseases in humans. Although the pathogenesis of asbestos-induced lung diseases is complex, a number of observations indicate that immune processes influence the development and resolution of both the inflammatory response and fibrotic lesions. For example, exposure to asbestos is associated with alterations in cellular and humoral-mediated immunity, including reduction of lymphocyte mitogenesis, delayed hypersensitivity responses, and primary antibody responses (Hartmann et al., 1984; Hartmann, 1985; Bissonette et al., 1989; Miller, 1992). In addition, immunodeficient mice resolve asbestos-induced inflammatory and fibrotic responses only with difficulty (Corsini et al., 1994), suggesting that immune mediators with anti-inflammatory or anti-fibrotic activity (e.g. IL-4 or INFγ) are involved. Furthermore, it is well established that alveolar macrophages and type II epithelial cells secrete inflammatory cytokines, chemokines, and growth factors in response to asbestos (Driscoll et al., 1990; Rosenthal et al., 1994), and these mediators are directly involved in the inflammatory responses (e.g. inflammatory cell recruitment) and fibrogenesis (e.g. fibroblast proliferation).

2.2.9.2 *Silica*

Experimental animals have been used extensively to define the pathogenesis of silicosis (Uber & McReynolds, 1982). Several immune changes have been demonstrated in guinea-pigs, including depression of humoral and cellular immunity and increased susceptibility to infectious agents. Similarly, mice exposed to silica showed decreased lipopolysaccharide-induced proliferation of B lymphocytes and depressed plaque-forming cell responses (Scheuchenzuber et al., 1985). Antibody responses to T-independent antigens, however, were less markedly depressed than responses to T-dependent antigens, suggesting an additional effect on T-cell control of humoral immunity. The effects of silica on cellular immunity depend on the dose and route of entry of antigens. The concanavalin A-induced proliferation response of spleen T lymphocytes was increased, whereas that of mesenteric lymph node T lymphocytes was depressed. The aberrations of humoral and cellular immunity induced by silica are thus complex, and it remains to be

established how these immune changes correlate with the induction of lung fibrosis or autoantibodies, the major adverse consequences of exposure to silica. In addition, silica is markedly toxic to macrophages and activates alveolar macrophages, granulocytes, and monocytes (Gusev et al., 1993). Infectivity models consistently show an increased susceptibility of silica-exposed rodents to infectious pathogens.

2.2.10 *Substances of abuse*

The immunotoxic consequences of exposure to substances of abuse are difficult to ascertain in most instances as confounding factors, such as intercurrent infections secondary to intravenous injection, may contribute to the observed changes. Recent research has provided evidence, however, that substances of abuse can directly affect the immune system (Descotes, 1986; Watson, 1990; Friedman et al., 1991a; Watson, 1993).

In rodent lymphocytes *in vitro*, D^9-tetrahydrocannabinol depressed the proliferative responses of T lymphocytes in a dose-dependent manner (Friedman et al., 1991b). Further to the early findings that opiates adversely affect immune competence (Cantacuzene, 1898), an increasing body of evidence shows that exogenous opioids have a variety of effects on cells of the immune system (Rouveix, 1993). At pharmacological concentrations, opiates suppress antibody production, lymphocyte proliferation, and delayed-type hypersensitivity and decrease NK cell activity in various animal models. In addition, phagocytosis is impaired. Opioid peptides can, however, also have a stimulatory effect on the immune system, depending on the experimental conditions. β-Endorphin affects cytokine production in rat and mouse T-cell cultures *in vitro*; e.g. it stimulates the synthesis of IL-2, IL-4, and INFγ, thereby inducing MHC class II expression on B cells (van den Bergh et al., 1993a,b, 1994).

In general, short-term exposure of mice, rats, and guinea-pigs to mainstream tobacco smoke either produces no significant immunomodulatory effect or a slight immunostimulation, which returns to normal shortly after cessation of exposure (Johnson et al., 1990). In contrast, subchronic or chronic (more than one year) exposure is generally immunosuppressive: cellular immunity, e.g. mitogen-induced lymphocyte proliferative response, and NK cell activity are impaired after long-term exposure to tobacco smoke. The humoral immune response is also depressed, as shown by decreased antibody titres, and animals exposed to cigarette smoke for extended periods are more susceptible to tumour and infectious challenge than naive animals.

2.2.11 *Ultraviolet radiation*

The earliest indication that ultraviolet radiation (UVR) affects the immune system came from studies of host resistance to UVR-induced tumours in mice (Kripke, 1974). Subsequent studies showed that low doses of UVR suppress contact hypersensitivity responses to chemical sensitizers (Toews et al., 1980) and that systemic immunosuppression (depressed contact hypersensitivity in unirradiated skin) occurs after exposure to higher doses (Jessup et al., 1978). Irradiated mice were also found to be less resistant to infection (Giannini, 1990). Other studies (Noonan & De Fabo, 1990) have determined that systemic suppression of immunoreactivity is not a function of the dose of UVR but rather of the interval between irradiation and immunization of the mice. Thus, induction of contact hypersensitivity responses in mice exposed to low doses of UVR was not affected when the animals were immunized through unirradiated skin immediately after exposure to UVR; however, sensitization was suppressed if three days were allowed to elapse between irradiation and immunization. It has also been shown that the dose of UVR required to induce 50% suppression of the immune response depends on the strain of mouse and the type of antigen used (Noonan & De Fabo, 1990; Noonan & Hoffman, 1994).

The mechanism of UVR-induced suppression of cellular immunity has not been elucidated, nor has a single initial event been identified that leads to suppression of immunoreactivity. Currently, induction of pyrimidine dimers in DNA (Kripke et al., 1992) and isomerization of urocanic acid (Noonan & De Fabo, 1992) are the leading contenders. Increased suppressor cell activity (Brodie & Halliday, 1991) and efferent lymphatic blockade, which inhibits lymphocyte homing, may be responsible for the UVR-associated accumulation of lymphocytes in lymph nodes in UVR-exposed areas (Spangrude et al., 1983) and have been proposed as possible causes of immunosuppression. Exposure to UVR has also been shown to alter the pattern of cytokine production by T cells, from a response dominated by Th1 (i.e. favouring delayed hypersensitivity responses) to one dominated by Th2 (i.e. favouring antibody production) (Araneo et al., 1989; Simon et al., 1990). Exposure to UVR has been reported to affect Langerhans cells directly, such that their interaction with T cells induces specific antigen tolerance in the Th1 subpopulation (Simon et al., 1990) and preferential activation of the Th2 population (Simon et al., 1991). This may be the reason that mice exposed to UVR are more susceptible to infection with the protozoan *Leishmania major* (Giannini, 1992), since resistance to infection with this intracellular parasite is dependent on the magnitude of the Th1 response

of the host (Reed & Scott, 1993). In addition, reduced resistance to *T. spiralis* was found in rats exposed to UVR on days 5–10 of infection (Goettsch et al., 1993). Altered cytokine production profiles may also be responsible for increased sensitivity to *Mycobacterium lepraemurium*, an intracellular pathogen that induces a chronic and eventually fatal infection in susceptible mice. In a comparison of susceptible (BALB/c) and resistant (C57Bl/6J) mice, Brett & Butler (1986) determined that resistance to infection is correlated with the ability of mouse lymphocytes to elaborate cytokines that activate macrophages, rather than with the actual development of a delayed hypersensitivity response to bacterial antigens. Jeevan & Kripke (1990) and Jeevan et al. (1992) reported that irradiation of BALB/c mice resulted in decreased resistance to infection, as measured by bacterial counts and length of survival after infection. Elevated bacterial counts were seen in animals exposed to doses of UVR that did not suppress the delayed hypersensitivity response to bacterial antigens, suggesting that the underlying mechanism of UVR-induced suppression of resistance to infection is independent of suppressed delayed hypersensitivity.

2.2.12 *Food additives*

There is little information about the effects of food additives on the immune system. An early study showed that the preservative methylparaben and the antioxidants butylated hydroxyanisole, butylated hydroxytoluene, and propylgallate suppress the in-vitro T-dependent antibody response, whereas vanillin and vanillic acid stimulate it (Archer et al., 1978). The significance of these findings *in vivo* has yet to be established.

The immunotoxicity of 'caramel colour', which covers a large number of complex products used as food colorants, has been investigated. One of the compounds in this group, 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI) (caramel colour III), has been found to be immunotoxic in rodents (Kroplien et al., 1985). THI induces a rapid reduction in the number of B and T cells in blood, spleen, and lymph nodes and morphological changes in the thymus of rats, with an increased number of mature medullary thymocytes and a decreased number of cortical macrophages. THI might reduce the migration of mature thymocytes into the periphery, as a decrease in the number of recent ER4^{+} thymic emigrants was found in the spleens of exposed rats (Houben et al., 1992). Functional studies indicate changes in Th cell function, an increased capacity to clear the Gram-positive bacterium *L. monocytogenes*, and modulation of the activity of adherent splenic cells (Houben et al., 1993). It has been hypothesized that THI exerts an

antivitamin B6 action by competing with pyridoxal 5′-phosphate for binding to the cofactor site of one or more pyridoxal 5′-phosphate-dependent enzymes.

2.3 Immunotoxicity of environmental chemicals in wildlife and domesticated species

Most of the concern about chemical contamination of wildlife populations has been focused on the aquatic ecosystem, and a growing body of literature has appeared on the effects of pollution on the health status of aquatic life. These studies deal mainly with the occurrence of tumours and infectious diseases in fish and marine mammals. These are multifactorial diseases in which perturbations of the immune system may play a part.

2.3.1 Fish and other marine species

2.3.1.1 Fish

Fish diseases are being monitored on a routine basis at various sites in North America and Europe. In Europe, most of the programmes are carried out under the auspices of the International Council for Exploration of the Sea. National and local studies have been directed to estuarine, marine, and brackish waters suspected of being polluted, such as in the vicinity of industrial areas and after major oil spills. The common diseases that are discussed in relation to pollution are certain skin diseases, such as lymphocystis, papillomas, fin rotting, and skin ulcers (Vethaak & ap Rheinallt, 1992), as they are easily identified grossly and are therefore potentially useful for biomonitoring. Since most diseases of fish have a viral or bacterial etiology, and elevated incidences have been correlated with chemical pollution, immunotoxicity may play a role. A causal relationship between chemical pollution and a disease state induced by immunosuppression cannot be finally established, however, since many confounding factors exist in the natural environment. Liver neoplasia and precursor lesions have been used to biomonitor environmental pollution in flatfish (Malins et al., 1988; Vethaak & ap Rheinallt, 1992; Vethaak, 1993); however, the role of the immune system is not evident.

Effects observed in field studies are modified or confounded by numerous factors, in particular for feral fish, for which there are deficient case histories and limited knowledge of their migratory patterns and biology (Vethaak, 1993). Extensive epidemiological surveys are required that include specific parameters that have been validated

in experiments under (more) controlled conditions (in mesocosms or the laboratory). Changes in disease patterns may suggest immune alterations, but this should be demonstrated. Since most diseases have a complex etiology, it will be difficult to establish the role of immunotoxic effects under field conditions. Circumstantial evidence can be obtained in these instances, although in the case of feral animals mesocosm or laboratory experiments must carried out in order to reach a final conclusion (Secombes et al., 1992; Vethaak, 1993).

The etiological components and their role in the pathogenesis of many diseases in fish in the field are, as yet, poorly understood, and laboratory experiments are often indispensable for background knowledge. Even when such scientific deficiencies are resolved, laboratory studies will still be needed, since function tests under controlled conditions yield the most reliable and sensitive methods of assessing immunological stress and must often accompany field studies, as mentioned above. Findings from laboratory situations do not necessarily imply effects in the field, however; in particular, when results from the laboratory are extrapolated to field situations, there is often a discrepancy between the levels of exposure.

2.3.1.2 *Marine mammals*

Marine mammals are of special interest to the discipline of immunotoxicology. As the highest predators in highly contaminated marine environments, these animals are exposed to a large number of environmental chemicals, some of which have been identified as potentially immunotoxic. Persistent lipophilic halogenated compounds such as PCBs, polychlorinted dibenzodioxins, polychlorinated dibenzofurans, and pesticides accumulate in the marine food chain and are thus biomagnified in marine mammals. The concentrations of PCBs in the blubber layer of marine mammals are higher than in any other wildlife species measured (Table 6). In times of food shortage and other stressful circumstances, these lipids are mobilized, thereby releasing their toxic burden.

Because of the high level of exposure of marine mammals, they may be expected to be the first wild animals to suffer from immunosuppression due to chronic exposure to environmental contaminants. Toxicological research over the last 20 years has identified environmental chemicals as the source of many disorders in marine mammals. In both field studies and controlled experiments, PCBs have been linked to reproductive problems. As early as 1976, a high incidence of premature parturition was seen in California sea-lions (*Zalophus californianus*), which was caused by a viral infection and was suggested to be linked

Table 6. Concentrations of polychlorinated biphenyls (PCBs) in herring and the blubber layer of marine mammals

Species	Source	Total PCBs (µg/g lipid)	Reference
Herring	Atlantic Ocean (United Kingdom)	0.0003–0.001	De Swart et al. (1994)
Herring	Baltic Sea (Sweden)	0.0035–0.0045	De Swart et al. (1993)
Weddell seal (*Leptonychotes wedelli*)	Weddell Sea (Antarctic)	0.07–0.09	Luckas et al. (1990)
Harbour seal (*Phoca vitulina*)	Atlantic Ocean (Iceland)	1–13	Luckas et al. (1990)
Harbour seal (*Phoca vitulina*)	Baltic Sea (Sweden)	21–140	Luckas et al. (1990)
Beluga whale (*Delphinapterus leucas*)	St Lawrence River (Canada)	15–700	Muir et al. (1990); Martineau et al. (1987)
Striped dolphin (*Stenella coeruleoalba*)	Mediterranean Sea (Spain)	100–2600	Kannan et al. (1993)

to higher levels of pollutants in animals that aborted as compared with mothers that gave birth to healthy pups (Gilmartin et al., 1976). Pathological changes in the uteri of seals in the highly polluted Baltic Sea, in some cases leading to sterility, could be correlated with increased levels of PCBs (Helle et al., 1976). In addition, several studies have linked skeletal deformities in grey seals (*Halichoerus grypus*) and harbour seals (*Phoca vitulina*) in the Baltic Sea to high levels of organochlorines in their environment (Bergman et al., 1992; Mortensen et al., 1992). In porpoises (*Phocoenoides dalli*) living in the north-western part of the North Pacific, a negative correlation was found between serum testosterone levels and DDE concentrations in blubber (Subramanian et al., 1987). In an experimental situation, two groups of harbour seals were fed fish containing different levels of pollutants. Seals that were fed fish from the heavily polluted western part of the Dutch Wadden Sea had significantly lower pup production than seals feeding on less polluted fish (Reijnders, 1986). In the same study, it was shown that the

seals fed polluted fish also had significantly reduced levels of vitamin A and thyroid hormone in their serum (Brouwer et al., 1989). No parameters of immune function were studied.

Such correlative observations in the natural environment, in combination with the results of semi-field studies, suggest that the current levels of contaminants may be adversely affecting certain marine mammal populations. The occurrence of a large number of epizootics among seals and dolphins inhabiting polluted coastal areas—among bottlenose dolphins (*Tursiops truncatus*) on the Atlantic coast of the United States in 1987 and in the Gulf of Mexico in 1990, striped dolphins (*Stenella coeruleoalba*) on the coast of France in 1989 and in the Mediterranean Sea in 1990–92, Baikal seals (*Phoca sibirica*) in Lake Baikal in 1987, and harbour seals in north-west Europe in 1988—led to both public and scientific discussions about the possible contribution of environmental pollutants to these disease outbreaks. Owing to the complexities of the relationships between toxicants and the immune system and the difficulties in obtaining samples fit for use in immunotoxicological studies, it has been impossible thus far to conclusively demonstrate immunosuppression in free-ranging marine mammals.

Another immunotoxicological experiment was carried out in which two groups of juvenile harbour seals (*Phoca vitulina*) were fed fish from the Baltic Sea or from the relatively unpolluted Atlantic Ocean. The diets were analysed for concentrations of potential immunotoxic chemicals: the estimated daily intakes of TCDD-like organochlorines were 270 and 35 ng/day for the two groups, respectively. Immunological function in the two groups was examined by measuring mitogen- and antigen-induced proliferative responses of lymphocytes, NK cell activity, serum antibody levels after immunization with primary antigens, and delayed-type hypersensitivity reactions. These techniques had to be validated for application to seals, as virtually nothing was known about the cellular immune system of marine mammals (De Swart et al., 1993, 1994). Seals fed herring from the contaminated Baltic Sea had significantly depressed immune function, as measured by decreased NK cell activity (Ross et al., in press) and T-cell mitogen-induced lymphocyte proliferation (De Swart et al., 1994), and significantly lower delayed-type hypersensitivity and antibody responses to immunization with ovalbumin (Ross et al., 1995). The functional changes were accompanied by increased numbers of neutrophils in the peripheral blood (De Swart et al., 1994). Since NK cells are an important line of defence against viruses, and lymphocytes (especially T cells) play a major role in the clearance of viral infections, functional suppression of these leukocytes may contribute to the severity of epizootic episodes among marine mammals.

These experiments not only provide the first demonstration of pollution-induced impairment of immune function in marine mammals but indicate that mammals in general can undergo such impairment as a consequence of chronic exposure to the levels of pollution found in their natural habitats. It is still difficult, however, to link the disease outbreaks among marine mammals directly to pollution-induced impairment of immune function.

2.3.2 *Cattle and swine*

The effects of antimicrobial, corticosteroid, and hormonal compounds have been investigated in cattle, mainly as lymphocyte proliferative responses and neutrophil functions *in vitro*. The results are in keeping with those obtained in humans (Black et al., 1992).

Few studies have dealt specifically with the direct immunotoxic effects of pesticides and environmental pollutants. No statistical difference was found between control and polybrominated biphenyl-exposed animals in the numbers of circulating total, T, and B lymphocytes, serum immunoglobulin levels, mitogen-induced proliferative responses of lymphocytes, antibody response to keyhole limpet mitogen, or cell-mediated response to purified protein derivative (Kateley & Bazzell, 1978). In contrast, peripheral blood lymphocytes from sows fed polybrominated biphenyls for 12 weeks had significantly decreased responses to mitogens (Howard et al., 1980).

The mycotoxin tricothecene produced by *Fusarium* and several other fungi was shown to reduce lymphoid tissue cellularity and serum IgA, IgG, and IgM concentrations and to impair neutrophil migration, chemotaxis, and phagocytosis in cattle exposed to high doses (Buening et al., 1982; Mann et al., 1983). Similarly, aflatoxin was reported to suppress the mitogen-induced proliferative response of bovine lymphocytes (Paul et al., 1977). Other mycotoxins, e.g. ochratoxin A and zearalenone, have been suggested to be immunosuppressive in cattle (Black et al., 1992).

2.3.3 *Chickens*

Chickens have been used in a number of immunological studies, as the unique bursa of Fabricius, the avian organ for B-cell development, underlies the need for a separate avian model in immunotoxicology. Exposure of adolescent chickens to cyclophosphamide decreased the levels of antibodies to various antigens without decreasing graft-versus-host reactivity (Lerman & Weidanz, 1970). Nutritional deficiencies in selenium or vitamin E have also been shown to impair

the humoral immune responses of adolescent chickens (Marsh et al., 1981). Exposure to cyclophosphamide *in ovo* results in decreased antibody forming capacity, decreased responsiveness to phytohaemagglutinin and concanavalin A, and decreased weights and altered morphology of the thymus, spleen, and bursa of Fabricius (Eskola & Toivanen, 1974). Peritoneal macrophages were not affected after exposure to cyclophosphamide *in ovo*, as judged by their number, superoxide anion production, and surface expression of Ia antigen and transferrin receptor (Golemboski et al., 1992). Dietert et al. (1985) showed that exposure to aflatoxin B1 *in ovo* did not alter humoral immunity; however, two parameters of cell-mediated, graft-versus-host, and cutaneous basophil hypersensitivity reactions were depressed. Methyl methanesulfonate decreased the bactericidal action of peritoneal macrophages for *E. coli* after exposure *in vitro* (Qureshi et al., 1989). TCDD impairs B-cell development in the bursa of Fabricius in chicken embryos (Nikolaidos et al., 1990).

2.4 Immunotoxicity of environmental chemicals in humans

Although limited, various lines of evidence derived from case reports, clinical studies, and well-designed longitudinal studies imply that environmental agents can affect the human immune system. While these data raise concern about potential health effects, they rarely refer to clinical disease, for a number of reasons. For example, there may be sufficient redundancy or reserve in the immune system that 'moderate' levels of immunosuppression do not result in disease. Alternatively, the clinical changes most likely to be associated with moderate immunosuppression, e.g. increased severity or frequency of pulmonary infections, do not occur. Well-designed clinical studies with adequate populations and appropriate monitoring, follow-up, and documentation of exposure have rarely been conducted. Examples of published reports that attribute immune changes in human populations exposed to environmental agents are summarized below. As the reader will note, these studies range from poorly defined to relatively large longitudinal studies.

2.4.1 Case reports

In an unsubstantiated study, a cluster of cases of Hodgkin's disease reported in a small town in Michigan (United States) was ascribed to chronic immune stimulation by mitogenic substances in the environment (Schwartz et al., 1978). Immunological studies of family members revealed a large number of individuals with altered ratios of T-lympho-

cyte subpopulations, autoantibodies, infections, recurrent rashes, and NK cell function. A report of a four-year study of workers engaged in the manufacture of benzidine, a human bladder carcinogen, suggested that individuals with depressed cell-mediated immunity (as judged by skin testing) had precancerous conditions and subsequent neoplasms (Gorodilova & Mandrik, 1978); no cases of neoplastic disease were registered in workers with normal immunological responses.

2.4.2 *Air pollutants*

The association between changes in immunological parameters and host resistance and inhalation of particulate materials and oxidant gases is well established (Folinsbee, 1992). For example, decreases in delayed-type hypersensitivity response, circulating T-cell numbers, and T-cell proliferation have been observed with, and sometimes preceding, asbestos-related diseases, i.e. fibrosis, asbestosis, and mesothelioma (Kagan et al., 1977a; Gaumer et al., 1981; Lew et al., 1986; Tsang et al., 1988). B-Cell responses are increased, however, as evidenced by increased serum and secretory (primarily IgA) immunoglobulins (Kagan et al., 1977b). Kagan et al. (1979) also reported an association between exposure to asbestos and B-cell lymphoproliferative disorders. Several studies have shown altered NK cell activity after exposure to asbestos (Kubota et al., 1985; Tsang et al., 1988). In studies of NK cell responses in asbestos workers, Lew et al. (1986) found that immune changes may occur independently of any early neoplastic process. Similarly, there have been multiple observations of abnormal antibody production, decreased cell-mediated immune responses, and decreased resistance to disease in people occupationally exposed to silica (Uber & McReynolds, 1982).

Oxidant gases have been associated with an increased prevalence of respiratory infections, particularly bacterial, and potential immune effects, but the data are less convincing than those from studies of rodents. The association between air pollution in industrialized areas and altered health status has been well established in epidemiological studies (French et al., 1973). A number of studies have linked exposure to air pollutants (ozone, nitrogen dioxide, sulfur dioxide, environmental tobacco smoke) with an increased incidence, severity, or duration of symptoms associated with respiratory infections (Lunn et al., 1967; French et al., 1973; Durham, 1974; Harrington & Krupnick, 1985; Neas et al., 1991; Schwartz et al., 1991; Schwartz, 1992; US Environmental Protection Agency, 1990), although several studies of nitrogen dioxide failed to show such an association (Speizer et al., 1980; Ware et al., 1984; Samet et al., 1993). In Ontario, Canada, increased air pollution from

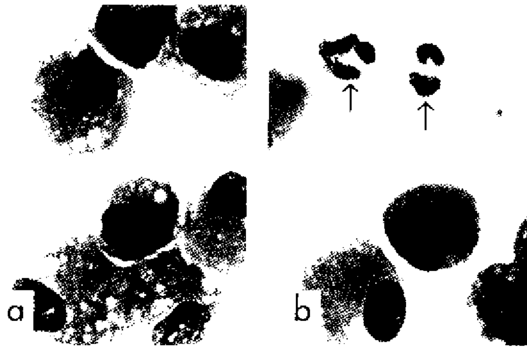
sulfur dioxide and ozone during the summer was directly related to hospital admissions for acute respiratory symptoms (Bates & Sizto, 1983). Goings et al. (1989) subjected young adult volunteers to 1, 2, or 3 ppm nitrogen dioxide for 2 h per day on two consecutive days and then administered influenza virus intranasally. Although no statistical differences were observed, the frequencies of infections in exposed volunteers (91%) were higher than the 56–73% in healthy individuals, suggesting that nitrogen dioxide may play a role in increasing susceptibility to infection. In assessments of air pollution at home, young children in households with gas stoves had a higher incidence of respiratory disease and decreased pulmonary function than children in households with electric stoves. This difference was related to increased levels of nitrogen dioxide in homes with gas stoves, which reached peak values of > 1100 mg/m^3 (Melia et al., 1977; Speizer et al., 1980). In contrast, Samet (1994) found no association between indoor levels of nitrogen dioxide and respiratory infections in children. A study of schoolchildren in Chattanooga (United States) showed an increased incidence of respiratory illness associated with atmospheric nitrogen dioxide levels (Shy et al., 1970).

Examination of hospital admissions in Massachusetts (United States) in 1980 and 1982 revealed a positive association between 1-h maximum ozone levels in the summer months and daily admissions for pneumonia and influenza (Ozkaynak et al., 1990). An effect of atmospheric pollution, including oxidant gases, was also seen on the number of influenza cases in Sofia, Bulgaria (Kalpazanov et al., 1976); however, no demonstrable adverse effect on the course of a rhinoviral infection was seen in young adult male volunteers after exposure to moderate levels of ozone (0.3 ppm for 6 h/day over a period of five days) (Henderson et al., 1988), and children living in areas with high ozone concentrations had lowered CD4:CD8 ratios of peripheral lymphocytes but no higher incidence of chest colds (Zwick et al., 1991). The phagocytic activity of alveolar macrophages (obtained by bronchoalveolar lavage) and other functions were impaired in human volunteers exposed to ozone (Devlin et al., 1991). The sensitivity of human and mouse macrophages to the effects of ozone is similar (Selgrade et al., 1995).

Not all epidemiological studies have showed a correlation between air pollution and respiratory disease. Some of the discrepancies from experimental studies may be due to the parameters used to assess enhancement of infection. In experimental studies, increases in viral titres in the respiratory tract tend to be taken as an indication that the exposure enhances infection, whereas epidemiological studies rely on symptoms, many of which could be related to enhanced inflammatory or even allergic responses to the virus in the absence of viral replication.

Controlled studies (in an environmental chamber) showed that acute exposure to ozone causes an inflammatory response in the lower airways of human subjects (Koren et al., 1989; Devlin et al., 1991). The inflammatory response was manifested by increases in various inflammatory indicators including polymorphonuclear neutrophils (Figure 34), proteins, fibronectin, IL-6, and tryptase (Koren et al., 1989, 1994).

Figure 34. Cytocentrifuge preparation of bronchoalveolar lavage fluid from a control person (a) and an individual exposed to ozone (b)



Note the presence of polymorphonuclear leukocytes (arrows) in the bronchoalveolar lavage fluid from the ozone-exposed person; May Grünwald-Giemsa stain.

The proinflammatory effects of ozone raise the possibility that it can increase the sensitivity of people with atopic asthma to challenge with a specific allergen. Several studies have investigated the effect of exposure to pollutants on subsequent reactivity to antigen in atopic human volunteers. Molfino et al. (1991) reported that exposure to 0.12 ppm ozone significantly increased bronchial responsiveness to antigen in some individuals. Although they acknowledged weaknesses in the design of the experiment and recommended that the findings be confirmed, their observations are in accordance with those of the majority of epidemiological studies. In contrast, Bascom et al. (1990) found no alteration in the acute response to nasal antigen challenge in allergic patients pre-exposed to ozone in comparison with exposure to air; however, they did report increased upper respiratory tract inflammation after exposure to ozone in these patients in the absence of antigen challenge. Similarly, the bronchial response to inhaled grass

pollen was unaffected by prior exposure to 0.1 ppm nitrogen dioxide (Orehek et al., 1981). The topic of sensitization and the role of ozone in exacerbating asthma has been reviewed (Koren & Blomberg, in press).

2.4.3 *Pesticides*

Pesticides can alter the human immune system. For example, women chronically exposed to low levels of aldicarb-contaminated groundwater had altered numbers of T cells and decreased CD4:CD8 ratios (Fiore et al., 1986). While this finding was not confirmed in studies in animals (see section 2.2.3.4), follow-up studies by Mirkin et al. (1990) confirmed the immune changes in those individuals still available for study, although the population was considerably smaller.

2.4.4 *Halogenated aromatic hydrocarbons*

A number of chemical accidents have resulted in human exposure to halogenated aromatic hydrocarbons. A feed supplement for lactating cows, inadvertently contaminated with polybrominated biphenyls, was used in more than 500 dairy herds and poultry farms in Michigan (United States) in 1973. Diary farm residents had reduced proportions of circulating T lymphocytes and reduced lymphoproliferative responsiveness *in vitro* (Bekesi et al., 1978); these changes persisted during follow-up (Bekesi et al., 1987). Silva et al. (1979), however, were unable to detect any immune abnormalities in a similarly exposed cohort.

In Taiwan, more than 2000 people were exposed in 1979 to rice oil contaminated with PCBs and polychlorinated dibenzofurans. The clinical features in many of the exposed individuals included chloracne, pigmentation of skin, liver disease, and respiratory infections. When the immune status of the exposed individuals was examined one year after exposure, decreased concentrations of serum IgM and IgA, but not IgG, were reported, in addition to decreased numbers of circulating Th cells. The proportions of Ts/Tc cells and B lymphocytes were within the control values. Suppression of delayed-type hypersensitivity to recall antigens (streptokinase, streptodornase, tuberculin), enhanced mitogen-induced lymphocyte proliferation, and increases in sinopulmonary infections have been reported in this population (Chang et al., 1981, 1982; Lu & Wu, 1985). Many of the effects were transient, since two years after exposure most of the clinical abnormalities and laboratory parameters had returned to normal. A similar incident occurred in Japan in 1978, resulting in the 'yusho' syndrome. The immune system was assumed to be affected because of an increased frequency of respiratory infections and lowered serum IgM and IgA concentrations

(Shigematsu et al., 1978). Another incident of intoxication with PCBs and polychlorinated dibenzofurans occurred after exposure to contaminated soot of fires in electrical equipment (Elo et al., 1985). The exposed people had serum PCB concentrations up to 30 mg/litre. The number of blood T cells was lower five weeks after exposure but had returned to normal values seven weeks later. Lowered CD4:CD8 ratios and lymphocyte proliferation after mitogen stimulation were also seen. Nine of the 15 most heavily exposed persons suffered from at least one infection of the upper respiratory tract. No overt long-term effects or chloracne were observed.

The existing data also suggest that neonates are particularly sensitive to the immunotoxic effects of PCBs. Thus, higher incidences of colds and gastrointestinal (vomiting, abdominal pain) and dermatological (eczema, itchy skin) manifestations were observed in breast-fed infants of women occupationally exposed to the PCBs Kanechlor 500 and 300 than in infants born to unexposed women. The incidence of these symptoms increased with increasing length of breast-feeding (Hara, 1985). Epidemiological studies of women who consumed contaminated fish from the Great Lakes indicated that the maternal serum PCB level during pregnancy was positively associated with the number and type of infectious illnesses suffered by their breast-fed infants, especially during the first four months of life. The incidence of infections in the infant was correlated strongly with the highest rate of maternal fish consumption (Swain, 1991).

A number of studies have been conducted of the immune status of people exposed to TCDD. In 1976, an accident occurred at a chemical plant in Seveso, Italy, in which high concentrations of TCDD were released into the local environment. An evaluation of 44 exposed children (20 with chloracne) showed no overt changes in immune status (Reggiani, 1980), although the adequacy of the control populations used has been questioned. In a study of residents of an area of Missouri (United States) with long-term exposure (average, three years) to low levels of TCDD in contaminated soil, no clinical symptoms were recorded, although a number of individuals showed changes in cell-mediated immunity, manifested as altered delayed-type hypersensitivity (Hoffman et al., 1986). In the follow-up investigation, however, the skin anergy was not confirmed (Evans et al., 1988). The serum concentration of the thymic hormone thymosin-a1, which has been related to the toxic action of this compound on the thymus, was reduced (Stehr-Green et al., 1989; Hoffman, 1992). Jennings et al. (1988) found an increased frequency of circulating antinuclear antibodies and immune complexes in TCDD-exposed workers.

2.4.5 *Metals*

Exposure to metals may also affect the immune system. Workers with elevated blood lead levels (30–90 μg/100 ml) had increased suppressor cell activity (Cohen et al., 1989), lowered lymphocyte proliferation after mitogen stimulation *in vitro* (Jaremin, 1983), decreased IgA concentrations in saliva, and lowered complement C3 levels (Ewers et al., 1982). These individuals also had an enhanced prevalence of respiratory infection (Ewers et al., 1982). The immunotoxic effects of lead may be dose-dependent, since neither humoral nor cellular parameters were affected after long-term, low-level exposure (Reigart & Graber, 1976; Kimber et al., 1986). In unrelated studies, the cationic heavy metal mercury was associated with immune complex disease in humans (Makker & Aikawa, 1979).

In contrast to the database on the immunotoxic effects of cadmium, lead, and mercury in experimental animals *in vivo* and the results of mechanistic studies *in vitro*, the data on the effects of heavy metals on the human immune system are scanty and refer mainly to occupational exposure. These studies nevertheless provide evidence that at least mercury and lead affect the immune system (Moszczynski et al., 1990a; Bernier et al., in press).

Significantly decreased levels of serum IgG and IgA, but not IgM, IgD, or IgE, were reported in workers occupationally exposed to metallic mercury vapours for 20 years in comparison with unexposed controls (Moszczynski et al., 1990b). These workers had blood mercury levels of < 50 μg/litre. Similarly, significantly decreased IgG and IgA levels were observed in workers with urinary mercury levels of 0.029–0.545 mg/litre (Bencko et al., 1990). Studies of a small number of people exposed to mercury in dental amalgams have shown increased levels of IgE (Anneroth et al., 1992), an increased incidence of asthma (Drouet et al., 1990), and development of contact dermatitis (Gonçalo et al., 1992). Total lymphocyte, CD4, and CD8 levels were higher in exposed people than in controls (Eedy et al., 1990).

Epidemiological data indicate that the main effects of occupational exposure to lead are on cellular aspects of the immune system and that humoral parameters remain relatively insensitive to such exposure. Thus, serum IgG, IgM, and IgA levels remained within the normal range in workers exposed for 4–30 years, with a mean blood lead level of 38.4 μg/dl, in comparison with a mean control level of 11.8 μg/dl (Kimber et al., 1986). Similarly, no effects were noted on serum IgG or IgA levels in a cohort exposed to lead oxides at a reported concentration within the plant of 266 μg/m^3, who had an estimated blood lead level of 64 μg/dl; however, the response of lymphocytes from the exposed

group to stimulation with phytohaemagglutinin and concanavalin A *in vitro* was significantly lower than that of controls (Alomran & Shleamoon, 1988). Decreased serum Ig levels were reported in occupationally exposed workers with a mean blood lead level of 46.9 µg/dl, but the duration of exposure was not reported (Castillo-Mendez et al., 1991). In another study, no significant effects were noted on serum immunoglobulin levels after exposure to lead, but the levels of secretory IgA, which plays a major role in the defence against respiratory and gastrointestinal infections, was significantly decreased in workers with blood lead levels of 21–90 µg/dl. The incidence of influenza infections per year was significantly higher in these workers than in the control group (Ewers et al., 1982).

Studies on the effects of lead on lymphocyte levels in occupationally exposed workers have produced inconclusive results. One study found an increase in absolute B lymphocyte counts and CD8 cells (Coscia et al., 1987), while a decrease in total T lymphocytes and the CD4 subset was reported in another set of workers (Fischbein et al., 1993).

2.4.6 *Solvents*

Certain organic solvents may induce immune changes in humans. Benzene-induced pancytopenia with associated bone-marrow hypoplasia, a classical sign of chronic exposure to benzene, results in an immunodeficiency state due to the reduced numbers of immunocompetent cells (Goldstein, 1977). Alterations in the numbers of certain lymphocyte subsets, e.g. CD3 and CD4 lymphocytes, have also been reported in workers exposed to solvents (Denkhaus et al., 1986), suggesting that the effects may be somewhat specific.

2.4.7 *Ultraviolet radiation*

Numerous reports have shown that UVR inhibits contact hypersensitivity of the skin to sensitizers such as dinitrochlorobenzene (DNCB) (O'Dell et al., 1980; Halprin et al., 1981; Hersey et al., 1983a,b; Kalimo et al., 1983; Sjovall et al., 1985; Friedmann et al., 1989; Yoshikawa et al., 1990; Vermeer et al., 1991; Cooper et al., 1992). The dose required to produce immunosuppressive effects in humans is similar to that in C57Bl/6 mice, the strain phenotypically most sensitive to UVR-induced immune suppression (Noonan & Hoffman, 1994).

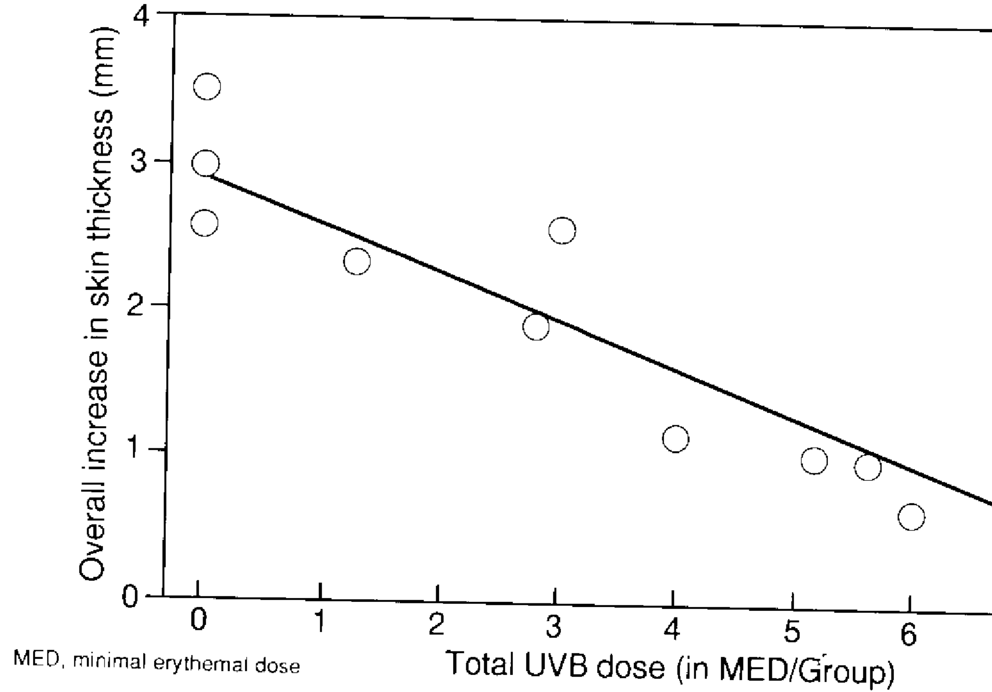
Human buttock skin was exposed to four daily doses of 144 mJ/cm^2 UVR, and the irradiated site was sensitized with DNCB immediately after the last exposure; the inner surface of the forearm was challenged 30 days later with DNCB and contact hypersensitivity assessed. Forty percent of the volunteers failed to develop contact

hypersensitivity and were designated sensitive, suggesting that, as in mice, susceptibility to UVR is genetically controlled. The sensitive phenotype also appeared to be a risk factor for the development of skin cancer (Yoshikawa et al., 1990). Suppression of contact hypersensitivity is seen in a similar percentage of black-skinned individuals, indicating that melanin cannot protect against this phenomenon (Vermeer et al., 1991). In another study, human buttock skin was exposed to 0.75 or two minimal erythemal doses (MED) of UVB (1 MED = 29.1–32.5 mJ/cm^2, depending on the individual) for four days and sensitized through irradiated skin immediately after the last exposure to DNCB; subjects were challenged with diluted DNCB at a distal site three weeks later. Some subjects were also exposed to four MED (moderate sunburn) and sensitized three days later with DNCB. Analysis of overall individual responses revealed decreased frequencies of fully successful immunizations in all UVB-exposed groups. The rate of immunological tolerance to DNCB (lasting up to four months) in the groups that were initially sensitized on skin that had received erythemagenic doses of UVB was 31%, whereas it was 7% in unirradiated controls (Cooper et al., 1992).

A dose–response relationship was established in the studies of Cooper et al. (1992) in a comparison of subjects with types I–III skin (fair to moderately fair) who received various doses or schedules of UVR from a bank of FS20 fluorescent sun lamps (rich in UVB) with respect to their ability to mount an immune response to DNCB. A linear inhibition of immune responsiveness was seen, the first detectable decrease occurring at 0.75 of the individual's MED, reaching complete inhibition of responsiveness for 95% of subjects when two MED were administered every day for four days before immunization. Similar inhibition occurred when DNCB was administered through skin that had been exposed to a single dose of four MED three days earlier. A dose–response curve for fair-skinned subjects was constructed by plotting the dose in total MED administered against the degree of immune response to DNCB (Figure 35). The 50% immune suppressive dose was calculated to be about 100 mJ/cm^2 of UVB.

Unresponsiveness to a contact sensitizer applied to UV-irradiated skin can thus be induced in a proportion of individuals after exposure to moderate levels of UVR, and at least some individuals become immunologically tolerant in a manner similar to experimental animals. Taken together, these data suggest that the systemic immunosuppression induced in mice by UVR also occurs in humans, possibly through a similar mechanism. UVR appears to alter antigen presentation and the expression of Langerhans (CDla^{-}DR^{+}) cells, which is followed by an influx of CDla^{-}DR^{-} monocytes that preferentially activate CD4^{-}

Figure 35. Dose–response curve for ultraviolet B (UVB) radiation-induced suppression of contact hypersens
in fair-skinned subjects



MED, minimal erythemal dose

(suppressor–inducer) cells, which induce maturation of $CD8^+$ Tc lymphocytes (Cooper et al., 1986; Baadsgaard et al., 1990). The UVR wavelengths responsible for induction of $CD1a^-DR^+$ cells are predominantly within the UVB band and to a lesser extent in the C band (Baadsgaard et al., 1987, 1989).

UVR from solarium lamps also suppressed NK cell activity in the blood of subjects exposed for 1 h per day for 12 days and tested one and seven days after exposure; the activity returned to normal by 21 days after exposure (Hersey et al., 1983a). The effects of UVR on NK cell activity are attributed to A radiation (Hersey et al., 1983b).

The depressed immune function observed in irradiated rodents reflects anecdotal observations in humans, i.e. that exposure to sunlight exacerbates certain infectious diseases, particularly those involving the skin. For example, it was noted at the turn of the century that smallpox lesions were worsened by exposure to sunlight (Finsen, 1901), and herpetic lesions and viral warts may be reactivated or exacerbated by sunlight (Giannini, 1990). It has also been hypothesized that sunlight affects susceptibility to infection with the bacteria that cause leprosy (Patki, 1991). Lesions of *Herpes simplex* virus type I and type II can be reactivated by exposure to UVR (Spruanoe, 1985; Klein, 1986). Using the criteria established by Yoshida & Streilin (1990) for the UVB-sensitive phenotype, Taylor et al. (1994) reported that 66% of individuals who have a history of herpes lip lesions provoked by exposure to sunlight were sensitive to UVB, in comparison with 40-45% of the general population and 92% of skin cancer patients. Exposure of immunosuppressed patients to sunlight can increase the incidence of viral warts caused by papillomavirus (Boyle et al., 1984; Dyall-Smith & Varigos, 1985). It is also known that UVR exacerbates the clinical course of systemic lupus erythematosus, an autoimmune disease (Epstein et al., 1965). The effects of UVR on the risk of infectious disease have been reviewed by Koren et al. (1994). In contrast, certain infectious diseases appear to be cured by sunlight; the most notable are erysipelas (Giannini, 1990), a skin disease caused by *Streptococcus*, and skin lesions caused by *Herpes zoster* virus.

2.4.8 Others

A large number of therapeutic drugs and drugs of abuse may also alter human immune function in humans. These include:

Therapeutic agents
Alkylating agents
- Nitrogen mustards: cyclophosphamide, L-phenylalanine mustard, chlorambucil
- Alkyl sulfonates: busulfan
- Nitrosoureas: carmustine (BCNU), lomustine (CCNU)
- Triazenes: dimethyltriazenoimidazolecarboxamide (DTIC)

Anti-inflammatory agents
- Aspirin, indomethacin, penicillamine, gold salts
- Adrenocorticosteroids: prednisone

Antimetabolites
- Purine antagonists: 6-mercaptopurine, azathioprine, 6-thioguanine
- Pyrimidine antagonists: 5-fluorouracil, cytosine arabinoside, bromodeoxyuridine
- Folic acid antagonists: methotrexate (amethopterine)

Natural products
- Vinca alkaloids: vinblastine, vincristine, procarbazine
- Antibiotics: actinomycin D, adriablastine, bleomycin, daunomycin, puromycin, mitomycin C, mithramycin
- Antifungal agents: griseofulvin
- Enzymes: L-asparaginase
- Cyclosporin A

Estrogens: diethylstilbestrol, ethinylestradiol

Substances of abuse
Ethanol
Cannabinoids
Cocaine
Opiates

Adapted from Dean & Murray (1990)

3. STRATEGIES FOR TESTING THE IMMUNOTOXICITY OF CHEMICALS IN ANIMALS

3.1 General testing of the toxicity of chemicals

The fact that substances used in various aspects of modern life can be simultaneously beneficial and harmful to human life creates a legislative and regulatory dilemma. In order to balance the desire to use the many new substances that enter the market every year and the economic benefit that is associated with their use on the one hand with the health and safety of the population on the other is an important challenge to governmental authorities. Legislative and regulatory efforts to minimize and control the risk of adverse effects on human health has resulted in a system for assessing and classifying the potential risk of exposure to chemicals. Potential adverse effects can be assessed in studies with experimental animals. In conducting such studies, attention must be paid to ethical and regulatory requirements for animal welfare and to good laboratory practice.

In assessing and evaluating the toxic characteristics of a chemical, its oral toxicity may be determined once initial information has been obtained by acute testing. Toxicity is routinely tested according to guidelines, one of which is guideline No. 407 of the Organisation for Economic Co-operation and Development (OECD) for testing of chemicals, the 'Repeated Dose Oral Toxicity - Rodent: 28-day or 14-day Study' (Organisation for Economic Co-operation and Development, 1981). This guideline has undergone three revisions, the most recent of which (January 1994) includes parameters of immunotoxicological relevance (see Table 7). Depending on the amount of a chemical to be produced and the expected exposure to the chemical, testing according to this guideline may in many countries provide the only information on its safety, including potential toxicity to the immune system. The information yielded by this type of testing is therefore decisive in determining how chemicals are used in society. Subsequent guidelines have been defined for use in follow-up studies if more exposure is expected or if there is a suspicion of toxicity on the basis of structural analogy with other known compounds. These include 90-day studies of oral toxicity, long-term studies, and studies of reproductive effects. Although such guidelines include more parameters of the immune system than OECD test guideline No. 407, detection of potential immunotoxicity may still not be adequately addressed. In practice, the best procedure is to carry out appropriate tests on a case-by-case basis,

Table 7. Parameters of OECD Test Guideline 407 that relate to the immune system

Current Guideline 407 (adopted 21 May 1981)	Proposal for updating Guideline 406 (February 1991)	Proposal for updating Guideline 407 (revision of January 1993)	Proposal for updating Guideline 407 (revision of January 1994)
Total and differential leukocyte count	Total and differential leukocyte count	Total and differential leukocyte count	Total and differential leukocyte count
	Weight of spleen and thymus	Weight of spleen and thymus	Weight of spleen and thymus
Histopathology of spleen	Histopathology of spleen, thymus, lymph node, and bone marrow	Histopathology of spleen, thymus, lymph nodes (one relevant to route of administration and a distant one to cover systemic effects), and bone marrow	Histopathology of spleen, thymus, lymph nodes (one relevant to the route of administration and a distant one to cover systemic effects), small intestine (including Peyer's patches), and bone marrow
Histopathology of target organs	Histopathology of target organs	Histopathology of target organs	Histopathology of target organs

OECD. Organisation for Economic Co-operation and Development

at increasing levels of complexity, when concerns are raised in more general toxicological studies.

An insight into the type of information that the OECD test guideline No. 407 yields is given below. In this test, the substance is administered orally in daily graduated doses to groups of experimental animals, one dose per group for 28 or 14 days. The preferred rodent species for this test is the rat, although others may be used. At least three doses and a control should be used. The highest dose should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation; the lowest dose should not produce any evidence of toxicity and should exceed a usable estimate of human exposure, when available. Ideally, the intermediate dose level(s) should produce minimal observable toxic effects.

In compliance with the guideline, the following examinations are carried out:

(a) haematology, including haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as clotting time, prothrombin time, thromboplastin time, or platelet count;

(b) clinical biochemistry of blood, including blood parameters of liver and kidney function. The selection of specific tests is influenced by observations on the mode of action of the substance. Suggested determinations are: calcium, phosphorus, chloride, sodium, potassium, fasting glucose, serum alanine aminotransferase, serum aspartate aminotransferase, ornithine decarboxylase, γ-glutamyl transpeptidase, urea nitrogen, albumin, blood creatinine, total bilirubin, and total serum protein. Other determinations that may be necessary for an adequate toxicological evaluation include analyses of lipids, hormones, acid–base balance, methaemoglobin, and cholinesterase activity. Additional clinical biochemistry may be used when necessary, to extend the investigation of any observed effects.

(c) pathology, including gross necropsy, with examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents. The liver, kidneys, adrenal glands, and testes are weighed wet as soon as possible after dissection to avoid drying. Liver, kidney, spleen, adrenal glands, heart, and target organs showing gross lesions or changes in size are preserved in a suitable medium for possible future histopathological examination. Histopathological examination is performed on the

preserved organs or tissues of the group given the high dose and the control group. These examinations may be extended to animals in other dosage groups, if considered necessary to further investigate changes observed in the high-dose group.

A properly conducted 28- or 14-day study will provide information on the effects of repeated doses and can indicate the need for further, longer-term studies. It can also provide information on the selection of doses for longer-term studies.

It is clear that the 1994 guideline is not suitable for adequate assessment of the potential adverse effects of exposure to a test chemical on the immune system, since the immunological parameters are restricted to total and differential leukocyte counts and the histopathology of the spleen. An evaluation of this test (Van Loveren & Vos, 1992) indicated that over 50% of the immunotoxic chemicals in a series of about 20 chemicals would not have been identified as such if the tests had strictly adhered to the guideline. In fact, it is even doubtful if chemicals indicated as immunotoxic only on the basis of guideline No. 407 would in practice have been picked up. For instance, in a toxicological experiment, a small but significant change in the percentage of basophilic leukocytes would by itself probably not be considered to be biologically relevant in the absence of any other parameter to suggest that an effect on the immune system might have been present.

These data indicate that extension of OECD test guideline No. 407 is necessary in order adequately to assess potential immunotoxicity. It is recommended that additional immunological parameters be included in this guideline in order to increase its power (Vos & Van Loveren, 1987; Basketter et al., 1994).

Guidelines also exist for follow-up studies if greater exposure is expected or if there is a suspicion of toxicity on the basis of structural analogy with other compounds. In these studies, potential toxicity to the immune system is generally addressed somewhat more extensively than in guideline No. 407. For instance, in a 90-day study of oral toxicity, the OECD guidelines prescribe that histopathological examination be done on the thymus, a representative lymph node, and the sternum with bone marrow, in addition to the spleen. Even with these additional parameters, it is highly questionable whether potential immunotoxicity is adequately assessed. For this purpose, a variety of tests is available, which are described in section 4. Depending on what is already known about the toxicity of the test compound, different panels of tests (also referred to as tiers) are selected for immunotoxicological evaluation. Usually, if little or no information is available, a dose range including high doses is used; lower, overtly nontoxic doses are chosen if some

knowledge is available about the physical and chemical properties, toxicokinetics, structure–activity relationships, and intended use.

3.2 Organization of tests in tiers

Immunotoxicity can be assessed in a tiered approach (Luster et al., 1988; Van Loveren & Vos, 1989). Generally, the objective of the first tier is to identify potentially hazardous compounds (hazard identification). If potential immunotoxicity is identified, a second tier of tests is carried out to confirm and further characterize the immunotoxicity.

Various approaches have been suggested for evaluating the potential immunotoxicity of compounds. Most are similar in design, in that the first tier is usually a screen for immunotoxicity and the second tier consists of a more specific confirmatory set of studies or in-depth mechanistic studies. Since the use of the tiers is usually tailored to the goals or objectives of the organization that proposes them, they differ in respect of the specific assays recommended and the organization of the assays into tiers.

3.2.1 *United States National Toxicology Program panel*

The tiers and assays originally adopted by the NTP, based on the proposed guidelines for immunotoxicity evaluation in mice reported by Luster et al. (1988), are shown in Table 8.

In this approach, the tier 1 assay is limited; it includes assays for both cell-mediated and humoral-mediated immunity and for innate (nonspecific) immunity with the inclusion of NK cell assays. It also includes immunohistopathology, which is part of the standard protocol for studies of subchronic toxicity and carcinogenicity conducted by the NTP. Tier II represents a more extensive evaluation and includes additional assays for assessing effects on cell-mediated, humoral, and innate immunity, in addition to host resistance. In this approach, animals are usually evaluated at only one time, so that the possibility for recovery or reversibility of immunological changes is not evaluated. A 14-day exposure period is employed routinely; however, 30- and 90-day exposures have been used, depending on the pharmacokinetic properties of the chemical being tested. The dose used in this tier system tends to be lower than those in several of the other approaches followed. In the NTP approach, dose levels are selected whenever possible that have no effect on body weight or other toxicological end-points. The approach has therefore focused on compounds for which the immune system is the most sensitive target. This is in marked contrast to other approaches, in which the highest dose is usually the maximum tolerated dose.

Table 8. Panel adopted by the US National Toxicology Program for detecting immune alterations after exposure of rodents[a] to chemicals or drugs

Parameter	Procedures
Screen (Tier I)	
Immunopathology	Haematology: complete and differential blood count Weights: body, spleen, thymus, kidney, liver Cellularity: spleen Histology: spleen, thymus, lymph node
Humoral immunity	Enumerate IgM antibody plaque-forming cells to T-dependent antigen (sheep red blood cells) Lipopolysaccharide mitogen response
Cell-mediated immunity	Lymphocyte blastogenesis to mitogens (concanavalin A) Mixed leukocyte response to allogeneic leukocytes
Nonspecific immunity	Natural killer cell activity
Comprehensive (Tier II)	
Immunopathology	Quantification of splenic B and T cell numbers
Humoral-mediated immunity	Enumeration of IgG antibody response to sheep red blood cells
Cell-mediated immunity	Cytotoxic T lymphocyte cytolysis; delayed hypersensitivity response
Nonspecific immunity	Macrophage function: quantification of resident peritoneal cells, and phagocytic ability (basal and activated by macrophage activating factor)
Host resistance challenge models (end-points)[b]	Syngeneic tumour cells PYB6 sarcoma (tumour incidence) B16F10 melanoma (lung burden) Bacterial models: *Listeria monocytogenes* (mortality); *Streptococcus species* (mortality) Viral models: influenza (mortality) Parasite models: *Plasmodium yoelii* (parasitaemia)

[a] The testing panel was developed using B6C3F1 female mice.
[b] For any particular chemical tested, only two or three host resistance models are selected for examination.

The assays that make up the NTP tier approach have undergone various revisions, partly on the basis of an immunotoxicological review of compounds evaluated in this tier structure (Luster et al., 1992). The mitogen assays were first moved from tier I to tier II and have now been dropped altogether: They were found to be insensitive and to add little when run in conjunction with other assays that have a proliferative component, such as the mixed leukocyte response and the plaque assay. Furthermore, the only macrophage phagocytic assay routinely carried out in immunotoxicological studies conducted for the NTP is evaluation of the functional activity of the mononuclear phagocyte system, which is an in-vivo assay for phagocytosis.

Studies by Luster et al. (1992) show that the potential immunotoxicity of a compound can be reasonably predicted with a few selected assays. As additional data become available, further changes to the NTP tiers will most likely be forthcoming.

3.2.2 *Dutch National Institute of Public Health and Environmental Protection panel*

The tier approach for immunotoxicological evaluation followed at the National Institute of Public Health and Environmental Protection (RIVM) in the Netherlands (Vos & Van Loveren, 1987) is shown in Table 9. This approach is based essentially on OECD test guideline No. 407, which suggests that the maximum tolerated dose be used as the high dose in the study. As a result, significantly higher doses are used than in the NTP approach in evaluating compounds for immunotoxicity. Additionally, the standard exposure period is 28 days, and the animal species routinely used is the rat instead of the mouse. This type of testing can therefore be performed in the context of studies in rats to determine the toxicological profile of a compound. At least three doses should be used, the highest having a toxic effect (but not mortality) and the lowest producing no evidence of toxicity. Moreover, immunotoxicity tests carried out in the context of such testing should not in any way influence the toxicity of the chemical (e.g. immunization or challenge with an infectious agent). In the NTP panel, the highest dose to which mice are exposed is chosen so that no overt toxicity, i.e. changes in body weight or gross pathological effects, is observed. As tests for immunotoxicity must be fairly sensitive in order to preclude false negatives, the NTP tier I includes functional assays. With a broader dose range that includes overt toxicity, potential immunotoxicity is more likely to be observed, without the inclusion of functional tests. If functional assays are to be included in the first tier, those tests that require sensitization of animals would require inclusion of satellite groups. In OECD test guideline No. 407 for testing chemicals, none of the other systems is approached functionally.

Table 9. Methods for detecting immunotoxic alterations in the rat evaluated by the Dutch National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands

Parameters	Procedures
Tier 1	
Nonfunctional	Routine haematology, including differential cell counts Serum IgM, IgG, IgA, IgE determination; lymphoid organ weights (spleen, thymus, local and distant lymph nodes) Histopathology of lymphoid tissues, including mucosa-associated lymphoid tissue Bone-marrow cellularity Analysis of lymphocyte subpopulations in spleen by flow cytometry
Tier 2	
Cell-mediated immunity	Sensitization to T-cell dependent antigens (e.g. ovalbumin, tuberculin, *Listeria*), and skin test challenge Lymphoproliferative response to specific antigens (*Listeria*) Mitogen responses (concanavalin A, phytohaemagglutinin)
Humoral immunity	Serum titration of IgM, IgG, IgA, IgE responses to T-dependent antigens (ovalbumin, tetanus toxoid, *Trichinella spiralis*, sheep red blood cells) by ELISA Serum titration of T-cell-independent IgM response to lipopolysaccharide by ELISA Mitogen response to lipopolysaccharide
Macrophage function	Phagocytosis and killing of *Listeria* by adherent spleen and peritoneal cells *in vitro* Cytolysis of YAC-1 lymphoma cells by adherent spleen and peritoneal cells
Natural killer function	Cytolysis of YAC-1 lymphoma cells by non-adherent spleen and peritoneal cells.
Host resistance	*Trichinella spiralis* challenge (muscle larvae counts and worm expulsion) *Listeria* challenge (spleen and lung clearance) Cytomegalovirus challenge (clearance from salivary gland) Endotoxin hypersensitivity; Autoimmune models (adjuvant arthritis, experimental allergic encephalomyelitis)

Ig, immunoglobulin; ELISA, enzyme-linked immunosorbent assay

It has been suggested that the NK cell assay be added to tier 1 (Van Loveren & Vos, 1992). Since the assay does not require animals to be sensitized or challenged, the same animals can be used without affecting other toxicological parameters, and thus an additional satellite group of animals is unnecessary.

3.2.3 *United States Environmental Protection Agency, Office of Pesticides panel*

The United States Environmental Protection Agency has proposed a tiered approach to the evaluation of biochemical pest control agents, which fall under the subdivision M guidelines for pesticides (Sjoblad, 1988). The proposed tiers are shown below. Tier 1 of this approach includes functional assays for evaluating humoral immunity, cell-mediated immunity, and innate immunity. Thus, while Tier 1 is considered by the Agency to be an immunotoxicity screen, it is much more encompassing than the first tier of the other approaches. By providing options in the selection of assays for tier 1, the approach can easily accommodate both the rat and the mouse as the laboratory animal species used. In this approach, the tier 2 studies provide information sufficient for risk evaluation, including information on the time course of recovery from immunotoxic effects and host resistance to infectious agents and tumour models. Additional functional tests would be required if a dysfunction were observed in tier 1 tests or if data from other sources indicated the compound could produce an adverse effect on the immune response.

Subdivision M guidelines: proposed revised requirements by the US Environmental Protection Agency for testing the immunotoxicity of biochemical pest control agents

I. Tier 1

A. Spleen, thymus, and bone-marrow cellularity

B. Humoral immunity (one of the following)
   1. Primary and secondary IgG and IgM responses to antigen; or,
   2. Antibody plaque-forming cell assay

C. Specific cell-mediated immunity (one of the following)
   1. One-way mixed lymphocyte reaction assay; or,
   2. Effect of agent on normal delayed-type hypersensitivity response; or,
   3. Effect of agent on generation of cytotoxic T-lymphocyte response

D. Nonspecific cell-mediated immunity
   1. Natural killer cell activity and
   2. Macrophage function

II. Tier 2

A. Required if:
1. Dysfunction is observed in tier 1 tests
2. Tier 1 test results cannot be definitively interpreted
3. Data from other sources indicate immunotoxicity

B. General testing features:
1. Evaluate time course for recovery from immunotoxic effects.
2. Determine whether observed effects impair host resistance to infectious agents or to tumour cell challenge.
3. Perform additional specific, but appropriate, testing essential for evaluation of potential risks.

This Agency has also suggested that immunotoxicological screening be conducted in evaluating conventional chemical pesticides (subdivision F guidelines; see below); however, unlike those of subdivision M, these guidelines are not designed as a tiered testing scheme. If the immunotoxicity screen listed in subheading I were added to subchronic and/or chronic studies in subdivision F, it would be a more effective screen for immunotoxicity than is currently available. If this proposed screen indicates that the immune system is a sensitive target, the Agency considers that it may be necessary to evaluate the risk for immunotoxic effects as under subheading II. Currently, these suggestions have not been promulgated as official guidelines or regulations.

Evaluations suggested by the US Environmental Protection Agency as appropriate additions to Subdivision F guidelines for immunotoxicity screening (subheading I) and possible additional data appropriate for risk evaluation of chemical pesticides (subheading II)

I. **Immunotoxicity screen**
- A. Serum immunoglobulin levels (e.g. IgG, IgM, and IgA)
- B. Spleen, thymus, and lymph node weights
- C. Spleen, thymus, and bone-marrow cellularity and cell viability
- D. Special histopathology (e.g. enzyme histochemistry, immunohistochemistry)
- E. More complete evaluation of 'premature' mortality of test animals, as possibly related to immunosuppressive effects

II. **Immunotoxicity risk evaluation**
- A. Host resistance to challenge with infectious agent and/or tumour cells
- B. Specific cell-mediated immune responses (e.g. mixed leukocyte response, delayed-type hypersensitivity response, cytotoxic T lymphocyte assays)[a]

C. Nonspecific cell-mediated immune responses (i.e. natural killer cell activity, macrophage function)[a]
D. Time course for recovery from adverse immunological effects

[a] Measures of specific and nonspecific cell-mediated immune responses that also may be considered useful in an immunotoxicity screen

3.2.4 *United States Food and Drug Administration, Center for Food Safety and Applied Nutrition panel*

The United States Food and Drug Administration is considering testing guidelines for evaluating the immunotoxic potential of direct food additives (Hinton, 1992). The multifaceted approach is included in the draft revision of the *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food* is (US Food and Drug Administration, 1993). The testing requirements are based on the 'concern level' of the substance, assigned on the basis of the available toxicological information or the substance's structural similarity to known toxicants and on estimated human exposure from its proposed use. A compound with high toxic potential and high exposure would be assigned a high initial 'concern level' (3), and one with low toxic potential and low exposure would be assigned a low initial level (1).

In general, substances will be evaluated for immunotoxic potential on a case-by-case basis. Two types of immunotoxicity tests and procedures are defined in this approach: Type 1 tests are those that do not involve perturbation of the test animal (i.e. sensitization or challenge). These are further divided into 'basic' tests, which include haematology and serum chemistry, routine histopathological examination, and determination of organ and body weights, and 'expanded' tests, which are logical extensions of the 'basic' tests and include those that can be performed retrospectively. Type 2 tests include injection of or exposure to antigens, infectious agents, vaccines, or tumour cells. In general, type 2 tests require a satellite group of animals for immunological evaluation. The sets of 'basic' and 'expanded' type 1 tests are defined as level-I immunotoxicity tests, and the sets of type 2 tests are defined as level-II tests. Some level-I tests can be used to screen for immunotoxic effects, while others focus on the mechanism of action or the cell types affected by the test substance. Level-II tests are conducted to define the immunotoxic effects of food and colour additives more specifically. The recommended testing scheme is shown below.

The functional tests generally require sensitization of exposed rats and controls and subsequent analysis of the responses to the sensitizing antigens. For this reason, functional tests are not readily conducted in the first tier of immunotoxicity testing. As guidelines for routine

Recommendations of the United States Food and Drug Administration for testing the immunotoxicity of direct food additives

Basic testing (rat model)

Complete blood count, differential white blood cell count;
Total serum protein, albumin:globulin ratio;
Histopathology, gross and microscopic (spleen, thymus, lymph nodes, Peyer's patches, and bone marrow);
Lymphoid organ and body weights

Retrospective level-I testing (possible in a standard toxicology study)

Electrophoretic analysis of serum proteins[a] (when positive or marginal effect is noted in basic testing);
Immunostaining of spleen and lymph nodes for B and T cells[a] (quantification of total immunoglobulins);
Serum autoantibody screen and deposition of immunoglobulins (micrometry for semiquantification 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 and thymus when indicated);
Quantification of total B and T cells (blood and/or spleen);
Mitogen stimulation assays for B and T cells (spleen);
Natural killer cell functional analysis (spleen);
Macrophage quantification and functional analysis (spleen);
Interleukin-2 functional analysis (spleen);
When indicated or for more complete analysis, other end-points such as total haemolytic complement activity assay in serum

Level-II testing with a satellite group or follow-up study for screening of functional immune effects

Kinetic evaluation of humoral response to T-dependent antigen (primary and secondary responses with sheep red blood cells, tetanus toxoid, or other);
Kinetic evaluation of primary humoral response to a T-independent antigen such as pneumococcal polysaccharides, trinitrophenyl-lipopolysaccharide, or other recognized antigens,
Delayed-type hypersensitivity response to known sensitizer of known T effector cell;
Reversibility evaluation

Enhanced level-II testing with a satellite group or follow-up study for evaluation of potential immunotoxic risk

Tumour challenge (MADB106 or other in rat);
PYB6 sarcoma (in mouse);
Infectivity challenge (*Trichinella, Candida* or other in rat; *Listeria* or other in mouse)

[a] Recommended for inclusion in basic testing

toxicology experiments preclude compromising the experiment by any agent other than the test chemical, the second tier of immunotoxicity testing, with immune function tests, requires a separate set of experiments. The antigens that are used to sensitize the exposed and control rats may be relatively simple antigens, such as ovalbumin or tetanus toxoid, or more complex antigens, such as sheep red blood cells, bacteria, or parasites. The responses can occur in various arms of the immune system, which consequently must be measured with different assays. For instance, humoral responses can be measured by determination of specific antibodies in serum; the appropriate tests for cellular responses are proliferative responses of lymphocytes to the specific antigens *ex vivo/in vitro* or delayed-type hypersensitivity responses to injection with antigen *in vivo*.

Not all functional assays require prior sensitization of the test animals, e.g. proliferative responses of lymphocytes *ex vivo/in vitro* to mitogens which are either specific for T cells, giving information on cellular immunity, or for B cells, providing data on humoral immunity. The phagocytic and lytic activity of macrophages and the nonspecific cytotoxic activity of NK cells can also be measured *ex vivo/in vitro*, without prior sensitization of the test animals. Both types of activity are examples of nonspecific defence mechanisms, directed to bacteria and certain tumour cells and to tumour cells and virally infected cells, respectively. Since measurement of these types of activity does not require prior sensitization of the host, such functional tests can be considered for inclusion in the first tier of testing for immunotoxicity in routine toxicology.

3.3 Considerations in evaluating systemic and local immunotoxicity

3.3.1 *Species selection*

Selection of the most appropriate animal model for immunotoxicology studies has been a matter of great concern. Ideally, toxicity testing should be performed with a species that responds to a test chemical in a pharmacologically and toxicological manner similar to that anticipated in humans, i.e. the test animals and humans metabolize the chemical similarly and have identical target organs and toxic responses. Toxicological studies are often conducted in several animal species, since it is assumed that the more species that show a specific toxic response, the more likely it is that the response will occur in humans. Data from studies in rodents on target organ toxicity at immunosuppressive doses for most immunosuppressive therapeutic

agents have generally been predictive of later clinical observations. Exceptions to the predictive value of rodent toxicological data are infrequent but occurred in studies of glucocorticoids, which are lympholytic in rodents but not in primates (Haynes & Murad, 1985). Although certain compounds exhibit different pharmacokinetic properties in rodents and in humans, rodents still appear to be the most appropriate animal model for examining the non-species-specific immunotoxicity of compounds, because of established toxicological knowledge, including similarities of toxicological profiles, and the relative ease of generating data on host resistance and immune function in rodents. Comparative toxicological studies should be continued and expanded, however, as novel recombinant biological compounds and natural products that enter safety testing will probably have species-specific host interactions and toxicological profiles.

The quantitative and possibly the qualitative susceptibility of an individual animal to the immunotoxicity of a selected agent can be influenced by its genetic characteristics, indicating not only a need to consider species but also strain. Rao et al. (1988) described two approaches for selecting appropriate genotypes for toxicity studies. The first is to select genotypes that are representative of an animal species, which by virtue of similar metabolic profiles may also exhibit a sensitivity similar to that of man, such as random-bred mice. A second approach is to attempt to identify genotypes that are uniquely suitable for evaluating a specific class of chemicals, such as the use of *Ah*-responsive rodent strains in studies with polyhalogenated aromatic hydrocarbons. In many cases, however, this approach requires considerable knowledge of the mechanisms of toxicity of the compound, which may not be available. One compromise has been to use Fl hybrids which have the stability, phenotypic uniformity, and known genetic traits of an inbred animal, yet have the vigour associated with heterozygosity. The description of the genetic relationships between inbred mouse strains on the basis of the distribution of alleles at 16 loci (Taylor, 1972) has made possible rational selection of appropriate Fl hybrids, such as the B6C3Fl mouse.

3.3.2 *Systemic immunosuppression*

Because of this complexity, the initial strategies devised by immunologists working in toxicology and safety assessment were to select and apply a tiered panel of assays in order to identify immunosuppressive or, in rare instances, immunostimulatory agents in laboratory animals (US National Research Council, 1992). Although the configuration of these testing panels varies according to the

laboratory conducting the test and the animal species used, they include measurements of one or more of the following: (i) altered lymphoid organ weights and histology, including immunohistology; (ii) quantitative changes in the cellularity of lymphoid tissue, peripheral blood leukocytes, and/or bone marrow; (iii) impairment of cell function at the effector or regulatory level; and/or (iv) increased susceptibility to infectious agents or transplantable tumours.

A variety of factors must be considered in evaluating the potential of an environmental agent or drug to adversely influence the immune system. These include appropriate selection of animal models and exposure variables, consideration of general toxicological parameters and mechanisms of action, as well as an understanding of the biological relevance of the end-points to be measured. Treatment conditions should be based on the potential route, level, and duration of human exposure, the biophysical properties of the agent, and any available information on the mechanism of action. Moreover, toxicokinetic parameters, such as bioavailability, distribution volume, clearance, and half-life, should be measured. Doses should be selected that will allow establishment of a clear dose–response curve and a no-observed-effect level NOEL). Although, for reasons explained earlier, it is beneficial to include a dose that induces overt toxicity, any immune change observed at that dose should not necessarily be considered to be biologically significant, since severe stress and malnutrition are known to impair immune responsiveness. Many laboratories routinely use three doses but generally conduct studies to define the range of doses before a full-scale immunotoxicological evaluation. If studies are being designed specifically to establish reference doses for toxic chemicals, additional exposure levels are advisable. In addition, inclusion of a 'positive control' group, treated with an agent that shares some of the characteristics of the test compound, may be advantageous when experimental and fiscal constraints permit.

The selection of the exposure route should reflect the most probable route of human exposure, which is most often oral, respiratory, or dermal. If it is necessary to deliver an accurate dose, a parenteral exposure route may be required; however, this may significantly change the metabolism or distribution of the agent from that which would occur following natural exposure.

3.3.3 *Local suppression*

Local immune suppression has received less attention than systemic immune suppression, and this is noteworthy, since the surface that is exposed to the environment, i.e. the skin, the respiratory tract, and the

gastrointestinal tract, are the major ports of entry of antigens and pathogens. While a variety of validated methods are currently available to detect chemical skin sensitizers in humans and experimental animals, there is no standard method to assess the potential of chemicals to induce local immunosuppression in the skin. Furthermore, although increasing evidence suggests that the consequence of skin immunosuppression would be an increase in neoplastic and infectious diseases of the skin, definitive data are still lacking. In contrast, considerable efforts are being deployed to develop sensitive models for monitoring skin irritants. For example, human keratinocyte cultures and keratinocyte–fibroblast co-cultures have been examined for end-points ranging from changes in cell viability to production and loss of various bioactive products. Few test systems are available for the gut and the respiratory tract.

4. METHODS OF IMMUNOTOXICOLOGY IN EXPERIMENTAL ANIMALS

This section comprises general descriptions of methods used for evaluating immunotoxicity.

4.1 Nonfunctional tests

4.1.1 *Organ weights*

It is routine practice in toxicology to weigh organs that are potentially affected by the compound that is being investigated. The immunological organs that are suitable for weighing in screening for potential immunotoxicity are: the thymus, which plays a decisive role in the development of the immune system and which is affected by many immunotoxicants; the spleen, which is the repository for many recirculating lymphocytes; and the lymph nodes, which are important for the induction of immune processes. Determination of the weight of draining lymph nodes (depending on the route of exposure, i.e. mesenteric nodes for oral exposure and bronchial nodes for inhalation) in addition to distant lymph nodes (such as popliteal lymph nodes for determining systemic effects) is the best. Mesenteric nodes, in particular, occur in a string within non-lymphoid fatty tissue, and care must be taken to remove this non-lymphoid tissue so that the weight can be adequately determined. The cellularity of these organs is another indication of the effects of chemicals on the immune system. Furthermore, cell suspensions can be prepared from lymphoid organs in order to assess the distribution of subpopulations of lymphoid cells and to test their functionality within the organs. Under OECD guideline No. 407, histopathological examination of lymphoid organs and tissues is crucial for detecting the effects of chemicals on the immune system. Therefore, upon termination of exposure to a compound in a toxicological experiment, organs such as the spleen should first be weighed; subsequently, they are divided into parts which are also weighed, and one or more parts are used for histopathological examination and the remainder to prepare cell suspensions that can be evaluated for distribution of lymphocyte subpopulations or can be assessed functionally.

4.1.2 *Pathology*

The histopathology of the thymus, spleen, and draining and distant lymph nodes, of the mucosal immune system (Peyer's patches in the gut or bronchus and nose-associated lymphoid tissue in the respiratory

tract), and of the skin immune system should be evaluated, depending on the route of exposure. The first level of evaluation should be of haematoxylin-eosin stained, paraffin-embedded slides. A more sophisticated level of evaluation is immunoperoxidase staining of special cell types.

Many monoclonal antibodies are available for mice, rats, and humans to detect differentiation antigens, cell adhesion molecules, and activation markers on haematolymphoid and stromal cells involved in immune responses. A list of some monoclonal antibodies that can be used in the identification of leukocytes and stromal cells in (frozen) sections of lymphoid tissue is presented in Table 10; a selection of these is reviewed below.

For a further description of these markers, and the cells that express them, reference may be made to the introductory section and to descriptions in the literature (Brideau et al., 1980; Bazin et al., 1984; Dallman et al., 1984; Dijkstra et al., 1985; Joling et al., 1985; Vaessen et al., 1985; Joling, 1987; Hünig et al., 1989; Kampinga et al., 1989; Portoles et al., 1989; Schuurman et al., 1991a).

These markers are usually stained in frozen tissue sections of 6–8 µm, fixed in acetone. A three-step immunoperoxidase procedure is most suitable: the first step includes the monoclonal antibody specific for the determinants to be studied (see above), the second step, rabbit anti-mouse immunoglobulin, and the third step, swine anti-rabbit immunoglobulin, the latter two antibodies conjugated to horseradish peroxidase. The peroxidase activity can be developed by 3,3-diaminobenzidine tetrahydrochloride with hydrogen peroxide as substrate, and the sections can be counterstained with Mayer's haematoxylin to facilitate evaluation. Negative controls are prepared by omitting the antibody in the first step or replacing it with an irrelevant one. Under these conditions, only the peroxidase activity of polymorphonuclear cells, when present, is visualized, and no immunolabelling is found.

In general, histopathological evaluation provides a semi-quantitative estimation of effects. The experienced pathologist can do this adequately in studies carried out 'blind', especially if the effects are clear. For more subtle effects, morphometric analysis is a valuable addition, especially when supported by software for assessing the values of parameters such as size, surface, and intensity of staining. The compartments of the immune system, i.e. specific T and B lymphocyte areas in spleen and lymph nodes and cortical and medullary areas of immature and differentiated thymocytes within the thymus, and the numbers of specialized cells per surface unit are parameters that are well suited for morphometric analysis.

Table 10. Some monoclonal antibodies to leukocytes and stromal cells used in immunohistochemical studies of tissue sections and flow cytofluorography on cell suspensions

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
T Cells					
CD1	gp43,45, 49,12	Ly-38		OKT6, a-Leu-6	Lymphocytes in thymic cortex, Langerhans cells in skin, interdigitating cells
CD2	gp50	Ly-37, NSM46.7, RM2-5	MRC OX-34, MRC OX-54, MRC OX-55	a-Leu-5, OKT11	All T cells in thymus and peripheral lymphoid organs, subset of macrophages (rat). Sheep erythrocyte receptor, leukocyte function antigen-2 (LFA-2). Ligand f or LFA-3 (CD58)
CD3	gp19-29	CD3-1,KT3, 145-2C11	IF4, G4.18	a-Leu-4, OKT3	T Cells in thymic medulla and peripheral lymphoid organs (T-cell receptor-associated, cytoplasmic in precursor T cells in thymus)
CD4	gp65	Ly-4, L3T4, YTS 177.9	MRC OX-35, MRC OX-38, (ER2), W3/25	a-Leu-3, OKT4	Lymphocytes in thymic cortex, about two-thirds of T cells in peripheral lymphoid organs, subset macrophages, microglia T helper/inducer and delayed-hypersensitivity phenotype. MHC class II binding, receptor for human immunodeficiency virus
CD5	gp65-62	Ly-1,Lyt-1	MRC OX-19, HIS47	a-Leu-1	Lymphocytes in thymic cortex (faint). All T cells in thymic medulla and peripheral lymphoid tissue, subset of B cells
CD6	gp120			Tü 33	T Cells in thymic medulla and peripheral lymphoid organs

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
T cells (contd)					
CD7	gp41			WT1,B-F12, a-Leu-9	Prethymic T-cell precursors, all T cells in thymus and fewer in peripheral lymphoid organs
CD8	gp32-33	Ly-2,3,Lyt-2,3, YTS 105.8	MRC OX-8	a-Leu-2, OKT8	Lymphocytes in thymic cortex, about one-third of T cells in peripheral lymphoid organs, splenic sinusoids (T cytotoxic/ suppressor phenotype, NK cells). MHC class I binding
CD24	p45,55,65	J11d,M1/69	SRT1	BA-1	B Cells in germinal centres and corona, myeloid cells, thymic cortex cells (rodents). Heat-stable antigen (HSA)
CD43	gp115	Ly-48	W3/13,HIS17	DFT-1, WR-14	(Pro)thymocytes, T cells, plasma cells, cells in bone marrow, polymorphonuclear granulocytes, brain cells. Leukosialin, sialophorin
CDw 90	p25-30	Thy-1	(ER4), MRC OX-7, HIS51	5F10	Thymocytes, T lymphocytes, connective tissue structures, epithelial cells, fibroblasts, neurons, subset of bone-marrow cells, plasma cells, stem cells (T-activation molecule)
		Thy-2			Thymocytes
	p40-55	H57-597	R73,HIS42	WT31, TαF1, TβF1	T-Cell receptor α–β chain. Mature T cells in thymic medulla and peripheral lymphoid tissue

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
T cells (contd)					
	p40-55	GL3,GL4, UC7-13D5	V65	CγM1,11F2, TCR δ1, δTCS1	T-Cell receptor γ–δ chain
	p41-55		MRC OX-44		Prothymocytes, lymphocytes in thymic medulla, T and B cells
	p41,47		MRC OX-2		Thymocytes, dendritic cells, B cells, brain cells
			ER3,ER7, ER9,ER10		Subset of thymocytes and peripheral T cells, subset of myeloid cells
			HIS44		Most lymphocytes in thymic cortex, small subset of medullary lymphocytes, erythroid cells, cells in germinal centre
			HIS45		Some lymphocytes in thymic cortex, most medullary thymocytes and peripheral T cells, subset of B cells. Quiescent cell antigen (QCA-1)
MHC class I		(Various antibodies to polymorphic and non-polymorphic epitopes)			All nucleated cells, including leukocytes and stromal cells; for T cells absent on thymic cortex cells (human)

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
B Cells					
CD9	gp24			BA-2	Germinal centres (faint); some cells in thymic cortex. Late pre-B cells
CD10	gp100			BA-3, W8E7	Germinal centres (faint); some cells in thymic cortex Common acute lymphoblastic leukaemia antigen (CALLA)
CD19	gp95			a-Leu-12, B4, FMC63	B Cells in germinal centres and mantles, follicular dendritic cells
CD20	p35	Ly-44		B1, a-Leu-16	B Cells in germinal centres and mantles, follicular dendritic cells
CD21	gp140			B2,BL13, HB-5	B Cells in germinal centres and mantles (faint), follicular dendritic cells (C3d receptor, CR2, receptor for Epstein-Barr virus)
CD22	gp135	Lyb-8.2, Cy34.1		a-Leu-14,To To 15,RFB4, SHCL-1	B Cells in germinal centres and mantles, cytoplasmic in precursor B cells
CD23	p45	Ly-42		a-Leu-20, Tü 1	Some B cells in marginal centres and mantles, activated B cells, subset of follicular dendritic cells (IgE Fc receptor)
CD24	p45,55,65	J11d,M1/69	SRT1	BA-1	B Cells in germinal centres and corona, myeloid cells, thymic cortex cells (rodents). Heat-stable antigen (HSA)

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
***B Cells* (contd)**					
CD37	gp40-45			BL14	B Cells in germinal centres and mantles
CD38	gp45			a-Leu-17, OKT10	Lymphocytes in thymic cortex, cells in germinal centres, plasma cells (immature lymphoid cells, plasma cells)
CDw75	p53?			LN1, OKB4	B Cells in germinal centre, in corona (faint), macrophages, epithelium
CD79a	p33,40			mb-1	B Cells, Igα chain
CD79b	p33,40			B29	B Cells, Igβ chain
		p200	(HIS14)		All B cells, including TdT^- precursors
		p200	(HIS22)		All B cells in corona, pre-B cells
MHC class II		(Various antibodies to polymorphic and non-polymorphic epitopes)			B Lymphocytes, activated T cells, monocytes/macrophages, interdigitating cells, Langerhans cells, epithelia, endothelia
					B Cells (surface); in germinal center $IgM^+IgG^+IgA^+$ and in anti-immunoglobulin corona IgM^+IgD^-, plasma cells (cytoplasmic)

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
Monocytes/macrophages, myeloid cells					
CD13	p130-150	ER-BMDM-1		a-Leu-M7, My7	Monocytes, granulocytes, dendritic reticulum cells (aminopeptidase N)
CD14	p55		ED9	UCH-M1, B-A8, a-Leu-M3	Monocytes, some granulocytes and macrophages
CD15	p170-190			a-Leu-M1	Granulocytes, some monocytes (lacto-*N*-fucose pentaosyl)
CD16	p50-70			a-Leu-11	NK cells, subset of T cells, neutrophilic granulocytes, activated macrophages. IgG-FcRIII, low affinity, complexed IgG
CD33	p67			a-Leu-M9, My9	(Precursor) granulocytes, macrophages, Langerhans cells. Myelin-associated protein
CD68	p110			Ki-M6,Ki-M7	Macrophages (specific)
	p160	F4/80			Monocytes–macrophages
	p32	Mac-2			Thioglycollate-elicited peritoneal macrophages
	p92-110	Mac-3			Peritoneal macrophages

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
***Monocytes/macrophages, myeloid cells* (contd)**					
		4F7			Dendritic cells in skin, bone marrow
			ED1		Monocytes/macrophages
			ED2,HIS36		Subset of macrophages (F4/80-like)
			ED3		Subset of macrophages, restricted, negative in thymus
			MRC OX-41		Granulocytes, macrophages, dendritic cells
			MRC OX-62		Dendritic cells (integrin-like)
			(IF119)		Dendritic cells
			HIS48		Granulocytes
				Mac-387	Macrophages
Natural killer cells					
CD16	p50-70			a-Leu-11	NK cells, subset of T cells, neutrophilic granulocytes, activated macrophages. IgG-FcRIII, low affinity, complexed IgG

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
Natural killer cells (contd)					
CD56	p220/135			a-Leu-19, B-A19	NK cells, monocytes, neuroectodermal cells NKH-1, isoform of neural cell adhesion molecule (NCAM)
CD57	p110			a-Leu-7, VC1.1	NK cells, subset of T cells, some B cells, some epithelial cells, monocytes, neuroendocrine cells, NKH-1
		a-asialo-GM1			NK cells, stromal components
		NK-1.1,2B4, 3A4, 5E6	3.2.3		NK cells (NKR-P1 gene family)
Follicular dendritic cells					
			ED5,ED6, MRC OX-2	Ki-M4,DRC-1	Follicular dendritic cells
Epithelial cells (thymus)					
		(Various anti-keratin antibodies)			Epithelium
		(ER-TR4),4F1	HIS38	TE-3,(MR3, MR6)	Thymic cortex epithelium
		(ER-TR5),IVC4	(HIS39)	TE-4,(MR19), RFD4	Thymic subcapsular or medullary epithelium

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
Complement receptors					
CD11b	p160	Ly-40, M1/70	MRC OX-41, MRC OX-42, WT.5	Mac-1, a-Leu-15	Granulocytes, macrophages, CD5+ B cells, C3b1R, CR3
CD21	gp140			B2,BL13	B Cells in germinal centres and mantles (faint), follicular dendritic cells (C3d receptor, CR2, receptor for Epstein-Barr virus
CD35	p220			To 5	Follicular dendritic cells, macrophages, B cells in corona (faint), renal glomerular epithelium. C3bR, CR1
IgG-Fc receptors					
CD16	p50-70			a-Leu-11	NK cells, subset of T cells, neutrophilic granulocytes, activated macrophages; IgG-FcRIII, low affinity, complexed IgG
CD32	gp140	Ly-17		3E1,CIKM5	B Cells, myeloid cells, macrophages; IgGFcRII, low affinity, complexed IgG
CD64	p75			32.2	Monocytes; IgG-FcRI, high affinity, monomeric IgG

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
β1-Integrin (CD29-CD49) family					
CD29	p130	9EG7		B-D15	Ubiquitous, not on erythrocytes; β1 chain of all CD49 antigens
CD49a	p200			TS2/7	Activated T cells, monocytes, smooth muscle cells. Very late antigen-1 (VLA-1), ligand of collagen, laminin
CD49b	p155			31H4,AK7, P1E6	T Cells, B cells, thrombocytes, fibroblasts, endothelium. Very late antigen-2 (VLA-2), ligand of collagen I, II, III, and IV, laminin
CD49c	p145			11G5,P1B5	B Cells, renal glomeruli, basal membranes. Very late antigen-3 (VLA-3), ligand of collagen, laminin, fibronectin, and invasin
CD49d	p150	R1-2, MFR4 B	P12520, MR?4	HP2/1,44H6, L25.3	Thymocytes, lymphocytes, monocytes, NK cells, eosinophilic granulocytes, erythroblasts. Very late antigen-4 (VLA-4), ligand of VCAM-1, fibronectin
CD49e	p160	MFR5, P12750		SAM-1	Monocytes, leukocytes, 'memory' T cells, fibroblasts, thrombocytes and muscle cells. Very late antigen-5 (VLA-5), ligand of fibronectin
CD49f	p150	GoH3		Go-H3,4F10	T Cells, thymocytes, monocytes, thrombocytes. Very late antigen-6 (VLA-6), ligand of laminin and invasin

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
β2-Integrin (CD11-CD18) family					
CD11a	p180	Ly-15, 2D7, M17/4	WT.1	YTH-81.5, B-B15, G-25.2	T and B cells, NK cells, erythroid and myeloid stem cells. Leukocyte function-associated antigen-1 (LFA-1) involved in cell adhesion, ligand for intercellular adhesion molecule (ICAM)-1 (CD54), ICAM-2 (CD102), ICAM-3 (CD50)
CD11b	p160	Ly-40, M1/70	MRC OX-41, MRC OX-42, WT.5	Mac-1, a-Leu-15	Granulocytes, macrophages, $CD5^+$ B cells. C3b1R, CR3
CD11c	p150			a-Leu-M5, S-HCL-3	Monocytes, macrophages, granulocytes (faint), activated lymphocytes. CR4
CD18	p95	YTS213.1, C71/16, M18/2	WT.3	BL5	All lymphocytes. β-Chain of CD11 antigens
		p160-95		ED7,ED8	CD11–CD18 molecule
Others					
Terminal deoxynucleotidyl transferase (TdT)					Immature (lymphoid) cells in bone marrow and thymic cortex (nuclear staining)

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
Others (contd)					
CD25	p55	Ly-43, AMT13, 7D4,3C7	MRC OX-39	Tac,a-IL2-R	Activated lymphocytes at scattered locations in thymus and T-cell areas in peripheral lymphoid organs. Interleukin-2 receptor α chain
CD122	p75	5H4, TM-β1		CF1,Mik-β2, Mik-β3	NK cells, T cells, B cells, monocytes. Interleukin-2 receptor β chain
CD26	p120	H194-112	MRC OX-61	134-2C2	(Activated) T cells. Dipeptidyl peptidase IV, in mouse T-cell activation molecule (THAM)
CD30	p105			Ki-1,Ber-H2	Sporadic cells in thymic (cortex) and T cell areas in peripheral lymphoid organs, some plasma cells. Activated lymphocytes,Hodgkin cells
CD34				MY 10,8G12 QBEND/10	Haematopoietic progenitor cells, capillary endothelium Human progenitor cell antigen (HPCA)
CD44	p65-85	Ly-24 IM7	MRC OX-49, MRC OX-50	a-Leu-44, F10-44-2	Prothymocytes, T cells, small B cells. Lymphocyte homing receptor. Phagocytic glycoprotein-1 (PgP-1), HCAM
CD45	p180-210	Ly-5	MRC OX-1, HIS41	T29/33	All leukocytes. Common leukocyte antigen

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
Others(contd)					
CD45R	p190–220	B220 MRC OX-32, HIS24	MRC OX-22, MB1,MT2	a-Leu-18,	All B cells, subset of T cells. Common leukocyte antigen. HIS24 restricted to strains of the RT7.2 allotype and labels all peripheral B cells except cells in marginal zone, pre-B cells
CD45RA	p205-220	14.8	MRC OX-33	HI100	B Cells, T cytotoxic–suppressor cells (faint), subset of thymocytes.In humans, also CD4+ subset (naive-virgin T cells)
CD45RO	p190-220			UCH-L1	T Cells in immature and memory stage. Common leukocyte antigen
CD54	p90	KAT-1 3E2	1A29	84H10,B-C14 HA58	Endothelial cells, many activated cell types. Intercellular adhesion molecule-1 (ICAM-1)
CD71	p95	YTA74.4,C2	MRC OX-26	B3/25	Proliferating cells in germinal centres, some cells in thymus and T-cell areas in peripheral lymphoid organs, stromal cells. Transferrin receptor
				Ki-67	Proliferating cells in germinal centres, some cells in thymus and T-cell areas in peripheral lymphoid organs. Proliferation antigen present in late G1, S, G2, and M phases

Table 10 (contd)

CD	Relative mol. mass	Mouse	Rat	Human	Reactivity
Others (contd)					
		PCNA	PCNA	PCNA	Proliferating cells in germinal centres, some cells in thymus and T-cell areas in peripheral lymphoid organs
		MEL14			Recirculating T and B cells. Lymphocyte homing receptor

MHC, major histocompatibility complex; NK, natural killer; Ig, immunoglobulin
CD nomenclature from: Clark & Lanier (1989); Knapp et al. (1989); Schlossman et al. (1994, 1995)
Antibodies within parentheses are not commercially available.

4.1.3 *Basal immunoglobulin level*

Serum immunoglobulin levels are often altered after exposure of rats to immunotoxic chemicals (Vos, 1980; Vos et al., 1982, 1984, 1990a; Van Loveren et al., 1993a). This is not surprising, as the total levels measured are a function of the humoral aspects of the immune system, which react to the antigens that the host encounters. For this reason, measurement of antibody levels is potentially valuable in screening for immunotoxicity. Since the amount of antibody in serum is a function of the antibody's half-life, the longer the study the more likely it is that an effect will be observed. Immunoglobulin levels can be influenced by the cleanness of the facility: studies conducted in facilities with excellent husbandry will have lower basal levels than those conducted in 'dirty' facilities where animals are constantly exposed to foreign antigens, including pathogens.

Measurement of basal immunoglobulin levels is useful only after subchronic or chronic exposure, i.e. with sufficient time for normal metabolic elimination. Basal levels of immunoglobulin decrease only when synthesis is reduced or prevented such that metabolized immunoglobulins are not replaced. The parameter therefore yields little information about possible mechanisms of immunotoxicity, and should rather be regarded as a screening parameter; this is in fact true for most non-functional tests. The IgM and G classes have usually been measured; however, since the two other classes (A and E) are biologically very important (for instance in mucosal immunity and allergic manifestations), they should also be measured.

Total IgM and IgG concentrations in serum can be analysed by means of a 'sandwich' enzyme linked immunosorbent assay (ELISA), as described by Vos et al. (1982). Total IgA and IgE concentrations can be analysed in an essentially similar way, except that the microtitre plates are coated with monoclonal anti-rat IgA (Van Loveren et al., 1988b) or monoclonal anti-rat IgE antibodies (MARE-1), respectively, and immunoglobulins bound to these antibodies in serum samples are detected by sheep anti-rat IgA or monoclonal anti-κ chains of rat immunoglobulins (MARK-1), conjugated with peroxidase.

Data from the ELISA are usually reported as percentages of control values, and a titration curve based on pooled sera is prepared; optimal dilutions of exposed and unexposed groups are then plotted from this curve. The deviation of the dilution of the test groups from the control groups is expressed, with the dilution in the control group set at 100%. While studies in rats indicate that measurement of basal immunoglobulin levels is useful in predicting the immunotoxic effects of compounds, studies conducted in mice at the NTP do not, and measurement of basal

immunoglobulins is not included in either tier of their testing panel (Luster et al., 1988). There are several possible reasons for the difference in the usefulness of basal immunoglobulin levels in rats and mice. First, in the studies of Vos and colleagues, cited above, the exposure period was routinely longer than the 14-day studies conducted within the NTP; since serum antibody level is a function of the antibody half-life, longer studies are more likely to detect an effect. Furthermore, the doses used at the NTP were often lower, by design, than those used in rats. A final possible explanation, which remains to be confirmed, is that immunoglobulin synthesis in rats is more sensitive than that in mice.

4.1.4 *Bone marrow*

Bone marrow is an important haematopoietic organ and a source of precursors for lymphocytes and other leukocytes. Changes in the bone marrow are therefore likely to result in alterations of immuno-competent cell populations, which may be long lasting or permanent and thus serve as an indicator of potential immunotoxicity. In a study to validate immunotoxicological parameters, bone-marrow cellularity was shown to be an indicator of the immunotoxicity of cyclosporin A, used as the model compound (Van Loveren et al., 1993a). Determination of cellularity in stained slides of bone marrow, evaluation of smears, and actual counts of the numbers of cells within bone marrow are practical. For this purpose, both ends of a femur are cut off, and bone-marrow cells are collected by flushing balanced salt solution through the femur with a 21-gauge needle. The concentration of nucleated cells is determined in a Coulter counter; a differential count of cells can be done visually in May-Grunvald Giemsa-stained cytospin preparations.

4.1.5 *Enumeration of leukocytes in bronchoalveolar lavage fluid, peritoneal cavity, and skin*

Mononuclear phagocytes in the alveoli of the lung play an important role in clearing inhaled particles, including microorganisms, from the lung. The numbers of cells and alterations in their function can be end-points of the toxicity of inhaled chemicals. In order to study these parameters, methods for harvesting the cells from the lungs should be easy to perform, guarantee the sterility of the cell harvest, and be standardized. Methods involve use of either a syringe (Blusse Van Oud Alblas & Van Furth, 1979) or a complex system of syringes, tubes, and valves (Moolenbeek, 1982). These methods often result in contamination of the harvested cell population; moreover, they are laborious and cannot easily be standardized since the syringe is operated manually.

In a more recently developed method (Van Soolingen et al., 1990), an excised lung is placed in a pressure chamber and connected to a cannula through which lavage fluid can be introduced into the lung and transferred from the lung into a test tube. This procedure is repeated several times to obtain an optimal yield.

Enumeration of mononuclear cells in the peritoneal cavity can also best be performed by harvesting these cells by lavage. Because of the architecture of this organ, three or four cycles of intraperitoneal injections of lavage fluid, followed by gentle massaging of the abdomen, and aspiration of the fluid with the syringe that was also used for injection suffice.

Langerhans cells in the skin can be enumerated with histopathological techniques. Frozen tissue sections are used, stained with immunoperoxidase techniques including markers for MHC class II antigens or specific markers, as indicated above. Morphometric analysis may provide a quantitative basis for this type of evaluation.

4.1.6 *Flow cytometric analysis*

Evaluation of phenotypic markers has proved to be one of the most sensitive indicators of immunotoxic compounds. The availability of fluorescent activated cell sorter (FACS) analysis units and fluorescent cell counter units in immunotoxicology laboratories has made analysis of cell populations routine. Determination of the phenotype of lymphoid cells is a non-functional assay, although it has often been inappropriately grouped with functional tests. The presence or absence of a particular marker on the surface of a cell does not reveal the functional capability of the cell. The usefulness of surface marker analysis for predicting potential immunotoxicity has been demonstrated. In studies conducted by Luster et al. (1992), a 91% concordance was found for correct identification of immunotoxic compounds on the basis of studies of surface markers alone.

As indicated above (section 4.1.2), numerous markers are expressed on the cells of the immune system. Essentially, the same reagents as used on tissue sections are applied on cells that have been isolated from tissues, body fluids, or lavage fluids in suspension (see above). Furthermore, both polyclonal and monoclonal antibodies are available for detecting these surface markers. While many of the markers have been used in immunological investigations, very few have been evaluated with a large number of immunosuppressive compounds. The markers that have been routinely used in studies of immunotoxicity conducted for the NTP in mice and the cell types they identify are shown in Table 11. The CD4:CD8 ratio in spleen has been shown to

concord best with the immunotoxicity of these surface markers (Luster et al., 1992).

Table 11. Phenotypic markers on lymphocyte subpopulations used in studies of immunotoxicity by the United States National Toxicology Program

Surface marker	Cell type
sIg^{+}	Pan B cells
Thy 1.2^{+} or $CD3^{+}$	Pan T cells
$CD4^{+}CD8$	T Helper/delayed-type hypersensitivity cells
$CD8^{+}CD4^{-}$	T Suppressor–cytotoxic cells
$CD8^{+}CD4^{+}$	Immature T cells

The identification of phenotypic markers in rats has not developed as rapidly as in mice; however, antibodies to rat cell surface markers are now becoming available commercially and are being used in immunotoxicological assessments (Smialowicz et al., 1990). The monoclonal antibodies currently used for this purpose are: OX4 or MARK-1 for B cells, W3/13 or OX 19 for T cells, R79 for the T cell receptor, W3/25 for CD4 cells, and OX 8 for CD8 cells.

In enumerating the cell types in lymphoid tissue, both percentages and absolute cell numbers should be reported. Of the two, absolute cell numbers are by far the most meaningful. Compounds that affect all populations equally and thus do not change the relative percentages of the various cell types may be missed if only percentages are evaluated. In addition, significant differences in the magnitude of an effect on one or more of the populations can be observed when the data are evaluated as absolute numbers and not as percentages. As indicated above, the absolute changes more closely reflect the events occurring in the animal and should thus be given priority in interpreting data.

FACS analysis is also being used to determine the activation state of various cell types, on the basis of changes in detectable activation markers. Some of the activation markers that have been studied are F4/80 (Austyn & Gordon, 1981),-Mac-1 (Springer et al., 1979), Mac-2 (Ho & Springer, 1984), transferrin receptor (Neckers & Cossman, 1983), and IL-2 receptor (Cantrell et al., 1988). While activation markers are of value in studying the mechanism of action of compounds, their usefulness as predictors of immunotoxicity has yet to be firmly established.

4.2 Functional tests

4.2.1 *Macrophage activity*

Phagocytic activity is the first line of defence against many pathogens. Macrophages can phagocytose many particles, including bacteria, and can lyse and inactivate them. Alterations in phagocytic activity are therefore important potentially adverse effects of chemicals on the immune system. The capacity to ingest particles *in vitro* can be measured, and activity *in vivo* can be measured by determining the clearance of bacteria, such as *L. monocytogenes*. This test is dealt with in section 4.2.10.1.

Several assays have been developed for evaluating various types of phagocytosis in mice and can also be used in rats, with slight modifications. Innate and non-immune-mediated phagocytosis by macrophages can be evaluated by determining the uptake of fluorescent latex covaspheres (Duke et al., 1985). Macrophages and peritoneal exudate cells are placed on a tissue culture slide and incubated with the covaspheres for 24 h on a rocking platform. The slides are then fixed with methanol. The slide chambers are evaluated under a fluorescent microscope, and macrophages with more than five latex covaspheres are counted as positive for phagocytosis. The results are expressed as percentage of phagocytosis, which is calculated as the ratio of macrophages positive for phagocytosis to total macrophages counted. In order to distinguish phagocytosed latex covaspheres from those that are merely associated with the macrophage surface, the cells are exposed for 30–60 s to methylene chloride vapour. By immersing the slides in this manner, the covaspheres that have not been phagocytosed are dissolved, while those inside the macrophage remain intact (Burleson et al., 1987). Phagocytosed fluorescent latex particles can easily be quantified under the fluorescence microscope. While this assay is straightforward, it is labour intensive, and reading the slides, shifting back and forth from the fluorescent field, and counting the macrophages is time-consuming.

A radioisotopic procedure, the chicken erythrocyte assay, can be used to evaluate both adherence to and phagocytosis of particles by macrophages. The phagocytic capacity is measured as an immunologically mediated (Fc receptor) response. Macrophages are added to each well of a 24-well tissue dish and allowed to adhere for a 2–3-h incubation period. Nonadherent cells are washed, and chicken erythrocytes labelled with ^{51}Cr are added to each well; then a subagglutinating dilution of antisera to chicken erythrocytes is added to each well and the plate incubated for 1 h. The plates are then washed to remove unbound erythrocytes; an ammonium chloride solution is

added to lyse adhered erythrocytes, and the supernatant is collected and counted to determine adherence of the erythrocytes to the macrophages. Next, both the macrophages and the phagocytosed chicken erythrocytes are lysed by addition of 0.1 N sodium hydroxide, and the solution is counted to determine the amount of phagocytosis. Three to six wells in each group do not receive ^{51}Cr and are used to evaluate the DNA content (Labarca & Paigen, 1980). The data are expressed as adherence counts per minute, phagocytosed counts per minute, and specific activity for adherence and phagocytosis. Specific activity is determined by dividing the number of adhered or phagocytosed counts per minute by the DNA content per well. The data must be expressed in terms of specific activity, since compounds that affect the macrophages' ability to adhere to the 24-well culture dish will significantly alter the results obtained.

While both the nonspecific and immune-mediated phagocytosis assays are useful for understanding the potential mechanisms of action of compounds, changes in phagocytic activity in these in-vitro assays have not been found to be predictive of immunotoxicity. For example, a single intratracheal exposure to gallium arsenide resulted in increased adherence and phagocytosis by chicken erythrocytes but decreased phagocytosis of latex covaspheres (Sikorski et al., 1989).

The phagocytosis assay that is most predictive of altered macrophage function is evaluation of the functional ability of the mononuclear phagocyte system. This is a holistic assay for measuring the capacity of the fixed macrophages of the mononuclear phagocyte system, where macrophages provide the first line of defence against both pathogenic and non-pathogenic blood-borne particles. The fixed macrophages of the mononuclear phagocyte system line the liver endothelium (Kupffer cells), the spleen, the lymph nodes (reticular cells), the lung (interstitial macrophages), and other organs such as the thymus and bone marrow. When the assay is conducted in mice, the animals are injected intravenously with ^{51}Cr-labelled sheep erythrocytes, and a 5-µl blood sample is taken from the clipped tail at 3-min intervals over a 15-min period. A final 30-min blood sample is taken, and 1 h after injection the animals are sacrificed and the liver, spleen, lungs, thymus, and kidneys are removed, weighed, and counted in a gamma counter. The 60-min time interval after injection of sheep erythrocytes was selected as the time of sacrifice since it represents the plateau for particle uptake by the selected organs (White et al., 1985). Blood clearance of the radiolabelled cells is expressed as vascular half-life and as a phagocytic index, which is determined by the slope of the clearance curve. Organ distribution is expressed as percent organ uptake and counts per minute per milligram of tissue (specific activity). The assay can detect

both stimulation and inhibition of the mononuclear phagocyte system. Bick et al. (1984) reported marked stimulation of the mononuclear phagocyte system after treatment with diethylstilbestrol; more recently, morphine sulfate was shown to decrease vascular clearance and hepatic and splenic phagocytosis significantly (LeVier et al., 1993).

4.2.2 *Natural killer activity*

NK activity against neoplastic and virus-infected targets has been clearly demonstrated *in vitro* and is thought to play an important role *in vivo* in providing surveillance against neoplastic cells and as a first line of defence against viruses (Herberman & Ortaldo, 1981). In humans, rats, and mice, most cells with NK activity can be identified by morphological (although the definition is not morphological) and functional characteristics (Timonen et al., 1981). Most of the cells that show NK activity are nonadherent, non-phagocytic lymphocytes and are morphologically associated with large granular lymphocytes (Timonen et al., 1982). Although cells with NK activity do not strictly belong to the T-cell lineage, they can express T cell-associated markers and express surface receptors, such as those for the Fc portion of IgG and the ganglioside asialo GM1. Some of these markers are also expressed by monocytes, macrophages, and polymorphonuclear leukocytes (Herberman & Ortaldo, 1981). Within 4 h, the cells can show nonantigen-specific cytotoxic activity *in vitro* and *in vivo* against certain (NK-sensitive) tumour cell lines and virus-infected cells.

The cells have enhanced cytolytic function after activation with a variety of stimuli, including viral infection (Stein-Streinlein et al., 1983), BCG (Tracey et al., 1977), IL-2 (Henney et al., 1981; Domzig et al., 1983; Lanier et al., 1985; Malkovsky et al., 1987), interferon, and interferon inducers (polyI:C) (Tracey et al., 1977; Oehler & Herberman, 1978; Djeu et al., 1979a,b). NK activity *in vitro* can be stimulated with IL-2 and interferon (Tracey et al., 1977; Djeu et al., 1979b). Anti-asialo GM1 antibody can strongly inhibit cytotoxic NK activity both *in vitro* and *in vivo* (Kasai et al., 1980, 1981; Yosioka et al., 1986). This antibody binds to the cell surface glycolipid GM1 and suppresses the lytic activity of effector cells. Large granular lymphocytes are found in several lymphoid organs. Many cells with high NK activity are found in spleen and peripheral blood (Rolstad et al., 1986); lymph nodes have less NK activity, and thymus and bone marrow show only marginal activity. NK activity can also be demonstrated in the bronchus-associated lymphoid tissue in the lungs. Moreover, large granular lymphocytes can migrate from the circulation into the extravascular tissue and can even be in contact with the lumen of the alveoli (Timonen et al., 1982;

Reynolds et al., 1984; Rolstad et al., 1986; Prichard et al., 1987). The presence of large granular lymphocytes associated with NK activity in the lungs is probably of great importance, because the lungs constitute a major site for neoplastic disease (metastatic spread) and viral infections. NK cells may also operate in certain types of bacterial infections. In experimental animals, suppression of NK cell activity increased the numbers of metastases after transplantation of tumours.

The clinical significance of altered NK cell activity in humans has not clearly been established. Asymptomatic individuals with low NK cell responses may be at some risk for developing upper respiratory infections and for increased morbidity (Levy et al., 1991); and extreme susceptibility to severe and repeated herpes virus infection was reported in an individual without NK cells (Biron et al., 1989). It is obvious therefore that exposure to toxic substances that alter NK activity can have biological consequences, and testing this activity is important in assessing potential immunotoxicity.

The procedure for determining NK activity is as follows: cell populations that exert NK activity (usually enriched peripheral blood mononuclear cells or spleen cells) are cultured with NK-sensitive target cells. A cell type frequently used for this purpose is the YAC lymphoma cell line, which has been applied to mice, rats, humans, and even seals. YAC lymphoma target cells are radiolabelled with ^{51}Cr, and lysis of the cells, resulting in release of chromium, within 4 h is used to estimate the cytolytic activity of the NK cells within the cell population. This assay has been used to demonstrate the effects of numerous compounds on NK activity in rats (e.g. TBTO, ozone, and HCB: Vos et al., 1984; Van Loveren et al., 1990c), mice (Luster et al., 1992), and harbour seals (Ross et al., in press).

4.2.3 *Antigen-specific antibody responses*

Most antibody responses require not only B cells, which, after maturation into plasma cells, produce antibodies, but also the help of T lymphocytes. A variety of T cell-dependent antigens can be used for this purpose, and an excellent one is tetanus toxoid. A typical immunization schedule in rats comprises intravenous immunization on day 0 followed by a booster on day 10. Primary and secondary IgG and IgM responses can then be measured in serum, taken on day 10 (just before the booster) and day 21, respectively. The primary IgM response is the immunoglobulin response that is least under the control of T cells. As tetanus toxoid is also used for human immunization, the responses to this antigen may be useful in extrapolating experimental data to humans. The responses can be determined in an ELISA (Vos et al., 1979b).

Another widely used T cell-dependent antigen is ovalbumin. This antigen can be and has been used to induce all classes of antibody responses, i.e. IgM, IgG, IgA, and IgE, that can be measured with the ELISA (Vos et al., 1980; Van Loveren et al., 1988b). The classical assay of specific IgE responses is the passive cutaneous anaphylaxis reaction. Serial dilutions are injected into the skin of rats, sensitizing local mast cells; the specific antigen is then injected intravenously, simultaneously with Evans blue. Mast cell products are released where IgE meets the antigen, and IgE is cross-linked on the membranes of the mast cells, leading to extravasation of Evans blue. The titre can be determined from the magnitude of the reaction at each dilution of IgE. ELISA techniques and the specific reagents to detect IgE in an ELISA that are now available make this test preferable.

Ovalbumin induces not only humoral responses but also delayed-type hypersensitivity. Sensitization to ovalbumin in Freund's complete adjuvant enhances responses and makes it possibile to assay both responses in one animal. Delayed-type hypersensitivity can also be directed to purified protein derivative, with responses induced by the adjuvant (Vos et al., 1980). At least in mice, however, immunization in complete adjuvant skews responses in the direction of Th1 responses, i.e. delayed hypersensitivity, and hence suppresses Th2-, IgE-, and IgA-dependent immune responses.

A few antigens can induce humoral immune responses without involvement of T lymphocytes. One example is trinitrophenol-Ficoll (lipopolysaccharide). Sensitization of animals to this antigen yields immunoglobulin responses that can be measured in an ELISA. This is a useful test for use in mechanistic studies to separate the effects of compounds on B and T cells.

4.2.4 *Antibody responses to sheep red blood cells*

4.2.4.1 *Spleen immunoglobulin M and immunoglobulin G plaque-forming cell assay to the T-dependent antigen, sheep red blood cells*

A widely used particulate T cell-dependent antigen is sheep red blood cells. Antibody titres induced after sensitization can be assayed with various techniques; one that is widely used is the plaque-forming cell assay, or antibody-forming cell response. This assay is relatively simple and can be conducted with inexpensive equipment found in most laboratories, but the optimal concentration of sheep red blood cells must be injected. As the antigenicity of red blood cells varies significantly from sheep to sheep, time must be invested to obtain cells

from a sheep that repeatedly gives a high response (≥ 1500 plaque-forming cells/10^6 spleen cells). The number of cells administered (about 2 x 10^8) should also be optimized for both mice and rats in the laboratory conducting the assay. The intravenous route is that preferred for sensitization; intraperitoneal injections can be used but significantly increase the potential for nonresponding animals as a result of a poor injection. Animals are sacrificed on day 4 after injection, and spleen cells are prepared by mincing the spleen between two frosted microscope slides, teasing it apart with forceps, or passing it through a small mesh screen; all of these methods are satisfactory, and that used to prepare single splenocyte cultures varies from laboratory to laboratory. An aliquot of cells is added to sheep erythrocytes and guinea-pig complement; these are placed in a microscope slide chamber when the Cunningham assay method is used (Cunningham & Szenberg, 1968), or, in the Jerne method, cells and guinea-pig complement are added to a test tube containing warm agar and after thorough mixing the test tube mixture is plated in a petri dish and covered with a microscope cover slip (Jerne et al., 1974). In either case, the preparations are then incubated at 37 °C for 3–4 h to allow plaques to develop. The plaques are counted under a Bellco plaque viewer. A plaque results from the lysis of sheep erythrocytes and is elicited as a result of the interaction of complement and antibodies directed against sheep erythrocytes, which are produced in response to the intravenous sensitization. As each plaque is generated from a single IgM antibody-producing plasma cell, the number of IgM plaque-forming cells present in the whole spleen can be calculated. The data are expressed as specific activity (IgM plaque-forming cells/10^6 spleen cells) and IgM plaque-forming cells per spleen.

By incorporating rabbit anti-mouse or anti-rat IgG antibody into the preparation of spleen cells, complement, and sheep red blood cells, the number of IgG antibody-forming cells present in the spleen can also be determined. This number is calculated by subtracting the number of IgM plaque-forming cells from the total number of both IgM and IgG plaque-forming cells. The optimal IgG primary response is observed five days after sensitization (Sikorski et al., 1989).

The T-dependent IgM response to sheep red blood cells is one of the most sensitive immunotoxicological assays currently in use. Luster et al. (1992) reported that the individual concordance of the plaque-forming cell assay for predicting immunotoxicity was the highest of all the functional assays (78%). Furthermore, use of this assay in combination with either NK cell activity or surface marker analysis resulted in pairwise concordances for predictability of more than 90%.

While the plaque-forming cell assay has been shown to be sensitive and predictive, the procedure does have its limitations. As indicated earlier, the effect of the test compound on the immune system is evaluated only in spleen cells, and effects on other antibody-producing organs and tissues are not determined. The assay is somewhat laborious, and it is preferable that several people participate, to help in removing spleens, preparing cell preparations, counting cells, and adding preparations to either microscope slide chambers or agar dishes. An additional drawback is that the assay must be conducted on the same day as the animals are sacrificed. This is in marked contrast to the ELISA, in which sera can be frozen and evaluated at a later date. While the slides and petri dishes can be placed in a cold room or refrigerator and counted the next day, this procedure is not recommended, as they tend to dry out to some extent, making viewing and discerning plaques more difficult.

4.2.4.2 *Enzyme-linked immunosorbent assay of anti-sheep red blood cell antibodies of classes M, G, and A in rats*

An alternative to the plaque-forming cell assay is ELISA of anti-sheep red blood cell antibody titres in serum. Antigen preparations made from ghosts of sheep erythrocytes by extraction with potassium chloride are used to coat the bottoms of the wells of 96-well microtitre plates. Serum samples from rats immunized with sheep erythrocytes are titrated onto these plates using specific polyclonal antibodies to rat IgM or IgG, to which peroxidase is conjugated. IgA has also been assayed, using monoclonal anti-rat IgA antibodies and polyclonal rat anti-mouse IgG conjugated with peroxidase. The ELISA of serum titres of IgM, IgG, and IgA to sheep erythrocytes is an easy, reliable method that can be used to detect the effects of chemicals on the immune system of the rat (Van Loveren et al., 1991; Ladics et al., 1995).

The assay measures titres of specific antibodies, in contrast to the plaque-forming cell assay which determines the number of cells that are actually responsible for production. The ELISA assesses the production of antibodies, either per cell or in terms of the total capacity of the host to produce these antibodies *in vivo*. In interpreting the effects of exposure to chemicals, account must be taken of the fact that the cells used in the assay are derived from specialized parts of the body, such as the spleen, and alterations in the numbers of antibody-producing cells in such an organ in rats immunized with sheep red blood cells cannot give information on other, inaccessible pools of antibody-producing cells. In the ELISA, alterations in titres due to exposure to chemicals indicate changes in the immune potential of the exposed

animals. In screening for the effects of chemicals on the immune system, therefore, ELISAs may be preferable, but for studies on specific immunosuppressive mechanisms, the plaque-forming cell assay, although labour- and time-intensive, is a powerful tool for obtaining information complementary to the data provided by the ELISA. Unfortunately, it is not always possible to perform the two assays with material from the same animal. The peak response in the plaque-forming cell assay in both rats (Fischer 344) and mice (B6C3F1) occurs on day 4 after sensitization, while the peak response in the ELISA occurs on day 6 for rats and day 4-5 for mice (Temple et al., 1993). In order to detect the effects of chemicals on the immune response to sheep red blood cells, it is preferable to choose the optimal conditions, or to follow the kinetics, of the response.

4.2.5 *Responsiveness to B-cell mitogens*

Responsiveness to lipopolysaccharide is another estimate of humoral immune response, as solely B cells respond to this mitogen. Although the responses of rats to this mitogen are less pronounced than those of mice, good results can be obtained, and the immunosuppressive effects of chemicals can be detected (Vos et al., 1984).

An alternative B-cell mitogen is *S. typhimurium* mitogen (STM), a water-soluble, proteinaceous extract derived from the cell walls of *S. typhimurium*; it is a more potent mitogen for rat B lymphocytes than lipopolysaccharide (Minchin et al., 1990). In both mice and rats, the polyclonal activation of B lymphocytes is a multistep process. In mice, mitogens alone can provide all the signals necessary for proliferation and differentiation; in the rat, STM stimulation induces B lymphocytes to proliferate without differentiating. The addition of lymphokines to STM-stimulated B cells also failed to stimulate them to differentiate (Stunz & Feldbush, 1986). Nevertheless, this mitogen is useful for evaluating effects on the proliferative ability of rat B lymphocytes. Smialowicz et al. (1991) showed a decrease in the STM response in Fischer 344 rats after oral exposure to 2-methoxyethanol.

Unlike the bell-shaped mitogen dose–response curves observed with T-cell mitogens, the proliferative response of B lymphocytes to both lipopolysaccharide and STM rises quickly at low concentrations of the mitogens and plateaus at higher concentrations. As a result, a single concentration on the plateau phase of the mitogen response curve is sufficient to evaluate the effects of a test compound on B-cell mitogen-driven proliferation. One of the reasons that the mitogen assays appear to be insensitive is that the cells must remain in culture for several days in order to obtain a peak response. As a result, they may recover from the immunomodulatory effects of the test compounds during this in-

vitro phase. This is a common problem with many ex-vivo/in-vitro assays, including the cytotoxic T lymphocyte and mixed leukocyte response assays; because of the short, 4-h period of the NK cell assay, this is less of a concern.

4.2.6 *Responsiveness to T-cell mitogens*

The proliferative ability of T lymphocytes after stimulation with mitogens can be measured by the uptake of ^{3}H-thymidine in a manner similar to that used to measure B-cell proliferation (Anderson et al., 1972). Concanavalin A and phytohaemagglutinin are T-cell mitogens in both rats and mice; pokeweed mitogen stimulates the proliferation of both T and B cells and thus lacks specificity. Although both concanavalin A and phytohaemagglutinin stimulate T lymphocytes, T cells responsive to concanavalin A have been reported to be less mature than those responsive to phytohaemagglutinin (Stobo & Paul, 1973). Multiple concentrations of these mitogens should be used to ensure that a peak response is obtained: both produce a bell-shaped dose–response curve, and too high a concentration can result in a suboptimal response.

Historically, mitogens have been included in the battery of tests for evaluating potential immunotoxicity, because the assay is one that can also be carried out in humans. Human studies, however, are conducted on peripheral blood, while most studies of rodent lymphocyte transformation are conducted using spleen or lymph node cells. Thus, the argument that the assay has clinical relevance is not well founded. Furthermore, as the response of lymphocytes is extremely robust, the assay lacks sensitivity. After a significant number of compounds were evaluated for potential immunotoxicity in mitogen assays, use of this assay was shifted from the tier 1 screen originally described by Luster et al. (1988) to the tier 2 comprehensive evaluation. Use of the mitogen assay has now been removed completely from studies conducted for the NTP, since other assays in which cellular proliferation is required (e.g. plaque-forming cell assay, mixed leukocyte reaction) were considered to be more sensitive, and the data obtained from the mitogen assays add little if any to an evaluation of the potential immunotoxicity of test compounds.

4.2.7 *Mixed lymphocyte reaction*

In the mixed lymphocyte reaction (also known as mixed lymphocyte culture), suspensions of responder T lymphocytes from spleen or lymph nodes are co-cultured with allogeneic stimulator cells. The

foreign histocompatibility antigen (MHC class I or class II molecules) expressed on the allogeneic stimulator cells serves as the activating stimulus for inbred populations. In noninbred populations, a pool of allogeneic cells can be used as stimulators. The assay analyses the ability of T cells to recognize allogenic cells as 'non-self' as a result of the presence of different MHC class II antigens on their surface. In response to the class II antigens, the spleen or node cells proliferate. Because a sufficiently large number of T cells in the mixed lymphocyte population respond to the stimulator population, the responder T cells need not be primed. Proliferation of the responder cells is one of the parameters for T-cell responsiveness to cellular antigens. If the allogeneic stimulator cell suspension contains T cells, their uptake of ^{3}H-thymidine must be prevented by γ-irradiation or mitomycin C, in order to preclude background thymidine uptake.

4.2.8 *Cytotoxic T lymphocyte assay*

The Tc lymphocyte assay is a continuation of the mixed lymphocyte reaction response in which the T lymphocytes further differentiate into cytotoxic effector cells under the influence of various cytokines. In mice, the assay is usually conducted using P815 mastocytoma cells as the sensitizer and target cell (Murray et al., 1985). Mice are exposed *in vivo* to the test agent, and spleen cells are then removed and placed in culture flasks with the P815 mastocytoma cells. After a five-day co-culture period, the spleen cells are harvested and added to fresh P815 mastocytoma cells which have been radiolabelled with ^{51}Cr as sodium chromate. After a 4-h incubation, the percentage cytotoxicity is determined by measuring the specific release of ^{51}Cr into the supernatant. The five days of culture are necessary for the T lymphocytes to differentiate into cytotoxic effector cells. Unfortunately, this extended period in culture may give the spleen cells sufficient time to recover from any adverse effects of the test compound, although such effects may have been present at the time the spleen cells were removed from the animal. This inherent limitation of the assay detracts from its usefulness in assessing the immunotoxicity of test compounds.

A holistic Tc lymphocyte assay has been described, in which the animal is sensitized after injection of the irradiated target cells (Devens et al., 1985). Inhibiting the ability of the sensitizing cells to proliferate either through irradiation or mitomycin C treatment before injection prevents development of Tc lymphocytes in the animal. Smialowicz et al. (1989) developed an assay in rats in which effector cells are generated in culture by incubating cells with lymph node cells from Wistar/Furth rats, and ^{51}Cr-labelled W/Fu-G1 tumour cells are used as the target

cells. The assay requires four days in culture and can be run simultaneously with the rat mixed lymphocyte reaction, thus providing information on the test compound's ability to affect proliferation and differentiation into effector cells.

4.2.9 *Delayed-type hypersensitivity responses*

Delayed-type hypersensitivity responsiveness is a reflection of the capacity of the cellular immune system to execute immune responses and especially those dependent on IL-2 and INFγ, which include attraction and activation of nonspecific mononuclear leukocytes (macrophages–monocytes). Many systems can be used, depending on the antigen. One is sensitization to BCG, followed by challenge with purified protein derivative, to which sensitivity is induced. Another example is ovalbumin, to which sensitization is most efficient if the ovalbumin is emulsified in complete Freund's adjuvant. In this system, delayed hypersensitivity can be measured to both purified protein derivative and ovalbumin (Vos et al., 1980). Another antigen is *L. monocytogenes*: This system is particularly interesting since it can be used in the context of experiments in which host resistance to this pathogen is also measured (Van Loveren et al., 1988a).

Delayed hypersensitivity responses can be measured after sensitization to *Listeria* by subcutaneous injection of the test antigen into the ears. Prior to and 24 and/or 48 h after challenge, the increment in ear thickness can be measured with a micrometer by a person unaware of the experimental group. The background ear swelling responses of similar, unimmunized control animals are subtracted from the swelling responses found in immunized animals.

Several delayed-type hypersensitivity assays have been developed and used for evaluating immunotoxicity in the mouse. Most have involved measuring swelling in either the footpad or the ear after sensitization and challenge with a protein antigen. Studies by LaGrange et al. (1974) demonstrated that sheep erythrocytes could elicit a delayed-type hypersensitivity response after a single injection into the foot pad; however, more sheep erythrocytes were needed to elicit the delayed-type hypersensitivity response than to produce the optimal humoral immune response. Foot pad swelling can be measured with a micrometer, as described for rats or by a more objective, isotopic procedure, as described by Paranjpe & Boone (1972) and Munson et al. (1982). The delayed-type hypersensitivity response to sheep erythrocytes was previously considered to be a good assay for detecting effects on cell-mediated immunity; however, the lack of persistence of the response (LaGrange et al., 1974; Askenase et al., 1977) and the possible contribution

of antibody to the response raised concern about the specificity of the assay when sheep erythrocytes are used as the eliciting antigen. Benzo[*a*]pyrene, a compound that selectively affects humoral but not cell-mediated immunity in adult mice, appears to decrease cell-mediated immunity when measured in the sheep erythrocyte assay but has no effect on delayed-type hypersensitivity when evaluated in the keyhole limpet haemocyanin assay. The effect in the sheep erythrocyte assay is observed at doses of benzo[*a*]pyrene that decrease antibody production, suggesting a significant antibody component of the swelling observed (White, 1992).

Keyhole limpet haemocyanin is another protein antigen used in evaluating delayed-type hypersensitivity responses. Holsapple et al. (1984) characterized the response to this antigen in the mouse, showing that it produced the classical delayed-type hypersensitivity response both with and without adjuvant. Two immunizations with keyhole limpet haemocyanin were required, however, to produce a response equivalent to one obtained with complete Freund's adjuvant. In these studies, animals were sensitized with subcutaneous injections of keyhole limpet haemocyanin in the shoulder area, with seven days between the sensitizations. They were then challenged with the same antigen injected intradermally into the central portion of the pinna of one of the ears. Increases in ear thickness were evaluated by both micrometer readings and radioisotopically. The unchallenged ear was used as an individual control for each animal, and a group of unsensitized but challenged animals was used to control for nonspecific and background effects. The results indicated that, whenever possible, the use of adjuvant in delayed-type hypersensitivity studies should be avoided. Despite the fact that complete Freund's adjuvant boosted the responses to keyhole limpet haemocyanin, it partially masked the dexamethasone-induced suppression of the response. In some cases, however, delayed-type hypersensitivity responses are difficult to induce without adjuvant.

The studies currently conducted in mice and rats with this assay are holistic assays for evaluating cell-mediated immunity. Since sensitization and challenge occur in the intact animal, all components of the immune system are present to respond in a physiologically relevant manner. This type of assay is much more valuable for evaluating the effects of compounds on cell-mediated immunity than are in-vitro assays such as the mixed leukocyte response or Tc cell assay. Luster et al. (1992) reported that the delayed-type hypersensitivity response assay in mice was highly predictive (100% concordance) of immunotoxicity when used in combination with the NK cell assay and the plaque-forming cell assay.

4.2.10 *Host resistance models*

4.2.10.1 Listeria monocytogenes

Relevant mechanisms of defence against *L. monocytogenes* include phagocytosis by macrophages and T cell-dependent lymphokine production which enhances phagocytosis (Mackaness, 1969; McGregor et al., 1973; Takeya et al., 1977; Pennington, 1985; Van Loveren et al., 1987). Humoral immunity is not relevant in protection against infection in this model. Clearance of *Listeria* after infection by, for instance, the intravenous or the intratracheal route can be assessed at various times after infection by determining the numbers of colony forming units in the spleen or lungs, respectively. This can be done by classical methods (Reynolds & Thomson, 1973) that involve the following steps: Serial dilutions of homogenates of the organs, prepared in mortars with sterile sea sand, are plated onto sheep blood agar plates; after a 24-h incubation at 37 °C, the colonies are counted to determine the number of viable bacteria in the organ. Differences in the numbers of bacteria retrieved from the organs are an indication of the clearance of the bacteria, i.e. the rate at which the host disposes of the bacteria after infection.

Histopathology after a *Listeria* infection can also be valuable. For instance, exposure to ozone before an intratracheal infection with *Listeria* affects pathological lesions due to the infection (Van Loveren et al., 1988a): Pulmonary infection with *Listeria* induces histopathological lesions characterized by foci of inflammatory cells, such as lymphoid and histiocytic cells, accompanied by local cell degeneration and influx of granulocytes. If rats are exposed to ozone for one week before infection, the lesions are much more severe than in unexposed animals and persist at times when either ozone-associated or infection-associated effects alone would have resolved. The quality of the lesions is also influenced by prior exposure to ozone: mature granulomas were found in *Listeria*-infected rats that were also exposed to ozone.

When mice are challenged with *Listeria*, mortality is the usual end-point monitored; however, clearance and organ bacterial colony counts can also be determined. *L. monocytogenes* is a Gram-positive bacterium. The resistance of mice to the organism is genetically regulated, and the susceptibility of the B6C3F1 strain, the strain designated by the NTP for immunotoxicity studies, comes from the C3H parent, since the C57Bl/6 mouse is resistant (Kongshavn et al., 1980). *Listeria* can easily be stored at –70 °C at a stock concentration of approximately 10^8 colony forming units per ml. In studies in mice, three challenge levels are

routinely selected to produce 20, 50, and 80% mortality in the vehicle control animals. Mortality is recorded daily for 14 days. Treatment groups consisting of 12 mice per group have been found to be useful for obtaining statistically meaningful data on host resistance. This assay is extremely reproducible when the organism is administered intravenously.

The *Listeria* assay can detect both protection from and increased susceptibility to chemicals and drugs. Morahan et al. (1979) used the model to demonstrate a dose-dependent decrease in host resistance after exposure to δ-9-tetrahydrocannabinol, the major psychoactive constituent of marijuana. The *Listeria* host resistance assay is that most often used in immunotoxicological assessment of compounds, and numerous examples of its use can be found in the literature. It is one of the primary models used by the NTP for evaluating immunosuppression. Since *Listeria* is a human pathogen, appropriate precautions are needed in conducting the assay.

4.2.10.2 Streptococcus *infectivity models*

Two species of *Streptococcus* have been used widely in bacterial host resistance models for immunotoxicological assessment. *S. pneumoniae* has been used primarily for evaluating systemic immunity. *S. zooepidemicus* has also been used to evaluate systemic immunity but is used extensively to evaluate the effects of drugs and chemicals on the local immunity of the pulmonary system.

S. pneumoniae is a Gram-positive coccus to which host resistance is multifaceted (Winkelstein, 1981). The first line of defence against this organism is the complement system. Activation of the complement system can result in direct lysis of certain strains of *S. pneumoniae*; however, owing to the nature of their cell wall, some strains are resistant to lysis by complement. Complement can still participate directly in the removal of these bacteria as a result of deposition of complement component C3 on their surface, which facilitates phagocytosis by polymorphonuclear leukocytes and macrophages. In the later stages of the infection, antigen-specific antibody plays a major role in controlling the infection. Thus, compounds that affect complement, polymorphonuclear leukocytes, B-cell maturation and proliferation, or the production of antibody can be evaluated in this system. *S. pneumoniae* is an excellent model for evaluating immunotoxicity, since it elicits multiple immune components which participate in host resistance, each of which can be a potential target for an adverse effect of a xenobiotic. To date, this model has had limited success in rats.

Preparation of *S. pneumoniae* for challenge is slightly more complicated than the procedures used for *Listeria*; however, the potential of the model for detecting immunotoxic compounds makes the additional steps worthwhile. Stock preparations of *S. pneumoniae* (ATCC 6314) are easily maintained at -70 °C in defibrinated rabbit blood, and aliquots of the stock preparation can be removed and grown in culture at various dilutions to obtain the desired challenge concentration. An alternative approach is to grow the organism in culture and to monitor the bacterial concentrations by measuring the turbidity of the culture. A 5-μl aliquot of the stock preparation is used to inoculate 50 ml of brain–heart infusion broth, which is incubated at 37 °C, and the turbidity of the overnight culture is determined with an Abbott Biochromatic analyser system or another instrument that can sensitively measure changes in culture turbidity. The overnight culture is diluted with fresh brain–heart infusion broth to yield an absorbence difference of 0.020–0.025. The turbidity of the subculture is monitored periodically, and when the optimal density reaches an absorbence difference of 0.080, the subculture is rapidly cooled in an ice bath and diluted to the desired inoculum level. The turbidity of each inoculum is checked in the analyser, and adjustments are made to obtain the preselected differences in absorbence. Routinely, one day after the last exposure, female mice are challenged intraperitoneally with 0.2 ml of the *S. pneumoniae* inoculum. If the inoculum is administered intravenously, extremely high challenge levels must be used, which may reflect the efficiency of the mononuclear phagocyte system to clear and kill the organism. Three innocula, each at a different concentration, are prepared to give a range of lethality (e.g. 20, 50, and 80%), and a sample of each is serially diluted and placed on blood agar plates to determine the number of colony-forming units administered to the animals. Owing to the rapid onset of infection, mortality is recorded twice daily for seven days. In studies by White et al. (1986), when female B6C3F1 mice were exposed daily for 14 days to 1,2,3,6,7,8-hexachlorodibenzo-*para*-dioxin or TCDD by gavage, they were found to have decreased host resistance to *S. pneumoniae*, which is consistent with the decrease in complement activity caused by these compounds.

Another species of *Streptococcus* that has been used as a host resistance model is *S. zooepidemicus*, a group C streptococcus (Fugmann et al., 1983). Exposure to *N*-nitrosodimethylamine was shown to decrease host resistance to this strain significantly. Infection with *S. zooepidemicus* may be dependent on an antibody-mediated response, since the time to death after challenge is considerably longer than with *S. pneumoniae*. Numerous studies have demonstrated that aerosolized *S. zooepidemicus*

is one of the most sensitive indicators of the toxicity of air pollution: Mice exposed for short periods to single or mixed pollutants before infection with an aerosol of *S. zooepidemicus* and then assessed for mortality over 20 days had increased mortality with increasing concentrations of ozone (Coffin & Gardner, 1972; Ehrlich et al., 1977), nitrogen dioxide (Ehrlich & Henry, 1968; Sherwood et al., 1981), sulfur dioxide (Selgrade et al., 1989), metal particulates (Gardner et al., 1977; Adkins et al., 1979, 1980; Aranyi et al., 1985), phosgene (Selgrade et al., 1989), and other volatile organic compounds (Aranyi et al., 1986). With many of these compounds, enhanced susceptibility to infection has been demonstrated at concentrations at or below the United States national ambient air quality standards or threshold limit values. With all of these compounds, enhanced mortality has been associated with failure to clear bacteria from the lung and suppression of alveolar macrophage phagocytic function. This model has recently been adapted to rats. In this species, both ozone (Gilmour & Selgrade, 1993) and phosgene (Yang et al., 1995) delayed clearance of bacteria from the lungs and enhanced the inflammatory response (polymorphonuclear leukocytes in lavage fluid) at concentrations that do not themselves produce inflammation; however, mortality does not occur in this species. While the bacteria have generally been administered as aerosols, Sherwood et al. (1988) showed that similar results could be obtained when they were administered intratracheally or intranasally. Since some strains of *Streptococcus* are pathogenic to humans, appropriate precautions must be taken when using this host resistance model.

4.2.10.3 *Viral infection model with mouse and rat cytomegalovirus*

Cytomegalovirus infections are widely distributed in humans, with about 60–90% of the population infected. Human cytomegalovirus infections occur in several forms, the most serious being congenital and perinatal infection and infection of immunosuppressed individuals. Less serious forms include post-perfusion syndrome and some cases of infectious mononucleosis; however, the vast majority of postnatal infections in immunocompetent individuals are clinically asymptomatic. More severe disease may occur in immunodeficient hosts, such as transplant patients (Naraqi et al., 1977; Pass et al., 1978; Marker et al., 1981; Rubin et al., 1981). Primarily on the basis of morphological considerations, cytomegaloviruses are classified as members of the family Herpesviridae. Because these viruses have a relatively protracted replication cycle, a slowly developing cytopathology characterized by cytomegaly, and a relatively restricted

host range, they are grouped into the beta Herpesviridae subfamily (Roizman et al., 1981; Roizman, 1982). The roles of several arms of the immune system in resistance to cytomegalovirus have been studied extensively in mice. The role of humoral immunity is not well understood. It was suggested initially that neutralizing antibodies do not play a pivotal role in recovery from cytomegalovirus infection in mice (Osborn et al., 1968; Tonari & Minamishima, 1983); however, the role of antibodies in neutralization of murine cytomegalovirus and in antibody-dependent cell-mediated cytotoxicity is now recognized (Manischewitz & Quinnan, 1980; Quinnan et al., 1980; Farrell & Shellam, 1991). Cytomegalovirus-specific Tc cells can be detected in cytomegalovirus-infected mice (Ho, 1980; Quinnan et al., 1980). NK cell activity appeared to be the most effective, especially during the initial stages of infection (Bancroft et al., 1981; Selgrade et al., 1982; Bukowski et al., 1984). Enhanced susceptibility to infection has been demonstrated in mice when macrophage function was blocked by silica, and transfer of syngeneic adult macrophages to suckling mice significantly increased their resistance to mouse cytomegalovirus infection (Selgrade & Osborn, 1974). An inverse correlation is seen between the virulence of mouse cytomegalovirus and its infectivity for peritoneal macrophages (Inada & Mims, 1985), suggesting that attenuated virus may be controlled, in part, by macrophages. Since the rat virus acts very much like the attenuated mouse virus, macrophages may be even more important in rats. Macrophages may facilitate the generation of latent infection (Booss, 1980; Yamaguchi et al., 1988).

Enhanced susceptibility to mouse cytomegalovirus has been demonstrated after treatment of mice with cyclophosphamide, cyclosporin A, nickel chloride, or DMBA. Treatment with benzo[*a*]-pyrene or TCDD did not affect susceptibility to this infection (Selgrade et al., 1982). Enhanced susceptibility was correlated with chemical suppression of virus-augmented NK cell activity during the first week of infection. In rats, exposure to immunotoxic agents such as organotin compounds led to altered resistance to rat cytomegalovirus (Garssen et al., 1995).

Experimentally, rodents can be inoculated intraperitoneally with a species-specific cytomegalovirus, and the concentration of the virus in tissue can be determined in a plaque-forming assay, which is a modification of the method described by Bruggeman et al. (1983, 1985). Rat embryo-cell monolayers are prepared in 24-well plates. Different organs (salivary gland, lung, kidney, liver, spleen), obtained at various times after infection, are homogenized in a tissue grinder and stored as 10% weight/volume samples at –135 °C until use. The confluent monolayers are then infected with 10-fold serial dilutions of the organ

suspensions. After centrifugation, the suspension is removed, and 1 or 0.6% agarose is added. After incubation at 37 °C in 5% carbon dioxide for seven days, the cells are fixed in 3.7% formaldehyde solution, the agarose layer is removed, and the monolayer is stained with 1% aqueous methylene blue. Plaques are counted under a stereoscopic microscope.

In PVG rats, cytomegalovirus is detectable eight days after infection, although the virus load is much higher on days 15–20. The viral load in the salivary gland is higher than that in other organs, i.e. spleen, lung, kidney, and liver. In contrast, in Lewis rats and BN rats the viral load in e.g. the kidney was higher than that in the salivary gland during the first week after infection (Bruning, 1985), perhaps due to a strain difference (Bruggeman et al., 1983, 1985). Total body irradiation of PVG rats with ^{60}Co one day before infection with cytomegalovirus increased the viral load in the salivary gland, lung, kidney, spleen, and liver over that in unirradiated PVG rats; histological analysis also indicated a higher viral load in the salivary gland of infected rats. The mucosal epithelium of the salivary gland contains enlarged cells with nuclear inclusion bodies; these could be detected in irradiated, infected rats only if the salivary gland was dissected and fixed 15 days after infection. These results are in agreement with those of Bruggeman et al. (1983), who found that γ irradiation also induced higher viral loads in the salivary gland of BN rats. Taken together these results indicate a role for cellular immunity in resistance to this virus in rats.

4.2.10.4 *Influenza virus model*

Influenza virus A2/Taiwan H_2N_2 has been used as a viral challenge in evaluating alterations in host resistance of mice after exposure to various compounds. Compounds that decrease host resistance to the virus are *N*-nitrosodimethylamine (Thomas et al., 1985b) and TCDD (House et al., 1990a). Compounds that do not to alter host resistance to this pathogen include ozone (Selgrade et al., 1988), benzo[*a*]pyrene, benzo[*e*]pyrene (Munson & White, 1990), methyl isocyanate (Luster et al., 1986), and Pyrexol (House et al., 1990b). Mortality is the end-point routinely measured in evaluating decreased host resistance to influenza virus, which is usually instilled intranasally (Fenters et al., 1979). Host resistance to this virus has been reported to be mediated by cell-mediated immunity (Ada et al., 1981), interferon (Hoshino et al., 1983), and antibody (Vireligier, 1975). This model had been suggested for use in evaluating compounds that affect humoral immunity; however, its inability to detect such compounds indicates that it is not suitable. A possible explanation for the discrepancy is that administration of the

virus by intranasal instillation may invoke local immune mechanisms in the lung and may not adequately reflect systemic immuno-competence. In several cases, enhanced mortality has been demonstrated in the absence of effects on viral titres in the lung (Selgrade et al., 1988; Burleson et al., in press), indicating that enhanced mortality does not always reflect effects on virus-specific immune defences.

Influenza virus has been used in evaluating immunotoxicity in rats, after adaptation. Studies by Ehrlich & Burleson (1991) showed that rats exposed to phosgene had significantly decreased host resistance. TCDD was shown to affect the resistance of rats to the adapted influenza virus RAIV (Yang et al., 1994).

As influenza virus is a human pathogen, appropriate precautions must be taken.

4.2.10.5 *Parasitic infection model with* Trichinella spiralis

Resistance to infection with the helminth *T. spiralis* has been evaluated in both mice and rats after exposure to a variety of chemicals. In humans, as in other carnivores, infection occurs by eating meat containing infectious larvae. The life cycle of the worm is as follows: Infectious larvae excyst in the acid–pepsin environment of the stomach, rapidly migrate to the jejunum, and penetrate host intestinal epithelial cells. Sexually mature parasites are present within three to four days after infection. The viviparous females produce larvae that migrate via the lymphatic and blood vessels to host muscle, where they encyst and are encapsulated within a host-derived structure. Encapsulated muscle larvae can survive for years within this structure.

An intense inflammatory response, comprised mainly of mast cells and eosinophils, accompanies intracellular infection in the intestine. T Cell-dependent immunity plays a crucial role in this inflammatory response (Manson-Smith et al., 1979; Vos et al., 1983b; Wakelin, 1993), which is responsible for the expulsion of adult parasites. Antibodies damage the reproductive structures of the female parasite (Love et al., 1976), have a major role in the rapid elimination of subsequent infections in rats (Appleton & McGregor, 1984), and sensitize migrating newborn larvae for destruction by granulocytes (Ruitenberg et al., 1983).

The number of encysted muscle larvae is typically much higher in immunosuppressed animals than in immunocompetent animals, due perhaps to delayed expulsion of adult worms from the intestine, decreased host control of parasite fecundity, decreased destruction of migrating larvae, or a combination of resistance defects. These end-points of host resistance to *T. spiralis* infection and class-specific antibody

titres can be measured by standard techniques (Van Loveren et al., 1994). Histological evaluation of the inflammatory infiltrate surrounding encysted muscle larvae has also been described (Van Loveren et al., 1993b).

It should be noted that direct effects of the chemical under study can affect the outcome of infection. For example Bolas-Fernandez et al. (1988) determined that cyclosporin A delays expulsion of adult parasites from the intestines of rats but does not increase the number of larvae encysted in host muscle. This was determined to be a direct effect of cyclosporin A on the fecundity of female parasites rather than on immunity to infection. Animals are infected by oral gavage with known numbers of larvae, isolated from infected donor muscle. Because infection is spread only by consumption of infected meat or freshly isolated larvae, there is little danger of the infection spreading to other animals housed in the same room. *T. spiralis* is a human pathogen and must be handled as such; normal laboratory practices are sufficient to prevent accidental infection.

T. spiralis infection has been used as a host resistance model in both rats and mice. In general, chemicals that suppress T-cell function suppress resistance to *T. spiralis* infection. Thus, TBTO (Vos et al., 1990b), diethylstilbestrol (Luebke et al., 1984), TCDD (Luebke et al., 1994, 1995), and the virustatic agent acyclovir (Stahlmann et al., 1992) had deleterious effects on resistance.

4.2.10.6 Plasmodium *model*

Two strains of *Plasmodium* have been used to evaluate the potential immunotoxicity of compounds. *P. yoelii* (17XNL) is a nonlethal strain that produces a self-limiting parasitaemia in mice. Resistance to this organism is multifaceted and includes specific antibody, macrophage involvement, and T cell-mediated functions (Luster et al., 1986). In this assay, animals are injected with 10^6 parasitized erythrocytes, and the degree of parasitaemia is monitored over the course of the infection by taking blood samples. In control animals, the peak response usually occurs 10–14 days after injection. The degree of parasitaemia can be evaluated by a variety of methods, e.g. manually, by counting parasitized erythrocytes in blood smears (Luebke et al., 1991). Host resistance to *P. yoelii* has been used to assess the immunotoxicity of benzidine (Luster et al., 1985b), diphenylhydantoin (Tucker et al., 1985), TCDD (Tucker et al., 1986), pyran copolymer (Krishna et al., 1989), gallium arsenide (Sikorski et al., 1989), and 2'-deoxycoformycin (Luebke et al., 1991).

P. berghei is lethal to mice and certain strains of rats and has been used in assessing immunotoxicity (Loose et al., 1978). Host resistance

depends on specific antibody production and ingestion and destruction of antibody-coated *Plasmodium* by phagocytic cells such as macrophages. T Lymphocytes may also be involved in host resistance to the organism (Bradley & Morahan, 1982). Mortality has been evaluated after injection of 10^6 *Plasmodium*-infected erythrocytes. Compounds that have been evaluated for immunotoxicity in this model system include 4,4′-thiobis(6-*tert*-butyl-*meta*-cresol) (Holsapple et al., 1988), dietary fish-oil supplement (Blok et al., 1992), and styrene (Dogra et al., 1992). Neither *P. yoelii* nor *P. berghei* is infectious in humans; infection of animals can occur only through parenteral injection of contaminated blood.

4.2.10.7 *B16F10 Melanoma model*

The B16F10 tumour cell line is a malignant melanoma that is syngeneic with the C57Bl/6 mouse, which is one of the parents of the B6C3F1 mouse. This tumour line was selected for its propensity to metastasize to the lung. The assay is an outgrowth of the work of Fidler and colleagues (Fidler, 1973; Fidler et al., 1978). NK cells and macrophages have been proposed to be involved in host resistance to this metastasizing tumour; however, T lymphocytes have also been shown to play a role (Parhar & Lala, 1987). This host resistance assay is referred to as an artificial metastasis model, since the tumour cells are administered by intravenous injection, usually into the tail vein, and lodge in the lung, which is the first capillary bed they encounter. The B16F10 tumour cells can be stored frozen and can easily be grown in culture before use. Routinely, 1–5 x 10^5 cells are injected intravenously into sentinel mice (i.e. untreated mice injected with the highest challenge level of tumour cells), and the tumour burden is monitored in order to select the optimal day of assay.

Two parameters are routinely used to assess tumour burden. One is DNA synthesis in the lungs of mice bearing tumours. Since background DNA synthesis in the lungs of mice without tumours is extremely low, any detectable rate is a result of the presence of a tumour. In order to measure synthesis, mice are pulsed intraperitoneally one day before sacrifice with 0.2 ml of 10^{-6} mol/litre of 5-fluorodeoxyuridine, followed 30 min later by 2 μCi of ^{125}I-iododeoxyuridine administered by the intravenous route. After sacrifice, the lungs are removed, placed in Bouin's fixative solution, and counted with a gamma counter. A second indicator of tumour burden is visual enumeration of tumour nodules after fixation in Bouin's solution. The visibility of the black nodules of the melanin-producing B16F10 tumour cells on the yellow background of the fixed lung tissue allows enumeration of up to 200–250 nodules on

the surface of the lungs. A good correlation has been shown between number of tumour nodules and radioactivity present in the lungs (White, 1992). Thus, if the tumour nodules become too numerous to count, the results of the study can still be determined from the radioassay. This system has been useful in demonstrating decreased host resistance after systemic exposure to the tumour promoter phorbol myristate acetate (Murray et al., 1985), intratracheal exposure to gallium arsenide (Sikorski et al., 1989), and exposure to nickel chloride (Smialowicz et al., 1985b); it has also been used to show enhanced host resistance after exposure to manganese chloride (Smialowicz et al., 1984) and 4,4'-thiobis(6-*tert*-butyl-*meta*-cresol) (Holsapple et al., 1988).

4.2.10.8 *PYB6 Carcinoma model*

The PYB6 tumour cell line is a fibrosarcoma originally induced with a polyoma virus in C57Bl/6 mice. Host resistance to the tumour includes NK cell activity and T cell-mediated killing (Urban et al., 1982). While PYB6 cells can easily be grown in culture, they should be passed through an animal before use in challenge studies for immunotoxicity (Luster et al., 1988). In studies with the PYB6 line, mice are injected in the thigh with 1–5 x 10^3 viable tumour cells and are then palpated weekly to detect the development of tumours at the injection site. The end-points evaluated include the incidence of tumours and time to tumour appearance; tumour size can also be measured. This assay has been useful in detecting decreased host resistance to many compounds, including Aroclor 1254 (Lubet et al., 1986), DMBA (Dean et al., 1986), and benzene (Rosenthal & Snyder, 1987).

4.2.10.9 *MADB106 Adenocarcinoma model*

A tumour model used to evaluate host resistance in rats is the MADB106 rat mammary adenocarcinoma, which is syngeneic with the Fischer 344 rat. NK cells appear to play a major role in host defence to this tumour (Barlozzari et al., 1985). In this model, survival time after injection of the cells is the usual end-point monitored. Compounds that decrease host resistance to the tumour can decrease both the percentage survival and the survival time of treated animals. Control rats begin to succumb to the adenocarcinoma two to three weeks after an intravenous injection of 2 x 10^6 tumour cells. Smialowicz et al. (1985b) showed a significant decrease in the survival of animals treated with a single intramuscular dose of nickel chloride, which was correlated with a decrease in NK cell activity.

4.2.11 *Autoimmune models*

Autoimmune models can also be used to investigate whether a compound exacerbates induced or genetically predisposed autoimmunity. These models are used mainly to elucidate the pathogenesis of autoimmunity and the effect of immunosuppression in immunopharmacology. Few studies have been reported, although the relevance of the model for extrapolation to humans may be good. A number of autoimmune models are available in rats and mice. Autoimmune phenomena can be either induced or occur spontaneously. In induced models, an autoantigen is isolated from a target organ obtained from another species (generally bovine), and the animal is immunized with this purified antigen in adjuvant. Examples in the rat (Calder & Lightman, 1992) are experimental encephalomyelitis elicited by bovine spinal cord antigen (Stanley & Pender, 1991), experimental uveitis elicited by bovine retinal S-antigen (Fox et al., 1987), and adjuvant arthritis elicited by *Mycobacterium* containing H37RA adjuvant (adjuvant arthritis) or collagen (Holmdahl et al., 1990; Klareskog & Olsson, 1990; Wooley, 1991). Autoimmune phenomena and associated organ pathology normally emerge in almost all immunized animals within two to three weeks. Depending on the effector reaction and the reversibility of the damage, the disease either stops when the damage is complete (e.g. uveitis, resulting in blindness of the animal) or the autoimmune reaction is transient and animals recover (adjuvant arthritis). In some models, animals subsequently experience a relapse around day 30 (experimental encephalomyelitis). The induction and development of autoimmunity in these animals are mediated by T cells that show the cytokine expression pattern (IL-2, INFγ) of the Th1 subset. The effector phase of disease symptoms is also mainly a T cell-mediated process, to which $CD4^+$ cells, $CD8^+$ cells, and macrophages contribute. Lewis ($RT1^l$) rats are particularly susceptible. These autoimmune models can be induced in other species, such as mice (Baker et al., 1990) and rhesus monkeys (Rose et al., 1991). They are generally accepted as models of human (organ-specific) autoimmune diseases, e.g. experimental allergic encephalomyelitis as a model of multiple sclerosis, experimental allergic uveitis as a model of idiopathic posterior uveitis, and adjuvant arthritis as a model of rheumatoid arthritis.

Autoimmunity can also be induced by metals (Druet et al., 1989; Bigazzi, 1992). A well-known example is glomerulopathy induced by mercuric chloride in BN ($RT1^n$) rats. The process is initiated by T cells with a cytokine synthesis pattern (IL-4) of the Th2 subset. The relative incidence of these cells is much higher in BB rats than in other strains. After the cells of the Th2 subset have been stimulated, there is polyclonal

stimulation of B lymphocytes, leading to synthesis of antibodies (including pathogenic antibodies) to the glomerular basement membrane. These antibodies subsequently mediate autoimmune destruction of renal glomeruli. This model is the best studied model of 'drug'-induced autoimmunity. Mercuric chloride elicits glomerulonephritis in other rat strains, in which glomerular destruction is not due to anti-glomerular autoantibodies but is mediated by immune complexes deposited in the glomerulus (Druet et al., 1989).

In spontaneous models, predisposition to the development of autoimmune phenomena and disease is determined by the genetic composition of the animal strain. Well-known examples are BB rats (Like et al., 1982; Guberski, 1994) and NOD mice (Lampeter et al., 1989; Leiter, 1993), which develop autoimmune pancreatitis and subsequently diabetes. Within the pancreas, the islets of Langerhans are infiltrated by T lymphocytes and macrophages; subsequent destruction of the islets results in diabetes. These spontaneous models are considered to be animal models of human diabetes (Dotta & Eisenbarth, 1989; Lampeter et al., 1989; Riley, 1989). Other examples are the systemic autoimmunity that emerges in certain mouse strains (Guttierez-Ramos et al., 1990) like (NZBxNZW)F1 mice (Theofilopoulos & Dixon, 1985) and the mixed lymphocyte reaction in *lpr* (Matsuzawa et al., 1990) and *gld* mice (Roths et al., 1984). The spontaneous pathology in these animals resembles various disease manifestations in human systemic lupus erythematosus and is similarly mediated by immune complexes that deposit in tissue. In (NZBxNZW)F1 mice, mainly lupus nephritis is induced by immune complexes; in the mixed lymphocyte reaction in *lpr* mice, both joint manifestations and glomerulonephritis are seen. The genes associated with autoimmune and immune complex disease are not known. In comparison with induced models, these models have the advantage of spontaneous, gradual development of autoimmune disease symptoms; however, this is a disadvantage in experimental design, as not all animals develop disease, and emerging disease develops at different times or ages.

In general, treatment of animals with immunosuppressive drugs that interfere with signal transduction (cyclosporin, FK-506, rapamycin) or cell proliferation (cytostatics like azathioprine, mizoribine, and brequinar), and anti-inflammatory agents (corticosteroids) inhibit the development of symptoms in these models. Exposure to immunotoxic chemicals may also lead to alterations in the course of disease emergence. For instance, HCB, which leads to immunoenhancement in rats, markedly enhanced the severity of allergic encephalomyelitis (Van Loveren at al., 1990c). In contrast, arthritic lesions were strongly suppressed in HCB-exposed Lewis rats, indicating that HCB has biologically significant

immunotoxic effects. Although the contrasting effects in the two autoimmune models are not yet understood, and clear dose–effect relationships have yet to be established, this type of information should be obtained for risk assessment.

4.3 Assessment of immunotoxicity in non-rodent species

While most immunotoxicological evaluations are conducted in mice or rats, use of other species is increasing.

4.3.1 *Non-human primates*

Various non-human primates, including *Macaca mulatta* (rhesus macaques), *M. nemestrina* (pig-tailed macaques), *Cercocebus atys* (sooty mangabeys), *M. fasicularis* (cynomolgus monkeys), and marmosets have been used in immunotoxicological studies. Many of the assays carried out in mice or rats can be adapted for use with non-human primates. Strategies and methods used in studies of humans have also been introduced in studies on non-human primates. Monoclonal antibodies generated to human leukocyte subsets can be used in phenotyping blood mononuclear cells of e.g. marmoset monkeys (*Callithrix jacchus*) (Neubert et al., 1990, 1991), although the possibility of such use differs, depending on the evolutionary distance of the non-human primate from humans. While most of the assays conducted in non-human primates involve serum or peripheral blood, some assays, such as those used to measure delayed-type hypersensitivity, are holistic, in that the animals are sensitized *in vivo* and then evaluated *in vivo* at the challenge site (Bugelski et al., 1990; Bleavins & Alvey, 1991).

The effects of chronic exposure to the PCB Arochlor 1254 on the immune response of rhesus monkeys have been evaluated by Tryphonas et al. (1989). In these studies, the lymphocyte response to concanavalin A and phytohaemagglutinin was evaluated, as were total serum immunoglobulin levels, antibodies to sheep red blood cells, and numbers of T and B cells in peripheral blood. In later studies (Tryphonas et al., 1991a,b), one-way mixed lymphocyte cultures, antibodies to pneumococcal antigens, phagocytic mononuclear cell function, NK function, haemolytic complement activity, and production of IL-1, tumour necrosis factor, thymosins, and interferon were evaluated in monkeys exposed to Arocolor 1254. Ahmed-Ansari et al. (1989) evaluated phenotypic markers and function in three species of non-human primate. The functional assays included NK cell activity, lymphocyte transformation, and antigen presentation. Extensive studies included the evaluation of more than 20 phenotypic markers or combinations of markers for each of the three monkey species. Use of the monkey as a

test species is likely to increase as more and more biotechnology and recombinant products are produced.

4.3.2 *Dogs*

While dogs are not the species of choice for immunotoxicological studies, they are used predominantly in assessing toxicological safety, and virtually all of the assays used for assessing immunotoxic potential have been adapted for use in dogs. These include evaluation of basal levels of IgA, IgG, and IgM (Glickman et al., 1988), allergen-specific serum IgE (Kleinbeck et al., 1989), mononuclear phagocyte function (Thiem et al., 1988), NK cell activity (Raskin et al., 1989), Tc cell activity (Holmes et al., 1989), and mitogen- and cell-mediated immune responses (Nimmo Wilkie et al., 1992).

4.3.3 *Non-mammalian species*

Non-mammalian species are also used extensively for evaluating the potential adverse effects of compounds and agents on the immune system.

4.3.3.1 *Fish*

Because of their environment, fish are an excellent model for studying the effects of water- and sediment-borne pollutants. There are several other good reasons for studying immunotoxicity in fish: many of their diseases are related to environmental quality, various environmental pollutants have immunotoxic potential, and many of the diseases have an immune component. Moreover, there is concern about the health status of aquatic ecosystems in relation to pollution, and fish will be useful target species for developing biomarkers (see box). Fish are easy to obtain, there is an extensive body of knowledge, and their economic interest (aquaculture) facilitates the finding of research resources. At present, immunotoxicology in fish is not as sophisticated as that in mammals. Screening and functional tests are being developed in the laboratory but cannot yet be applied in the field.

A wide range of species is used for field and laboratory studies. The choice of species depends on its biology (migratory or local, marine or freshwater; sediment-dwelling or pelagic) and on experience in the laboratory. A lack of consistency, e.g. becuse of a limited number of species (as in mammalian immunotoxicology) makes this field of research diffuse and extended and may result in limited progress; nevertheless, a variable or consistent effect over a variety of species is certainly a valuable observation. Some species seem to be preferred,

Candidate biomarkers for immunotoxicity in fish

Tier 1: Screening tests

- Conventional haematology, including total and differential blood cell counts, surface markers (flow cytometry), and macrophage density and morphology: easy, nonspecific
- Serum immunoglobulin concentrations in naive (unstimulated) fish: easy, limited specificity
- Lymphoid organ weight (mainly spleen, occasionally thymus): impracticable
- Histopathology of the thymus, spleen, and kidney: possible, can be specific

Tier 2: Functional assays

- Humoral immune response (agglutination, enzyme-linked immunosorbent assay): possible, can be specific
- Cellular immune response (allograft rejection in scale, skin, or eye): possible, can be specific
- Macrophage functions (phagocytosis, bacterial killing, migration, chemiluminescence): limited specificity
- Host resistance (bacterial infections): possible, can be specific, relevant

such as trout, salmon, and carp, which are practical, owing to their size, for sampling blood and tissues for laboratory studies. Smaller species, such as guppies (*Poecilia reticulata*) and medaka (*Oryzias latipes*), have secured a niche in aquatic toxicology owing to the ease of husbandry and relatively low cost; moreover, because of their small size, whole animals can be used for histopathological examination (Wester & Canton, 1991), but their application in immunotoxicology may be limited because of difficulty in obtaining adequate blood and tissue samples. For studies of saltwater species, bottom-dwelling flatfish are commonly used in field studies and, to a certain extent, in studies of mesocosms and in the laboratory. In Europe, the flounder (*Platichthys flesus*) and dab (*Limanda limanda*) are popular target species since they are susceptible to certain recognizable diseases and are commonly available.

A compehensive variety of parameters is listed by Anderson (1990) and Weeks et al. (1992). A modified set based on those lists and assays used in rodent immunotoxicology is presented in the box above and classified as tier 1 (screening tests) and tier 2 (functional assays). Parameters commonly mentioned in the literature are discussed below.

Blood cell counts and differential counts: As leukocytes play a major role in specific and nonspecific humoral and cellular immune responses, this parameter is used as a measure of the status of the defence system, in particular in tier 1 testing. It is relatively easy to test blood samples drawn from live animals, but many environmental factors unrelated to defence may modify leukocyte status (Anderson, 1990). The use of monoclonal antibodies directed against individual cell types may improve their identification (Bly et al., 1990; Van Diepen et al., 1991). Another possible parameter is the haematocrit; however, it has no known specificity for any immune function, although it may be considered as a general indicator of stress.

Nonspecific defence: Other indicators of nonspecific defence have been proposed as indicators of immunological stress. These include acute-phase proteins (Fletcher, 1986), the levels of which appear to be stress hormone-dependent; and lysozyme and ceruloplasmin activity, which are reduced in carp exposed to trichlorphon *in vivo* (Siwicki et al., 1990).

Morphology: The spleen is easy to excise and weigh in animals of adequate size and could thus serve as a biomarker, although it is not commonly reported in the literature. One reason may be that a major and variable portion of the spleen consists of storage blood or erythropoietic tissue (Fänge & Nilsson, 1985); the lymphoid tissue is poorly developed and is mainly associated with melanomacrophage centres (Zapata, 1982, 1983; Fänge & Nilsson, 1985; Van Muiswinkel et al., 1991); and, after immunization, only a small proportion of the plaque-forming cells is found in the spleen, in contrast to the kidney pronephros (Van Muiswinkel et al., 1991). The role of the spleen in most fish species thus seems to be limited, although melanomacrophage centres are abundant.

Experimental immunotoxicology in mammals has demonstrated that the weight of the thymus, a primary and exclusively immunological organ, is a sensitive indicator of thymic effects. This parameter is not commonly used in fish. One reason is that the thymus has a complex location in some species, which makes clean dissection nearly impossible. Other reasons are inconsistencies in thymic morphology, histopathology, and morphometry (Ghoneum et al., 1986; Wester & Canton, 1987). The latter paper described studies in which guppies exposed to TBTO showed dose-dependent atrophy of the thymus, as seen in rats (Krajnc et al., 1984). Both species also showed a concomitant increase in 'neutrophils', which suggests functional compensation. This response could not be reproduced in medaka (Wester et al., 1990), stickleback, or

flounder (P. Wester, unpublished results), probably indicating species specificity. Thymic lymphocyte function can also be tested *in vitro*.

Macrophage function tests: Macrophages are an important cell population for both specific (antigen processing and presentation) and nonspecific (phagocytosis and destruction) defence. They are considered to be a relatively primitive defence mechanism and are therefore of major importance to lower animals (Ratcliffe & Rowly, 1981). Much effort has been devoted to establishing macrophage parameters as biomarkers for immune effects in fish; a possible reason for this preference is the fact that these cells are fairly easy to obtain, e.g. by peritoneal washing or removal of kidney pronephros, and many function tests do not require sophisticated techniques or species-specific reagents or markers (Mathews et al., 1990). The tests include determination of chemotaxis, phagocytosis, pinocytosis, and chemiluminescence. Zelikoff et al. (1991) studied the applicability of trout peritoneal macrophages for immunotoxicology, stressing the need for systematic baseline information. In addition to the tests listed above, they studied the morphology and spread of resident and stimulated peritoneal macrophages and concluded that these cells share many morphological and functional properties with their mammalian counterparts and may thus be useful indicators in immunotoxicology. Many case studies in fish species have been published, demonstrating the sensitivity of one or more parameters to chemicals, including PAHs (Weeks & Warinner, 1986; Zelikoff et al., 1991) and pentachlorophenol, *in vitro* (Anderson & Brubacher, 1993).

Melanomacrophage centres: Melanomacrophage centres, or macrophage aggregates, are widely distributed throughout the fish body, in particular in spleen, liver, and kidney. They are composed of clusters of swollen, rounded cells (macrophages) that stain pale-tan to black. This parameter must be determined histopathologically. Their occurrence and morphology have been described (Agius, 1985), but their function is not yet fully understood. The presence of pigments (haemosiderin, lipofuscin, and ceroid) indicates storage of effete biological material (erythrocytes, biomembranes) (Wolke, 1992). The melanin present may be a generator of the bactericidal hydrogen peroxide (Roberts, 1975), and the presence of antigens indicates a role in immune reactions, e.g. antigen presentation. An increase in melanomacrophage centres can be found with age and after stress (Blazer et al., 1987), as confirmed in field studies (Vethaak & Wester, 1993). Moreover, a large number of relatively small, pale centres was seen in animals caught in late winter, when conditions are more stressful, including spawning with associated migration and starvation (Vethaak & Wester, 1993). The presumption

that the small size and pale appearance are indicators of recent development is supported by the observation that in liver tumours composed of relatively young, fast-growing tissue melanomacrophage centres are usually absent or definitely smaller. As a consequence, when these centres are used as general parameters of stress, the study groups must be matched for age.

Because they are characteristic for fish and because of the multiplicity of their functions, these structures deserve special attention in the context of defining biomarkers for immunotoxicity. In addition, they are easy to monitor, since they do not require special preparation other than routine histological procedures, including morphometry. Since melanomacrophage centres can be considered primitive analogues of the mammalian lymph follicle (Payne & Fancey, 1989), it has been suggested that their presence indicates immune capacity or function, although their role in this context has not yet been established and the implications of a change in this parameter for the integrity of the defence systems remains unclear. The density of melanomacrophage centres in liver or spleen has been successfully correlated with environmental sediment (Payne & Fancey, 1989) and along a gradient of pollution in the North Sea (Bucke et al., 1992). Other studies have reported an increase in melanomacrophage centre density after contact with chemical contaminants (Blazer et al., 1987; Secombes et al., 1992), which may indicate accumulation of cytotoxic waste or immune stimulation.

At present, macrophage function and melanomacrophage centres are the most widely used and promising indicators of the effects of environmental stress (Blazer et al., 1987). Their relationship to other components of the immune system remains to be clarified, however, in tests with immunotoxicants.

Humoral immune response: Determination of circulating immunoglobulin levels in serum is useful for testing the net result on an immunological pathway *in vivo*. The response can be measured in 'naive' animals (total immunoglobulin) or after exposure to an antigen, e.g. to verify the efficacy of vaccination in aquaculture. Sheep red blood cells can be used as a standard antigen, and the immune response can be measured by agglutination tests. ELISA tests, which are sensitive and specific, can also be used (Arkoosh & Kaattari, 1990). A related test is the haemolytic plaque assay which identifies antibody-producing cells (splenic lymphocytes) (Anderson, 1990), but which has been used to only a limited extent in fish.

Specific lymphocyte stimulation tests: Functional tests widely used in mammalian immunotoxicology, in which lymphocytes are stimulated

in vitro by exposure to mitogens such as lipopolysaccharide, phytohaemagglutinin, and concanavalin A, can also be used in fish. Proliferation is monitored by measuring the incorporation of ^{3}H-thymidine into DNA. The test is not antigen-specific but provides information on the capacity of the entire B (lipopolysaccharide) or T (phytohaemagglutinin, concanavalin A) cell population. It is used to only a limited extent in fish immunotoxicology, although Faisal & Hugget (1993) gave an elegant demonstration of significant suppression of this parameter in spot (*Leiostomus xanthurus*) under field conditions; this was shown to be related to site and pollution in controlled laboratory experiments.

Specific cellular immune responses: Tests described in the literature to measure cellular immune responses are scale or skin allograft rejection, a relatively simple test (Zeeman & Brindley, 1981), and eye allograft rejection (Khangarot & Tripathi, 1991); delayed rejection was seen in carp after exposure to copper. These tests are applied to only a limited extent.

The tests described above are mainly tier 2 tests. The tests most often used in immunotoxicology, however, are those for host resistance (challenge by infections or tumours). The results of such tests are rarely reported in the literature and have not been validated. For ultimate proof of immunotoxicity, all phases of a test (maintainence, exposure, and infection) must be conducted under strictly controlled laboratory conditions. When suitable (often species-specific) pathogens are standardized, such tests are valuable and necessary for estimating the practical consequences of suspected immunotoxicity. Although the incentive for undertaking immunotoxicological studies in fish is usually epidemiological observation of a suspected toxic component, ultimate challenge experiments must be carried out before a final conclusion about immunotoxic mechanisms can be drawn.

More emphasis has been given to the development of biomarkers than to their application in the field, for several reasons, including the lack of specificity and the lack of association between effects at the level of the biomarker and the population (Mayer et al., 1992). Some comments on and some needs in this field are as follows:

- Immunological biomarkers in fish have great potential: many have not yet been fully explored, probably owing to practical limitations of lack of specificity and predictivity.
- The number of animal species should be limited in order to concentrate research, which often requires species-specific knowledge and reagents. Standardization could be achieved by choosing well-defined inbred strains of fish (e.g. carp or trout).

- A tiered approach is highly recommended for obtaining knowledge on the specificity of the biomarker.
- More knowledge is needed on the epidemiology, mechanisms, and etiology of diseases in fish, and particularly the predictive value of immune parameters and the influence of hormesis.
- In terms of relevance for the organism, a test that monitors the net result of a cascade of reactions (e.g. specific antibody production, host resistance) is more predictive than a single, nonspecific cell parameter (e.g. macrophage activity *in vitro*).
- In identifying potential biomarkers for immunotoxicity, evidence should be available that the levels tested in the laboratory are relevant for field conditions and that the effect is directly related to the immune system.

4.3.3.2 *Chickens*

Another non-mammalian species that has been studied extensively with regard to the structure and function of its immune response is the chicken. It is therefore not surprising that the chicken has emerged as the predominant avian model for assessing compounds for potential immunotoxicity. Humoral responses to different antigens have been assessed routinely (Lerman & Weidanz, 1970; Marsh et al., 1981). The weights of the thymus, spleen, and bursa of Fabricius have been used, in combination with decreased antibody responses *in vivo* and lymphocyte responses to phytohaemagglutinin and concanavalin A *in vitro* (Eskola & Toivanen, 1974). Graft-versus-host and cutaneous basophil hypersensitivity have also been used to detect immunosuppression in chickens (Dietert et al., 1985). The availability of chicken cell lines (Sung et al., 1992) will facilitate studies of the mechanisms of action of compounds on the immune responses of this avian model.

4.4 Approaches to assessing immunosuppression *in vitro*

The complexity of the immune system and the requirement of many agents for metabolism and distribution to produce an immunotoxic response has resulted in the almost exclusive use of animal models *in vivo* for immunotoxicity assessment. Culture systems have been used extensively, however, to study the mechanisms by which agents produce immunosuppression.

Since most of the assays used for assessing immunotoxicity are ex-vivo/in-vitro tests, they are easily adapted to completely in-vitro assays for assessing immunosuppression. The direct addition of compounds in various assays, including those involving NK cells,

lymphocyte proliferation, mixed leukocytes, and Tc lymphocytes, has been used to determine the mechanisms by which compounds alter the immune response at the cellular and subcellular level. Similarly, the action of benzene and its metabolites on bone marrow has been studied extensively *in vitro* (Gaido & Wierda, 1987), and the effects of TCDD on thymocytes have been well studied in thymic epithelium co-cultures (Greenlee et al, 1985). One of the most useful in-vitro assays for studying immunosuppression is the T-dependent antibody response to sheep erythrocytes. This assay, also known as the Mishell-Dutton assay (Mishell & Dutton, 1967), has been used extensively in studying the cellular target of immunotoxicants. It is the in-vitro counterpart of the in-vivo plaque-forming cell assay, but sensitization with sheep erythrocytes takes place in splenic cell culture and the plaque response is measured on day 5 after addition of the erythrocytes. The Mishell-Dutton assay has been used to study the structure-activity relationships of various immunosuppressive compounds (Kawabata & White, 1987; Davis & Safe, 1991). Since T cells, B cells, and macrophages are needed for the response and an adverse effect on any of these cell types can produce immunomodulation, it has proved to be a sensitive assay for evaluating compounds *in vitro* for immunosuppressive activity. Furthermore, since the various cell types that participate in the response can easily be separated, individually treated, and then reconstituted in the culture system, it is an excellent assay for determining which cell type is adversely affected by the compound. Using this approach, White & Munson (1986) demonstrated that asbestos suppresses the response by affecting macrophages; Shopp & Munson (1985) showed that the primary action of phorbol ester on the antibody response occurs through an effect on B cells; and Johnson et al. (1987) found that *N*-nitrosodimethylamine affects primarily B cells.

As indicated above, one of the limitations of in-vitro systems is that exogenous metabolic activation systems are often required. While lymphocytes can metabolize some compounds, such as benzo[*a*]pyrene, to active metabolites (Ladics et al., 1992), other potent immunosuppressive compounds such as cyclophosphamide require a metabolic activation system. Such preparations usually consist of a 9000 x *g* supernatant of liver (S9). Using S9 preparations, Tucker & Munson (1981) showed that cyclophosphamide could be activated to an immunosuppressive form *in vitro*. Similarly, naphthalene could be metabolized to an immunosuppressive metabolite (Kawabata & White, 1990). An alternative approach to the S9 activation system is a hepatocyte co-culture system, which has been shown to be capable of activating several parent compounds to their immunosuppressive metabolites (Yang et al., 1986).

Predictive in-vitro systems based on immune cells of human origin are particularly attractive, given the uncertainties of extrapolating the results of experimental studies to humans and the accessibility of immune cells in human peripheral blood. Although many of the immune cells obtained from human blood are immature forms, the large numbers and diverse populations (i.e. polymorphonuclear leukocytes, monocytes, NK cells, T cells, and B cells) that can be obtained provide an attractive alternative or adjunct to conventional studies in experimental animals. As a consequence, a number of studies have been conducted to compare the functional response of human and rodent lymphocytes to putative immunosuppressive agents *in vitro* (Cornacoff et al., 1988; Luo et al., 1992; Wood et al., 1992; Lang et al., 1993). Although these studies were hampered by the lack of assays to assess primary antigen-specific immune responses in human lymphocytes, a relatively good interspecies correlation has been observed in the limited responses available. Furthermore, several of these assays have been successfully modified to include co-culture with primary hepatocytes (Kim et al., 1987) to allow for chemical metabolism.

4.5 Future directions

4.5.1 Molecular approaches in immunotoxicology

A promising avenue for early detection of immunotoxicity may be measurement of the expression of various interleukins. Cytokines are involved both in regulation of the immune system and in pathological phenomena, hence alterations in their pattern of expression may be early indicators of immunotoxicity. Such testing can be done at the level of mRNA expression, on mRNA extracted from lymphoid tissue taken from exposed animals, or in tissue sections, so that the alterations can be evaluated in the context of morphological indications of the toxic effects. The signal of the cytokine that is being tested must therefore be strong enough to be picked up in material from exposed animals whose immune system has not received other stimuli, i.e. sensitization or infection. This may not be true for all cytokines; ex-vivo stimulation of cells that are part of the immune system may be necessary, although the tests then become more laborious and must in fact be considered functional assays, like tests for mitogen responsiveness.

Very sensitive analysis can be done with the semiquantitative polymerase chain reaction, which is a powerful technique for elucidating early kinetic changes of cytokine expression, before translation and secretion (Saiki et al., 1985). In addition, since immunosuppressive agents can enhance or inhibit the ultimate production and secretion of

cytokines at various stages such as transcription, the splicing of mRNA, translation of mRNA into polypeptides on ribosomes, post-translational processing, and secretion, potential molecular targets can be dissected by such techniques. Several other molecular approaches may be used, including northern blotting, dot–slot blotting, in-situ hybridization, and antisense oligonucleotides for inhibiting the translation of specific mRNAs.

4.5.2 *Transgenic mice*

The development of molecular genetic techniques has allowed not only the isolation and analysis of specific genes but also the manipulation of embryonic genes. Transgenic technology can be used in immunology to generate mice that lack virtually any genetic control mechanism or specific cell subpopulations. As a consequence, complex systemic responses can be dissected into individual components, and the mechanism by which immunosuppressive agents exert their affects can be better understood. Two strategies are used to induce genetic aberrations in transgenic mice (Bernstein & Breitman, 1989). One involves the introduction of genes that produce toxins, such as diphtheria toxin or the A subunit of ricin, into targeted cell subpopulations. The second strategy involves the thymidine kinase (*tk*) gene from *Herpes simplex* virus: When certain nucleotide analogues are administered and are metabolized exclusively by viral thymidine kinase, the metabolites are lethal only to cell subpopulations that express the *tk* gene. Both approaches are inducible systems for killing cells *in vivo*. Although gene ablation techniques can be used to generate mutant animals that lack specific cells *in vivo*, a small proportion of cells appeared to escape from targeted cell death in virtually every study using bacterial toxins or viral *tk* genes. While this may cause problems in determining the qualitative roles of ablated cell populations, these techniques hold promise for understanding the selective toxicity of drugs and environmental agents on the immune system.

Other promising avenues are the use of animals transgenic with respect to certain specificities of the TCR. If a gene that encodes for a certain antigen specificity is introduced into the genome, that specificity may be the only one that is expressed by the T cells. The effects of immunotoxicants that affect the (positive and/or negative) selection process that takes place in the thymus could be studied elegantly with such models, when either undesired specificities (which should be negatively selected) or desired specificities (which should be positively selected) are introduced.

4.5.3 *Severe combined immunodeficient mice*

Another approach that may warrant further exploration is the use of severe combined immunodeficient CB-17 *scid/scid* (SCID) mice grafted with human immune cells. Xenogeneic lymphoid cells and/or tissues can be successfully transferred to SCID mice (McCune et al., 1988; Namikawa et al., 1990; Barry et al., 1991; Greiner et al., 1991; Surhe & Sprent, 1991). SCID mice have been grafted with human fetal lymphoid tissue in order to study human haematopoiesis (McCune et al., 1988) or with human peripheral blood lymphocytes to allow production of human immunoglobulins, including secondary antibody responses (Mosier, 1990). SCID mice have also been used to study autoimmunity and potential antiviral therapeutics. While these animal models still have limitations (Pollock et al., 1994), they may ultimately provide predictive models for examining potential immunosuppressive agents.

In particular, SCID mice co-implanted with human fetal thymus and liver tissue fragments (SCID/hu mice) offer the possibility of studying the human thymus *in vivo* in an isolated xenogeneic environment (McCune et al., 1988; Namikawa et al., 1990) and the effects of immunotoxicants on these grafts. This system is particularly interesting with regard to those immunotoxicants for which the thymus is one locus of action. The placement of human fetal thymus under the SCID mouse renal capsule, followed by an intravenous injection of fetal liver cells (McCune et al., 1988), and co-implantation of human fetal liver and fetal thymus under the renal capsule of SCID mice (Namikawa et al., 1990) have resulted in reconstitution of SCID/hu mice; the fetal thymic implants increased in size, and were found to be vascularized. The architecture and antigenic distribution of these thymic grafts were virtually indistinguishable from those of normal, age-matched human thymus. Human stem cells were found to home to and differentiate within the grafted human thymus, and phenotypically mature and functional human T cells were found in the peripheral circulation of these mice (McCune et al., 1988; Krowka et al., 1991; Vandekerckhove et al., 1991). As such, the SCID/hu model can be helpful in immunotoxicological research on the human thymus. When data obtained in experimental animals are extrapolated to the human situation, a 'control' model, between the SCID/hu mouse model and the intact laboratory animal (rat), is desirable in order to test for possible differences in thymic behaviour, because of its location under the kidney capsule: Thymic blood flow and therefore the toxicokinetic behaviour of the thymus may differ. For this reason the SCID/ra model was developed, by implanting rat fetal thymus and liver tissue fragments under the SCID mouse renal capsule. The outcomes of exposure of rats

and SCID/ra mice can be compared and the influence of thymus location and mouse metabolism on extrapolation from SCID/hu to humans can then be determined.

Implantation of fetal rat thymus and liver tissue yields thymic grafts that are virtually indistinguishable from normal, age-matched rat thymus (De Heer et al., 1993). After implantation of rat fetal thymus and liver tissue, the thymic grafts increase considerably in size. Histologically, the SCID/ra thymic graft bears a close resemblance to normal rat thymus, and the (immuno)histology of the SCID/hu and SCID/ra mouse thymic grafts is comparable. Differences are found, however, in peripheral reconstitutions of SCID/hu and SCID/ra mice: Whereas large numbers of circulating donor rat T cells are found in the blood and peripheral lymphoid organs of SCID/ra mice, only a small number of donor T cells are found in the SCID/hu. This implies that the data for extrapolating immunotoxic data from rats to humans must be confined to thymic effects. With this restriction in mind, the outcome of experiments with SCID/hu and SCID/ra mice can be used to compare the sensitivity of the human and rat thymus and can thus yield important information for the process of human risk assessment.

4.6 Biomarkers in epidemiological studies and monitoring

There is a difference between assays of the immune system and biomarkers. Many validated tests can indicate alterations to the immune system, including its function, so that most assays can be helpful for hazard identification. Not every validated assay of the immune system is a biomarker, however. The IPCS (1994a) definition of a biomarker is one that indicates exposure (and is specific for exposure), indicates susceptibility to adverse effects, and/or is predictive of disease associated with exposure. Biomarkers should be used to characterize risk due to exposure, on the basis of identification of the hazard.

Within this strict definition, it is clear that not many biomarkers are available for immunotoxicity (as is true for other systems), especially for assessing immunotoxicity or individual susceptibility to immuno-toxicity. Some assays may be useful in epidemiological studies. In any event, more epidemiological studies are needed to obtain a better view of the usefulness of biomarkers for detecting immunotoxic events and hence the possible health risks that may be associated with exposure to immunotoxicants.

4.7 Quality assurance for immunotoxicology studies

In many countries, studies to support the safety of a compound or drug must be conducted in accordance with the requirements for 'good

laboratory practice' of the agency that is evaluating the material. Immunotoxicological studies conducted to support the safety of a new drug or chemical should follow at least the 'spirit' of good laboratory practice. The OECD has published their principles of good laboratory practice, with supporting publications on their application, and these have been adopted into legislation in a number of countries. IPCS (1992) has published a monograph, *Quality Management for Chemical Safety Testing*, covering the important aspects of good laboratory practice in a nonregulatory context and quality control of chemical analyses.

In the United States, good laboratory practice for conducting nonclinical laboratory studies for submission to the Food and Drug Administration has been detailed. The standards for studies on pesticides submitted to the Environmental Protection Agency were also published, as were the procedures to be followed in conducting studies submitted on compounds covered by the Toxic Substance Control Act. Each of these sets of standards is periodically updated by the respective agencies, and studies must be conducted in accordance with the most recent updates. While there are some differences in the wording of the standards, they are generally similar.

Good laboratory practice includes written protocols for evaluating potential immunotoxicants and the establishment of standard operating procedures for assays. Each laboratory must run the assays frequently enough to establish historical control values, and the results of any study conducted to evaluate a compound for immunotoxicity must be judged in the context of the historical control values for the laboratory and appropriate controls. Incorporation of positive control compounds in the study design provides additional confidence that the assays are being conducted correctly, particularly when the tested compound shows no effect.

The selection of assays to be used in evaluating compounds for immunotoxicity remains a subject of active discussion. Other parts of this document address this issue in detail. Regardless of which assays are used, however, they must be standardized and be recognized as validated and meaningful. Significant advances have been made in the standardization and harmonization of assay procedures for assessing immunotoxicity, mainly as a result of the willingness of leading laboratories in the field to share their standard operating procedures openly with other laboratories. Published papers and books on immunotoxicity test methods also contribute to the standardization process. As a result of studies by Luster et al. (1988), the assays used by the NTP for evaluating potential immunotoxicants have been accepted as validated assays in mice.

4.8 Validation

An important requirement of tests for evaluating immunotoxicity is that they be validated. While there is no agreed definition of validation, tests must meet certain requirements. In toxicology, validation is the process by which the reliability and relevance of a test to identify human health risk is established (Balls et al., 1990). A flow diagram of a proposed validation process and its end-point, the acceptance of a method by regulatory authorities for submission of toxicological data, is presented in Figure 36.

Four parameters must be considered in determining the validity of a testing method: specificity, sensitivity, accuracy, and precision. Specificity is based on the rate of false-positive results generated. Sensitivity is determined by the ability to identify true-positive results. These two parameters determine the level of predictability or relevance. In order to determine specificity and sensitivity, the method is evaluated with a set of compounds of known positive and negative immunosuppressiveness. This approach was used by the NTP in evaluating the predictability of various assays (Luster et al., 1988). In a subsequent study, the potential immunotoxicity (defined as a dose-related effect on any of two immunotoxicological parameters with no effect on body weight) was determined for 51 chemicals in mice, using a variety of general and functional immunological parameters (Luster et al., 1992).

Accuracy is determined by the ability to measure the intended end-point truly. Precision is the ability to reproduce results from experiment to experiment or between laboratories. In the NTP studies in mice (Luster et al., 1988), four laboratories participated in the inter-laboratory validation process. A number of international studies are in progress on the precision of several assays, using the rat as a model for immunotoxicological evaluations, in an attempt to bring the level of acceptance of immunotoxicological studies in rats to the level that has been achieved for mice. Most of these studies are multinational and represent an interaction of industry, government, and academia to achieve this common goal.

A comparative study in Fischer 344 rats with cyclosporin A (White et al., 1994) encompasses nine laboratories in Canada, Europe, and the United States. The primary focus of the study is on the use of functional assays for detecting immunosuppression; lymphoid organs and tissue are weighed and examined histopathologically in several of the laboratories. The functional assays used in this protocol include the plaque-forming cell assay or ELISA to sheep erythrocytes, splenocyte proliferative assays to concanavalin A and STM, the NK cell assay, and the mixed leukocyte response. Splenocyte surface markers were also

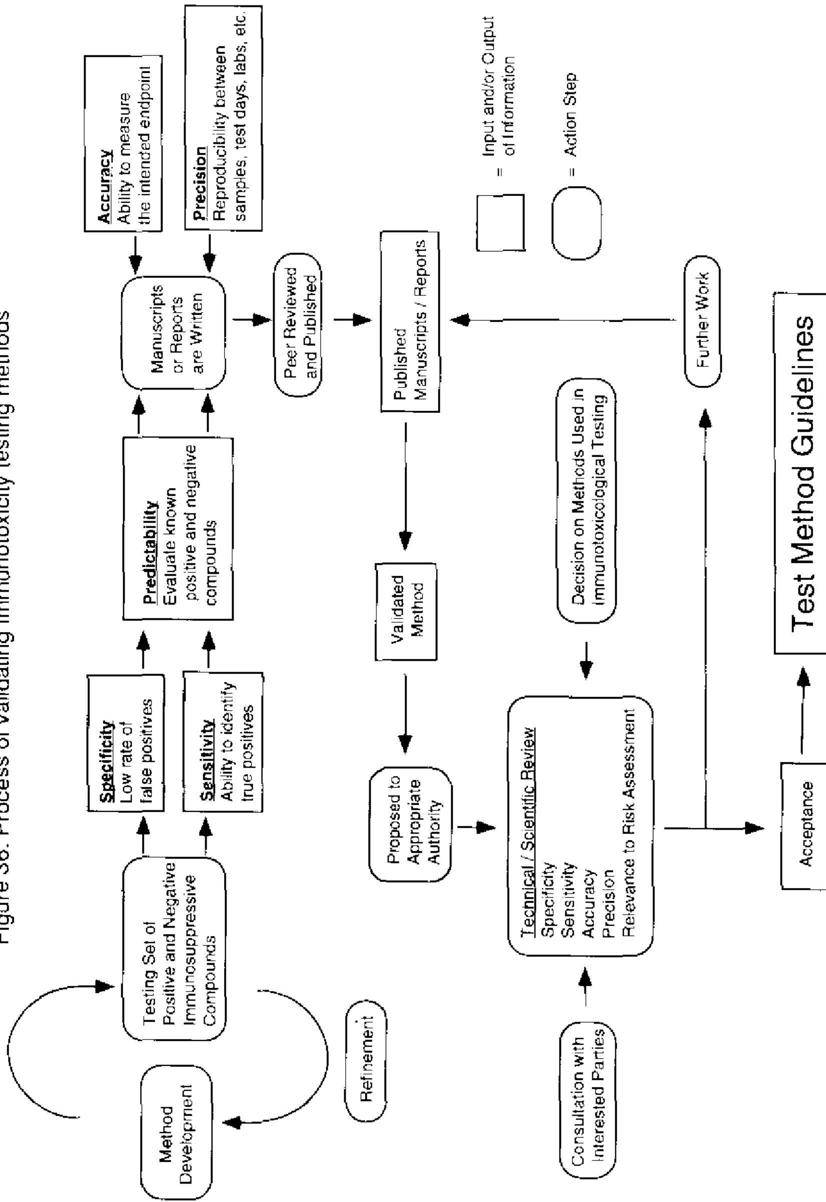


Figure 36. Process of validating immunotoxicity testing methods

analysed. The study design was similar to that used by the NTP, with a 14-day exposure and administration by oral gavage. The preliminary results demonstrated excellent reproducibility of the results for the plaque-forming cell assay, splenocyte proliferative assays to concanavalin A and STM, and the mixed leukocyte response. Differences were observed between the laboratories in the results of the test for NK cell activity.

The IPCS–European Union international collaborative immunotoxicity study in rats is also in progress. The study involves 20 laboratories in Canada, Europe, Japan, and the United States; its design is based on the OECD test guideline No. 407 for a 28-day toxicity study. The study focuses primarily on the ability to detect immunotoxic compounds on the basis of organ weights, pathological findings, and 'enhanced pathology', which includes additional evaluation of lymphoid tissues not currently required by test guideline No. 407. Functional assays were also conducted; the core assays included the plaque-forming cell assay or ELISA to sheep erythrocytes, splenocyte proliferative assays (concanavalin A and STM), and the NK cell assay. The study was conducted in two phases. In the first phase, azathioprine was used as the test compound, various strains of rat were used, and each laboratory established its own doses on the basis of a predetermined maximum tolerated dose. In the second phase, cyclosporin A was the test compound, only three strains of rat were used, and a more structured protocol was followed. A report on phase I of the study has been drafted, and the data from phase II are currently being analysed. All of the laboratories found that azathioprine is immunosuppressive, even though several strains of rats were used, different dose levels were administered, and no standard protocol was followed. The preliminary results with cyclosporin A show good agreement between the laboratories for the plaque-forming cell assay but some differences for the splenocyte proliferative assays (concanavalin A and STM) and the NK cell assay.

A third interlaboratory study in rats has been organized by the German Bundesgesundheitsamt, in Berlin, with German and French participants. The design is also based on OECD test guideline No. 407 for a 28-day repeated-dose study in Wistar rats. Cyclosporin A was selected as the test substance. The live phase of this study has been completed, the data are being analysed, and the final report is being prepared.

Information obtained from studies of predictability, accuracy, and precision, such as those described above, must undergo peer review before publication. A major goal of the validation process is to determine which methods should be recommended in the testing guidelines of

regulatory agencies. Figure 36 shows each input and output of information and the action steps. The process of developing and obtaining acceptance of testing guidelines is based on three major inputs: (1) publication of reports in peer-reviewed journals; (2) guidelines for deciding whether a method is valid; and (3) implementation of test methods that are interpretable by scientists involved in assessing biologically relevant risks and the results of which can be incorporated into quantitative dose–response analyses. The proposed process is built around the generation of these major inputs. Two of the issues that will arise in the development of guidelines are: 'How many and what type of compounds should be included in the validation process?' and 'Should the compounds be shown to be immunosuppressive in both humans and mice?'

5. ESSENTIALS OF IMMUNOTOXICITY ASSESSMENT IN HUMANS

5.1 Introduction: immunocompetence and immunosuppression

An immune response in the fully mature, immunologically competent individual provides protection against a myriad of infectious agents and environmental hazards. The immune system acts as a self-restoring (homeostatic) system which can quickly return to normal levels of function after periods of marked stimulation and response. This self-regulation allows the individual to recover from or circumvent the toxic effects of many potentially damaging environmental hazards. There are many well-known clinical conditions of inherited deficiency in immunological function; some result in specific defects in antibody formation, others consist of T-cell and/or metabolic defects, while others include impairment of both B- and T-cell function. These conditions, known as primary immunodeficiency disorders, are due to definable, inheritable genetic defects. Clinical studies of these disorders have demonstrated the importance of the immune system to host defence and individual survival, showing that individuals with partial or absolute defects in T-cell function rarely survive beyond infancy or early childhood. In contrast, individuals with defects in B-cell function, resulting in a deficiency in antibody formation, may suffer from a variety of chronic, recurrent infectious diseases and diminished health but can survive with appropriate therapy when the underlying disorder is recognized. Study of these genetic immunodeficiency states has also provided considerable information about the functions of human B and T cells which would otherwise not have been determined.

Impairment of the function of a key component of the immune system results in a diminished immune response (immunosuppression) or immunodeficiency. Acquired immunodeficiency states were recognized only sporadically until the late 1970s, when a syndrome appeared that spread rapidly through certain groups and produced a generalized type of immunosuppression known as acquired immunodeficiency syndrome (AIDS). AIDS was found to be due to retroviruses that infect and destroy Th ($CD4^+$) cells in humans (Fauci et al., 1991). CD^+ lymphocytes have been identified in experimental studies as the key cells in the recognition and secondary processing of antigens. Thus, progression of AIDS is associated with progressive loss of Th cells and increased frequencies of infection by bacterial, fungal, viral, and parasitic agents and of certain types of neoplasms.

5.2 Considerations in assessing human immune status related to immunotoxicity

The assessment of immunotoxicity in humans exposed to potentially immunotoxic compounds is much more complicated than in experimental animals. Issues such as logistics, appropriate controls, magnitude and pattern of exposure, and confounding parameters such as medication, drug abuse, and illness must be considered. Other considerations that should be taken into account in comparing human immune status with that in laboratory animals in relation to immunotoxicity are as follows:

(1) The human population is heterogeneous and genetically disparate; it can be considered as 'wildlife'. Inbred laboratory animals are, by definition, genetically identical; outbred laboratory animals typically have a larger genetic variability than inbred animals but a variability that is much smaller than that in wildlife populations. Genetic constitution, which accounts for the variability, has consequences for the antigen recognition capacity of the immune system, especially for the T-lymphocyte population. Antigen recognition by T cells is restricted to the MHC haplotype of the individual and therefore differs between (allogeneic) individuals. Inbred, and most outbred, laboratory animals are much more alike in antigen recognition capacity than wildlife populations. For instance, the repertoire of certain inbred mouse strains lacks part of the spectrum of T-cell specificity, as seen by the absence of T cells that express distinct 'variable gene families' in the repertoire of TCR specificities. Such 'gaps' in the repertoire have thus far not been detected in the outbred human population by similar methods of detection (Hu et al., 1993), perhaps because each individual in an outbred population expresses the MHC products of both parents and can in principle multiply the repertoire of MHC-restricted reactions by a factor of 2 (including MHC I and MHC II).

Interindividual variability has obvious consequences for immunotoxicity, in which the response to the chemical or drug underlies the mechanism of toxicity. Its effect on direct toxicity is presumably less, but the manifestation of toxicity is often reduced immune reactivity (e.g. increased incidence of infections) and hence determined by individual reactions to antigens.

(2) The human population, like populations of 'wildlife' animals, is continuously exposed to environmental stimuli. It is well known that it is not necessary that each member of a population be protected ('immune') but that a certain proportion of protected individuals must

be reached in order to achieve 'herd immunity'. That is to say, the whole population is protected when a certain percentage of individuals is immune. In contrast, when this percentage falls below the required incidence (which differs for different infectious agents), the population as a whole loses its protected state, and an infectious epidemic can result. This situation easily arises in small groups in a country where vaccination is minimal. Well-known examples are the outbreaks of poliomyelitis in the Netherlands and the hepatitis A virus outbreaks in China. This phenomenon should be taken into account in epidemiological studies and associated laboratory investigations (e.g. antibody levels to microorganisms) in assessing immunotoxicity in human populations.

(3) Most of the human population is continuously exposed to environmental stimuli and maintains its ability to respond to foreign material from the pool of immunological memory. From the first postnatal period through to adulthood, the T-cell repertoire is generated by the thymus; later, this generation of cells is reduced to a low level. Strict MHC restriction implies that the T-cell population cannot easily change specificity, e.g. by somatic mutation of the genes that encode the TCR. There is some evidence from immunophenotyping, both in mice and humans, that the T-cell population shifts gradually during life, from naive (committed) T cells to memory T cells. Within the B-cell population, the situation is different. Here, the repertoire changes continuously, due to somatic mutation presumably associated with 'affinity maturation' in lymph nodes. This phenomenon may result in the emergence of B cells with a strong affinity for stimuli and the disappearance of low-affinity cells. It is not known whether neoantigens or pathogens are recognized by 'affinity matured' or memory B cells or by naive B cells. In the absence of information on this aspect, it can be suggested that most of the immune capacity of adults is deployed for memory reactions, whereas in young people the contribution from the naive pool is higher. This is reflected in infectious epidemics, when microorganisms like influenza change to phenotypes that cannot be recognized by the memory pool, an aspect to be kept in mind when choosing immune tests to be used in evaluating immunotoxicity. Primary responses, like those to keyhole limpet haemocyanin antigen, are considered to be more sensitive than secondary responses, like those to tetanus toxoid. Another example of this effect is the composition of the recall antigens used in testing delayed-type hypersensitivity (Borleffs et al., 1993). Both primary and secondary antibody responses, however, are valuable for evaluating the intrinsic naive and memory immune capacity of individuals, although secondary responses are less sensitive to immunological insults.

(4) For a number of infectious microorganisms, the immune response does not result in complete elimination of the invader but rather in its 'silent' integration into the genome. Certain viruses, like herpes viruses, cytomegalovirus, and Epstein-Barr virus, are dealt with in this way. An individual is considered to be a carrier of the virus (postinfection status) on the basis of the presence of antibodies. In other words, individual postinfection is a continuous defence against these viruses, often with sufficient capacity to keep the virus in a silent form. In diminished immune capacity, this natural protection can be lost, and infections can re-occur after viral reactivation. Primary infection and reactivation therefore have different pathogeneses, although the subsequent disease may be characterized by the same symptoms. This situation is well known clinically, when high doses of immunosuppressive drugs are given for long periods. The relevance of this observation in immunotoxicity testing must be established.

(5) Ex-vivo diagnosis in humans is often restricted to haematological investigations, so that only information on the circulatory pool of cells and plasma factors is obtained. For example, the distribution of immunoglobulins differs in the intravascular and extravascular spaces. Only about 1% of the total lymphocyte pool is present in blood (10^{10} cells out of the total of about 10^{12}), and this population represents only the recirculating pool of cells and not the tissue-bound cells that participate actively in immunological responses. Investigations of peripheral blood cells can be somewhat misleading: for instance, patients infected with the human immunodeficiency virus (HIV)-1 may show severe depression of CD^- cells in blood but less reduction in CD cells in lymphoid tissue (Schuurman et al., 1985).

It is considerably more complex to establish immune changes in humans than in animals, since noninvasive tests are limited, the levels of exposure to an agent (i.e. dose) are difficult to establish, and the responses in the population are extremely heterogeneous. With respect to the latter, the variation in immune responses (genetic or environmental) can exceed a coefficient of variation greater than 20–30%. Because many of the immune changes in humans that follow exposure to chemicals may be sporadic and subtle, recently exposed populations must be studied and sensitive tests for assessing the immune system be performed. Since many of the immune tests used in humans have a certain degree of overlap (redundancy), it is also important that a positive diagnosis not be based on a change in one test but on a profile (pattern) of changes, similar to that observed in primary or secondary immunodeficiency diseases. For example, low CD:CD8 ratios are often accompanied by changes in skin reactions to recall antigens. The Clinical Immunology Subcommittee of WHO and the International

Union of Immunological Societies published methods for examining changes in the human immune system and described their pitfalls (Bentwich et al., 1982, 1988); however, most of the tests were selected on the basis of observations in patients with primary immunodeficiency diseases. Such individuals suffer from severe recurring infections, and their degree of immunosuppression is probably considerably greater than that induced by chemicals. Thus, some of the methods may be of limited value for examining potential chemical-induced immunosuppression, and further evaluation of methods is needed.

In view of the difficulties in identifying chemical-induced immunosuppression in humans, establishment of exposure levels (e.g. in blood or tissue) would not only be useful but would in many instances be essential for determining a cause–effect relationship. Clinical disease may not necessarily have to be present in order for immunosuppression to be meaningful, for several reasons. Firstly, there are uncertainties about the extent of the reserve capacity of the immune system and whether the relationship between immune function and clinical disease shows a linear or a threshold response. In a linear relationship, even minor changes in immune function would be related to an increase in disease, if the population examined is large enough. While the relationship at the low end of the dose–response curve is unclear, at the high end of the curve (i.e. severe immunosuppression), clinical disease is readily apparent. This is exemplified by increased incidences of the opportunistic infections that occur in AIDS patients. Secondly, clinical disease may be difficult to establish, as neoplastic diseases may involve a 10–20-year latency before tumour appearance, and increased infection rates are difficult to ascertain in epidemiological surveys (e.g. increased numbers of cases of severe common cold).

5.3 Confounding variables

The normal population has a wide range of immunological responses, with no apparent health impact. In addition to the underlying population variability, certain host characteristics and common exposures may be associated with significant, predictable alterations in immunological parameters. If not recognized and effectively addressed in study design or statistical analysis, these confounding factors may severely alter the results of population studies.

Examples of factors associated with measurable alterations in immunological parameters are age, race, sex, pregnancy status, acute stress and the ability to cope with stress, coexistent disease or infection, nutritional status, lifestyle, tobacco smoking, and some medications. The effect of acute stress on the immune system is mentioned in section

1.2.1.5. Protein caloric restriction and deficiencies of trace elements such as zinc have also been associated with immune deficiency (Chandra, 1992; Good & Lorenz, 1992; Chandra, 1993). Periodic influences, ranging from daily to seasonal, also exist; some are relatively minor, but others are of a magnitude that may rival the expected effect of a low-level exposure to a toxic agent. They are therefore of primary concern in large epidemiological studies. For example, African Americans have, on average, serum IgG levels that are 15–20% higher, neutrophil counts that are 10–15% lower, and a proportion of circulating B cells that is significantly higher than those of Caucasians, with no discernible health implications. Cigarette smoking is associated with a significant decrease in IgG level and an increase in leukocyte count, independent of ethnic differences. Therefore, it is imperative that population norms and reference ranges be supplemented by detailed analysis of potential confounding factors. Study designs should include considerations of matching, stratification, and subgroup analysis to control for these potential effects. As new immunological assays are developed, normative data will be required, particularly for ethnic minorities, children, the aged, and certain groups potentially at high risk, such as pregnant and lactating women.

Certain endocrine diseases and conditions may be associated with significant alterations in immune function (e.g. adrenal dysfunction). Some medications, such as corticosteroids, phenytoin, and nonsteroidal anti-inflammatory agents, may depress a variety of immune functions. Questionnaires and population surveys should allow the collection of sufficient information to make it possible to consider these factors in data analysis and interpretation.

An increasingly important consideration in any analysis of immune function, particularly in relation to immune deficiency, is the potential presence of HIV infection, which causes widespread alterations in virtually all elements of the immune system. Even a small proportion of unrecognized HIV-infected individuals in a study population may significantly affect the results and the interpretation of data. When immunological studies indicate decreased immune parameters consistent with HIV infection, testing for the virus should be considered; otherwise, interpretation of the results of immunological tests of immune dysfunction, particularly among populations with potentially high rates of HIV infection, may be severely limited.

In assessing human immunotoxicity, it is useful to establish the presence of infectious, allergic, or autoimmune diseases in order to ensure completeness and to rule out additional confounding variables. A clue to the type of immunological defect is often provided by the kind of infection observed. For example, patients with impaired

humoral immunity have an increased incidence of recurrent infections with encapsulated bacterial pathogens (e.g. *Pneumococcus* and *Haemophilus influenzae*), which can induce chronic sinopulmonary infection, bacteraemia, and meningitis. In contrast, if cellular immunity is intact, the patients will have less severe infections with fungal and viral agents. Abnormalities of T cells and impairment of cell-mediated immunity predispose individuals to infections with a wide variety of agents, including viruses that cause disseminated infections (e.g. *Herpes simplex* virus, varicella-zoster virus, and cytomegalovirus), fungi that cause mucocutaneous candidiasis, and parasitic organisms including the protozoan *Pneumocystis carinii*.

5.4 Considerations in the design of epidemiological studies

An important factor in assessing the usefulness of an epidemiological study for risk assessment is its design. The commonest design used in immunotoxicology is the cross-sectional study, in which exposure and disease status (in this case, changes in immunological function) are measured at one time or over a short period. The immune function of 'exposed' subjects is compared with that of a comparable group of 'unexposed' individuals. Important considerations in using the data provided by such studies in risk assessment have been discussed (E. Ward, unpublished manuscript):

(1) What is the relationship between changes in immune function and human health risk?
(2) Are the selection procedures for study subjects adequately documented?
(3) Is there evidence that the exposed group was actually exposed to the substance of interest?
(4) Has the possibility that other exposures, of either the entire population or individuals, been accounted for?
(5) Are the 'exposed' and 'unexposed' populations comparable with respect to factors other than the exposure of interest?
(6) Have major individual aspects (such as illness and use of medications) that may influence the outcome of tests for immune function been accounted for?
(7) Has inter- or intralaboratory variability been controlled for? Was the laboratory that ran the tests for immune function able to distinguish between samples from 'exposed' and 'unexposed' individuals?

5.5 Proposed testing regimen

Biological research involving human subjects must be conducted in accordance with ethical standards and involve scientific procedures designed to ensure the safety of the subjects (Council for International Organizations of Medical Sciences, 1993). Below are shown a testing scheme proposed by WHO for preliminary evaluation of individuals exposed to immunotoxicants, an approach developed by a working group organized by the United States Centers for Disease Control and Agency for Toxic Substances and Disease Registry, and that proposed by a panel of the United States National Academy of Science (US National Research Council, 1992). The three approaches have many similarities.

Assays suggested by WHO for assessing immunotoxicity in all persons exposed to immunotoxicants

1. Complete blood count with differential counts
2. Antibody-mediated immunity (one or more of following):
   - Primary antibody response to protein antigen (e.g. epitope-labelled influenza vaccine)
   - Immunoglobulin concentrations in serum (IgM, IgG, IgA, IgE)
   - Secondary antibody response to protein antigen (diphtheria, tetanus, or poliomyelitis)
   - Natural immunity to blood-group antigens (e.g. anti-A and anti-B)
3. Phenotypic analysis of lymphocytes by flow cytometry
   - Surface analysis of CD3, CD4, CD8, CD20
4. Cellular immunity
   - Delayed-type hypersensitivity skin testing using Multitest Biomerieux®
   - Primary delayed-type hypersensitivity reaction to protein (keyhole limpet haemocyanin)
   - Proliferation to recall antigens
5. Autoantibodies and inflammation
   - C-Reactive protein
   - Autoantibody titres to nuclei, DNA, mitochondria and IgE (rheumatoid factor)
   - IgE to allergens
6. Measure of nonspecific immunity
   - Numbers of natural killer cells (CD56 or CD60) or cytolytic activity against K562
   - Phagocytosis (nitroblue tetrazolium or chemiluminescence)
7. Clinical chemistry

Screening panel recommended for human studies by the United States Centers for Disease Control and Agency for Toxic Substances and Disease Registry

- Complete blood count with differential counts
  - absolute lymphocyte count
  - granulocyte count
  - platelet count
  - absolute eosinophil count
  - examination of peripheral smear
- Immunoglobulins
  - IgG
  - IgA (optional)
  - IgM (optional)
- C-Reactive protein
- Autoantibody screening panel
  - Antinuclear antibody
  - Rheumatoid factor
  - Anti-thyroglobulin antibody
- Peripheral blood leukocyte surface markers
  - CD2/CD3
  - CD4/CD8
  - CD8/CD3
  - CD19/CD20
- Clinical chemistry in serum
  - Blood urea nitrogen
  - Cholesterol
  - Creatinine
  - Total bilirubin
  - Alkaline transaminase
  - Alkaline phosphatase
  - Total protein (albumin:globulin ratio)

Tests recommended by the panel of the United States National Academy of Sciences for studies of persons exposed to immunotoxicants

Tier 1 (all persons exposed to immunotoxicants)

I. Humoral immunity
- Immunoglobulin concentrations in serum (IgM, IgG, IgA, IgE) and immunofixation electrophoresis
- Natural immunity: Antibody levels to ubiquitous antigens (e.g. anti-A and anti-B group substances in individuals of non-AB blood type)

- Secondary antibody responses to proteins (e.g. diphtheria, tetanus, poliomyelitis) and polysaccharides (e.g. pneumococcal, meningococcal)

Note: In immunization studies, live microorganisms should not be given to persons suspected of being severely immunocompromised.

II. Lymphocytes
- Enumeration of B and T cells in blood
- Surface analysis of CD3, CD4, CD8, CD20
- Secondary delayed-type hypersensitivity reaction (e.g. candida, diphtheria, tetanus)
- Alternative: Multiple antigen skin test kit

III. Autoantibody titres (to red blood cells, nuclei, DNA, mitochondria, IgE (rheumatoid factor)

Tier 2 (all persons with abnormal Tier 1 test results and a fraction of the total exposed population to be determined by a statistician)

I. Humoral immunity
- Primary antibody response to protein and polysaccharide antigens

Note: A panel of antigens should be developed that can be used in sequential studies on a given individual, since a particular antigen can be used only once to assess a primary response.

II. Cellular immunity
- Proliferative response to mitogens (phytohaemagglutinin, concanavalin A) and possible antigens such as tetanus; primary delayed-type hypersensitivity reaction to keyhole limpet haemocyanin

Note: Here, too, a panel of standard antigens is needed for sequential testing; these could be the same as those used to assess primary antibody responses.

III. Natural killer cells, monocytes, and other T- and B-cell markers
- CD5, CD11, CD16, CD19, CD23, CD64; class II MHC on T cells by two-colour flow cytometry for co-expression of class II and a T-cell marker such as CD3

IV. Serum levels of cytokines (e.g. IL-1, IL-2, IL-6) and of shed or secreted cellular activation markers and receptors (e.g. CD25).

V. Class I and II MHC antigen typing

Tier 3 (to be considered for persons with abnormalities in Tier 2 tests or for a random fraction of the entire population in Tier 2)

- If a proportion of CD16 cells of Tier 2, III, is abnormal: nonspecific killing of a tumour cell line to test for natural killer function

- If primary delayed-type hypersensitivity reaction in Tier 2, II, is abnormal: cell proliferation in response to phorbol ester and calcium ionophore,anti-CD3 antibody, and a staphylococcal enterotoxin B (experimental)
- Generation of secondary cell-mediated immune reactions (proliferation and MHC-restricted cytotoxicity) *in vivo*, e.g. with influenza virus (experimental)
- Immunoglobulin subclass levels in serum (IgA1, IgA2, IgG1-4)
- Antiviral titres (e.g. influenza, parainfluenza, cytomegalovirus, human immunodeficiency virus) in serum (no deliberate immunization)

5.6 Assays for assessing immune status

A plethora of tests has been developed to assess immunity in humans (Bentwich et al., 1982, 1988), which are described in laboratory manuals (Lawlor & Fischer, 1988; Miller et al., 1991; Coligan et al., 1994). Many of these tests are now commercially available in kits. A systematic approach to the evaluation of immune function, which is based on simple screening procedures, followed by appropriate specialized tests of immune function, usually permits the definition of an immune alteration. The tests should include evaluation of the B-cell system, of the T-cell system, and of nonspecific resistance (polymorphonuclear leukocytes, monocytes and macrophages, NK cells, the complement system). Although some exogenous agents can alter several elements of the human immune system, others have a primary effect on a single element. For example, low doses of cyclosporin A selectively affect T cells by acting on the production of IL2 and IL2 receptors. Conversely, the anticonvulsive drug phenytoin acts primarily on the humoral immune system, leading to a selective deficiency of IgA.

A number of immune function assays recommended for inclusion in a simple screening panel for assessing human immune function after potential exposure to xenobiotics believed to affect the immune system are described below. It should be noted that there are many indicators of altered immune function in humans which may not be specific markers for exposure, immune disease or susceptibility (IPCS, 1994a; IPCS/Department of Health, 1995).

5.6.1 *Total blood count and differential*

A complete blood count, with differential absolute counts of lymphocytes, granulocytes, eosinophils, and platelets, are basic components of immunotoxicology. These tests are useful in defining the general health status of a population, since they are relatively well standardized over most age, sex, and race groups. Such counts are also essential for interpreting the results of ex-vivo/in-vitro functional tests,

described below, since functional tests reflect a combination of numbers of cell types and activity per cell.

The absolute lymphocyte count is critical: Higher absolute counts are found in children than in adults, but lymphocyte counts consistently below 1500/mm^3 are indicative of lymphocytopenia, and a higher count signals a defect in the T-cell system or effects on the bone marrow. Lymphocytopenia can occur not only in primary immune deficiency but also as a result of viral infections, malnutrition, stress, autoimmune diseases, and haematopoietic malignancies. Examination of bone marrow may be indicated to exclude some other factors once lymphocytopenia has been confirmed. Additional assessment of cell-mediated immunity and direct measurement of T-cell parameters, such as lymphocyte phenotypic markers, may also be indicated.

Review of peripheral smears for morphological abnormalities of the white and red cells adds useful information for interpreting raw cell counts, such as the atypical lymphocytosis of many acute viral infections. The absolute eosinophil count can be very helpful in delineating allergic disorders, vascular collagen diseases, and parasitic manifestations.

5.6.2 *Tests of the antibody-mediated immune system*

Evaluation of antibody-mediated immunity involves measurement of serum concentrations of immunoglobulins and assessment of antibody formation after immunization or measurement of 'natural antibodies'.

5.6.2.1 *Immunoglobulin concentration*

Several methods are available for measuring the concentrations of the five major classes of immunoglobulin, IgM, IgG, IgA, IgD, and IgE, in serum, including single radial diffusion, double diffusion in agar gel, immunoelectrodiffusion, radioimmunoassay, ELISA, and automated laser nephelometry. Single radial diffusion is widely used. Gel diffusion methods are very sensitive to differences in diffusion constants and thus to differences in molecular size.

The serum concentration of each of the major immunoglobulins should be determined, with the exception of IgD (which occurs predominantly on the cell surface). The determinations must be well standardized because antisera vary in quality. Since serum immunoglobulin concentrations vary with age and environment, appropriate norms must be used. Patients can manifest a deficiency in all immunoglobulin classes (common variable hypogammaglobulinaemia), or they may have a deficiency in only a single class (IgA deficiency as a primary defect or after phenytoin therapy).

The concentration of immunoglobulins cannot be used as the sole criterion for a diagnosis of immunodeficiency. Diminished immunoglobulin concentrations can result from loss into the gastrointestinal tract as well as from decreased synthesis. An indication of loss can be obtained by measuring serum albumin, which is usually lost concomitantly.

5.6.2.2 *Specific antibodies*

Antibody-mediated immunity can be assessed from antibody responses to common specific antigens (basal levels). Humoral immunity after immunization can be assessed in the same way. The response to antigenic stimulation with both protein and polysaccharide antigens must be defined if immunodeficiency is strongly suspected. Failure to respond to one or more classes of antigen has been observed in patients with normal or high levels of immunoglobulins, regardless of whether they have an isolated immunoglobulin class or subclass deficiency. Specifically, patients with the Wiskott-Aldrich syndrome may have normal or even elevated immunoglobulin concentrations, yet have multiple infections because of their failure to mount a specific immune response, especially when they are exposed predominantly to polysaccharide antigens.

Natural antibodies: Isohaemagglutinins are naturally occurring antibodies to blood group A and B antigens found in all normal individuals except those with type AB red cells. By three years of age, 98% of normal persons with type A, B, or O blood have isohaemagglutinin titres of at least 1:16. Patients with the Wiskott-Aldrich syndrome may have normal immunoglobulin levels yet lack isohaemagglutinins as an indicator of their antibody-deficient state. Other natural antibodies that can be assayed include heterolysins (e.g. against sheep or rabbit red blood cells) and antistreptolysin.

Antibody response to immunization: In order to test for T cell-dependent antibody responses, commercially available diphtheria–tetanus vaccine can be given in recommended doses. Blood is taken two weeks after each injection and tetanus and diphtheria antibodies are determined. In patients who have been immunized with diphtheria–tetanus or diphtheria–pertussis–tetanus vaccine, one booster injection is given before determination of antibodies. In testing for T cell-independent antibody responses, commercially available pneumococcal vaccine can be given in recommended doses. Three doses of killed poliomyelitis vaccine (1.0 ml intramuscularly, at intervals of two weeks) can also be used as the immunogen. Blood is taken two weeks after the last

injection, and antibody is usually determined by virus neutralization. There is strong consensus that quantification of a primary immune response (antibody and/or cellular) after immunization is not only a very relevant test but also very sensitive. Although such tests are not routine in clinical immunology, they have been used successfully for determining immune integrity. While keyhole limpet haemocyanin has been used as the antigen, beneficial immunizations such as influenza vaccine linked to a marker epitope can also be used.

5.6.3 *Tests for inflammation and autoantibodies*

5.6.3.1 *C-Reactive protein*

Inclusion of an acute-phase reactant marker is helpful for clinical interpretation of other laboratory biomarkers such as autoantibodies. The concentration of C-reactive protein rises and falls to baseline values in direct proportion to tissue damage and is thus a sensitive indicator of the presence of a generalized inflammatory state. It offers the best example of an accepted, standardized procedure that can be used in large population studies, because it is less subject to transport variables than other procedures.

5.6.3.2 *Antinuclear antibody*

Antinuclear antibody is a common autoantibody that may be associated with various rheumatic disorders and, classically, systemic lupus erythematosus. Several commercial kits are available to detect the presence of autoantibodies. Progressively greater titres increase the specificity for a disease. Positive sera should be titred at dilutions of > 1:40 to 1:640.

5.6.3.3 *Rheumatoid factor*

Rheumatoid factor is an autoantibody to immunoglobulin (usually IgM) that occurs in a high percentage (50–70%) of individuals with classical rheumatoid arthritis but may also develop in a variety of other disorders, including infections and immunological diseases. Positive sera should be titred at dilutions of > 1:40 to 1:640.

5.6.3.4 *Thyroglobulin antibody*

This antibody occurs in association with a variety of thyroid disorders but may be found without detectable thyroid dysfunction. Positive sera should be titred at dilutions of > 1:40 to 1:640.

5.6.4 *Tests for cellular immunity*

A variety of tests are commonly used to assess cell-mediated immunity, including enumeration of T cells and T-cell subsets, identification of delayed skin reactions, and measurement *in vitro* of stimulation of lymphocytes to proliferate and form blast cells. Other tests are available to measure T-cell effector or regulatory function *in vitro*. As for humoral immunity, a series of simple tests is available to screen for defects in cell-mediated immunity. The proportion of circulating T cells in a mononuclear cell preparation can be determined by immunofluorescence with CD2 or CD3 monoclonal antibodies. Normally, T cells constitute 55–80% of peripheral blood lymphocytes. The normal values reported for absolute numbers of circulating T cells are 1620–4320/mm^3 during the first week up to 18 months of life and 590–3090/mm^3 after 18 months of age (Fleisher et al., 1975).

5.6.4.1 *Flow cytometry*

Antibodies that may be used in immunological phenotyping are listed in Table 10 in section 4.1.2. Subsets of T cells have been defined with monoclonal antibodies specific to cell-surface antigens. The association of a particular T-cell subset with a given function has caused some confusion in the analysis of immunological data for patients with immunodeficiency states, as discussed in more detail in section 1.2.2.1. For example, CD-positive cells have commonly been associated with helper functions, and CD8 cells have been associated with cytotoxic functions. This approach is an oversimplification, which became evident with the finding that CD and CD8 cells recognize foreign antigens in the context of MHC class II and class I antigens, respectively. Thus, the CD population contains helper cells, memory cells, and cytotoxic cells for targets bearing MHC class II molecules. The CD8 population contains cytotoxic cells and also cells that can recognize antigens presented by macrophages and cells that can augment the interaction of CD-positive cells with B cells. Abnormalities in the number of CD or CD8 cells, or their ratio, can be associated with abnormalities in the ability to recognize antigens and regulate T-cell function that can lead to immune incompetence or autoimmunity.

5.6.4.2 *Delayed-type hypersensitivity*

The ability of patients to manifest pre-existing T-cell immunity has been evaluated *in vivo* with a series of skin antigens that normally produce a delayed-type cutaneous hypersensitivity response. Because delayed cutaneous hypersensitivity, a localized immunological skin response, depends on functional thymus-derived lymphocytes, it is

used in detecting T cell-mediated immunodeficiency. A device (Multitest®, Institut Merieux, Lyon, France) is available that enables the simultaneous intradermal injection of seven standardized antigens and so overcomes the inconvenience of giving seven separate injections of antigens and the technical difficulty of ensuring their intradermal rather than subcutaneous injection. The Multitest®, consisting of eight tined, preloaded heads, delivers a glycerol control and the seven test antigens dissolved in glycerol. The size of the induration produced by each antigen should be measured at 48 h in two diameters: reactions of less than 2 mm are scored as negative. No reaction should be seen with the glycerol control. The test includes as antigens: tetanus, diphtheria, streptococcus, tuberculin, *Candida*, *Trichophyton*, and *Proteus*.

Recall of delayed-type hypersensitivity as a test for cell-mediated immune competence was assessed with the Multitest® device in 254 subjects. When 77 subjects were tested concurrently with the Multitest® and a conventional panel of six antigens (Frazer et al., 1985), similar results ($r = 0.65$) were obtained with the two systems. The reproducibility of the Multitest® among three observers, who assessed the aggregate size of reactions in 45 subjects independently, was high ($r = 0.89$); the correlation for the reaction score in 24 subjects tested twice, three months apart, was also high ($r = 0.88$), demonstrating the suitability of the test for serial studies of immune function.

5.6.4.3 *Proliferation of mononuclear cells* in vitro

A common test of lymphocyte function is measurement of the capacity of lymphocyte subsets to enlarge and convert into blast-like cells that synthesize DNA and incorporate thymidine after stimulation *in vitro*. In this test, lymphocytes can be activated by antigens (e.g. purified protein derivative, *Candida*, streptokinase, tetanus, or diphtheria); allogeneic cells are also used in the one-way mixed lymphocyte culture to stimulate T-cell proliferation. T-Cell blast transformation can be assessed directly by measuring blastogenesis and proliferation of cells, expression of activation antigens (e.g. CD69 or CD25 and HLA DR), and release of mediators. The blastogenic response is assessed as incorporation of ^{3}H-thymidine, usually for 16–24 h, followed by cell precipitation on filter paper and liquid scintillation counting. Non-isotope assay alternatives are also available.

The responses to various antigenic stimuli by different types of responding cells must be interpreted with caution. The mixed leukocyte reaction is the result of T-cell reactivity to MHC-encoded peptides displayed on the surface of B cells and monocytes. The T cells in the population of normal irradiated or mitomycin C-treated lymphocytes used as the stimulators can secrete factors that induce blastogenesis in

the patient's lymphocytes. As this can be misleading, it is preferable to use B-cell lines or T cell-depleted normal cells as the stimulators.

5.6.5 *Tests for nonspecific immunity*

5.6.5.1 *Natural killer cells*

The differences between NK cell function, phenotyping for NK cells, and the cytology of large granular lymphocytes are described in section 1.2.2.1. The identification of NK cells remains problematic owing to this apparent heterogeneity. The cells can be evaluated in ex-vivo/in-vivo tests with enriched peripheral blood mononuclear cells. The proportion of NK cells can be identified with appropriate monoclonal antibodies (see Table 10, p. 166). Functional assays of NK activity involve the ability of the appropriate mononuclear cells to kill specific NK targets, such as the K562 cell, in which cell-mediated cytolysis *in vitro* is quantified by release of ^{51}Cr from the target cells.

5.6.5.2 *Polymorphonuclear granulocytes*

Some defects of phagocytic function affect polymorphonuclear phagocytes. Neutrophil function depends on movement in response to chemotactic stimulus, adherence, endocytosis, and destruction of the ingested particles. Phagocyte mobility depends on the integrity of the cytoskeleton and contractile system. Defects in intracellular killing of ingested microorganisms usually result from failure of the 'respiratory burst' that is critical to production of superoxide radicals and hydrogen peroxide. The organisms cultured from lesions of patients with this type of defect generally contain catalase and include staphylococci, *E. coli*, *Serratia marcescens*, fungi, and *Nocardia*. Patients with defects in mobility, adherence, and endocytosis usually have infections of the skin, periodontitis, and intestinal or perianal fistulae; patients with normal endocytosis and defective killing tend to have chronic granulomas. Measurement of nitroblue tetrazolium dye reduction or chemiluminescence by actively phagocytosing leukocytes has been accepted as a standard measure for the adequacy of the respiratory burst. Recently, methods have been developed to measure the production of reactive oxygen intermediates by flow cytometry (Emmendorfer et al., 1994). Kits are commercially available for assessing phagocytic capacities and the production of reactive oxygen intermediates by phagocytes. Assays for bacterial killing yield highly variable results, depending on the bacterial species used in the assay, and are not recommended for routine use. The activation state of neutrophilic granulocytes can be assessed by flow cytometry with the antibodies CD11b, CD14, CD16, CD54, and CD64 (Spiekermann et al., 1994).

Activation of platelets can also be assessed by flow cytometry (Tschöpe et al., 1990)

5.6.5.3 *Complement*

The classic complement system consists of nine components (C1–9) and a series of regulatory proteins (C1 inhibitor, C4 binding protein, and properdin factors). Many biological activities important in the inflammatory response and in host resistance to infection occur at various points in the classical and alternative pathways of complement activation. Three clinical states should raise a suspicion of deficiency in a complement component: systemic lupus erythematosus, recurrent infections of the type seen in hypogammaglobulinaemia in patients with normal immunoglobulin levels, and severe *Neisseria* infection. Laboratory measurement of serum haemolytic complement (CH50) is a useful test. Serum haemolytic complement is usually absent and rarely above 10% of the normal value in inherited complement deficiencies, with the exception of hypercatabolism of C3. More detailed analysis of the complement system requires functional and antigenic measurements of the individual components, usually best performed in laboratories that specialize in the complement system.

5.6.6 *Clinical chemistry*

Assessment of clinical abnormalities in standard serum chemistry, such as liver function, renal function, glucose, and albumin, is indicated to facilitate reasonable interpretation of specific changes in the immune system as secondary to another condition.

5.6.7 *Additional confirmatory tests*

After activation, mononuclear cells from peripheral blood express the genes that encode a series of interleukins and colony-stimulating factors. Activated T cells and monocytes synthesize and secrete IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, interferon, other cytokines such as tumour necrosis factor, and cytokine receptors. These cytokines are involved in the growth and differentiation of T and B cells, eosinophils, and basophils. The supernatants of mononuclear cells from peripheral blood stimulated by soluble phytohaemagglutinin can be assessed for IL-2 by determining their capacity to stimulate ^{3}H-thymidine uptake by mouse IL-2-dependent, cultured T-cell lines. Other biological assays, radioimmunoassays, and ELISAs have been developed to quantify the production of lymphokines and colony-stimulating factors. With molecular cloning techniques, messenger RNA transcription by each of the lymphokines can be quantified after appropriate lymphocyte

activation. In the assessment of lymphokines, T cells or peripheral blood mononuclear cells are usually activated with concanavalin A, pokeweed mitogen, or insolubilized CD3 antibodies; then, the appropriate assays are performed to quantify the specific lymphokine(s) produced in the culture media. Different patterns of lymphokine secretion have been observed with different subsets of T cells: INFγ and IL-2 are produced by the Th1 subset, while IL-4, IL-5, and IL-10 are produced by the Th2 subset, as originally documented for cloned CD T-cell lines from mice (Mosmann & Coffman, 1989). Thus, an assay of the pattern of lymphokine production could be used in pinpointing the action of an immunotoxicant on a particular subset of immune cells.

6. RISK ASSESSMENT

6.1 Introduction

Publications on immunotoxicology published by IPCS and the European Union (Berlin et al., 1987; Dayan et al., 1990), the United States Office of Technology Assessment (1991), and the United States National Research Council (1992) demonstrate the growing interest and concern within scientific and public communities on the capacity of environmental agents to perturb normal immune processes. The incorporation of experimental data on toxicant-induced alterations in the immune system into evaluations of drugs, chemicals, and biological agents for human risk assessment has thus become increasingly common. For example, in the United States, the Environmental Protection Agency (Sjoblad, 1988) and Food and Drug Administration (Hoyle & Cooper, 1990; Hinton, 1992) indicate the benefits of testing the immuno-suppressive potential of biochemical pest control agents, antiviral drugs, and food additives. Furthermore, the Environmental Protection Agency has established reference doses (an estimate of the daily exposure of the human population that is likely to have no appreciable risk of deleterious effects during a lifetime), on the basis of data on the immunotoxicity of several compounds, including 1,1,2-trichloroethane, 2,4-dichlorophenol, and dibutyltin oxide. The United States Agency for Toxic Substances and Disease Registry has derived 'minimal risk levels' for arsenic, dieldrin, nickel, 1,2-dichloroethane, and 2,4-dichlorophenol on the basis of immune end-points.

Risk assessment is a process whereby relevant biological, dose–response, and exposure data for a particular agent are analysed in an attempt to establish qualitative and quantitative estimates of adverse outcomes (Scala, 1991). Such data are sometimes used in the development of standards for regulating the manufacture, use, and release of chemicals into the environment (Kimmel, 1990). As defined by the United States National Academy of Science (US National Research Council, 1983), risk assessment comprises four steps: hazard identification, dose–response assessment, exposure assessment, and risk characterization. The process of assessing the risk of both cancer and non-cancer end-points, including immunotoxicity, may be adapted to this form.

The first step, hazard identification, involves a qualitative evaluation of available human and animal data to determine whether a chemical agent poses a potential hazard. Consideration is given to dose, route, and duration of exposure. The precise quantitative relationships between changes in immune function or in the histological appearance of lymphoid organs and host resistance to infectious agents or neoplastic

diseases are unclear. It can be assumed that any significant difference from appropriate controls in the ability of the immune system to respond to a challenge may evolve into an adverse effect and thus present a potential hazard. This applies to adaptive as well as nonadaptive responses.

After hazard identification, 'dose–response' is assessed. For non-cancer toxicity, a no-observed-adverse-effect-level (NOAEL) is established for an adverse response of interest. This process is no different in immunotoxicology and is the same as for the other target organ systems. The NOAEL value is either obtained from the dose–response curve or is estimated from the lowest-observed-adverse-effect-level (LOAEL). Once the NOAEL has been determined, safety and uncertainty factors can be applied to allow for various uncertainties, such as species or interspecies variability, irreversible effects, and chronic exposure. The use of safety factors, however, should be flexible and should allow incorporation of any relevant information on the mechanism of action of the chemical under review. Ideally, however, dose–response relationships should be established from human epidemiological data that include the exposure levels expected on the basis of human contact with the agent in the environment. As illustrated in an assessment of developmental toxicity (Kimmel & Gaylor, 1988), use of the risk assessment and management paradigm of the United States National Academy of Sciences (US National Research Council, 1983) to non-cancer end-points such as immunotoxicity, offers some serious challenges. For example, since the presence or absence of an effect is based upon whether a statistically and/or biologically significant response is observed at a certain dose or doses, and since multiple assays are routinely conducted, the NOAEL will depend heavily on the sensitivity of the assay. The differences may be particularly exaggerated when continuous responses (i.e. the results of most immune function tests) are compared with categorical data, the latter being routinely expressed as proportions. For example, in experimental animals, changes in many immune functions may be statistically significant when they vary by as little as 15–25% from the control values. In contrast, host resistance, when expressed categorically (e.g. tumour frequency), must change by 80% to reach a comparable degree of significance, assuming group sizes of about 15 animals and effective doses of 20% in the control group. Furthermore, immune tests are often, but not always, interdependent (Luster et al., 1992), and individual or combinations of tests might have to be ranked in order of sensitivity and degree of interdependence before dose–response is assessed. This has not been done in the past.

Exposure assessment (step 3) is done in parallel with hazard identification and dose–response assessment (Scala, 1991). It often

involves field measurements and other estimates of human exposure, such as the composition and size of the population, biological or clinical effects and types, and the magnitude, frequency, and duration of exposure to the agent. Many of these parameters are difficult to determine accurately in a longitudinal study; even measurements of body burden can be misleading since the concentrations at the target organ (e.g. lymphoid tissue) are not determined. The problems in immune testing in humans are similar to those in testing other organs and systems and include differences in individual responses due to unique sensitivities (e.g. age, pregnancy, genetics) and confounding factors (e.g. smoking, stress, drugs).

Risk characterization (step 4) is the aggregation of the three previous processes. It provides an estimate of the incidence of adverse effects in a population and the potential health problems. As part of risk characterization, the strengths and weaknesses of each component of the assessment are considered, with assumptions, scientific judgements, and, to the extent possible, estimated uncertainties. Most assessments of the risks presented by chemical agents have focused on the estimated incidence of cancer after lifetime exposure to a chemical at some unit dose, assuming that there is essentially no threshold for carcinogenicity. The assessment of non-cancer end-points, such as disorders of the neurological, developmental, and reproductive systems, is somewhat similar to that of cancer, in that it involves calculations that include both assumptions and uncertainties. For example, considerations in risk assessment include ranking the value of epidemiological against experimental data, extrapolations from high to low doses, from subchronic to chronic exposure, and from animals to humans, and appropriate use of mechanistic and pharmacokinetic data. Data from immunotoxicology, like those from developmental toxicology (Schwetz & Tyl, 1987), do not easily lend themselves to the mathematical models used in cancer risk assessment, which usually involve non-threshold models for genotoxic carcinogens. For more accurate assessment, mechanistic models will be required which include the concept of 'threshold'. It is assumed that threshold levels exist below which no adverse immunological effect can be demonstrated. Complex mixtures of chemicals, in which each chemical occurs at a subthreshold concentration, may reach or exceed a threshold for immunotoxicity (Germolec et al., 1989), although problems associated with mixtures of compounds are not unique to the field of immunotoxicology.

The approaches currently used by the US Environmental Protection Agency (1986) in extrapolating the risk for developmental toxicity have been outlined, and similar guidelines have been developed by IPCS (1994b). One method is to apply uncertainty factors to the NOEL or

NOAEL for the most sensitive animal species tested. The uncertainty factor is usually composed of a 10-fold factor to account for interspecies differences and a 10-fold factor for intraspecies variability. If no NOEL is available, an additional 10-fold factor may be applied to the lowest-observed-effect level (LOEL). Another approach is to calculate a margin of safety, which is the NOEL divided by the estimated level of human exposure from all potential sources. The margin of safety can then be evaluated for adequacy to protect human health. There are several drawbacks to both of these approaches, the primary one being that they use only one point on the dose–response curve (NOEL or LOEL) and ignore the rest of the data. Also, since the variability around the NOEL and LOEL is usually not taken into account, these approaches may rely on poor studies, i.e. studies that result in a higher NOEL because of their limited ability to detect small changes over the background.

Since the purpose of risk assessment is to make inferences about potential risk to human health, the most appropriate data are those derived from studies of humans; however, adequate data are seldom available, and most risk assessments are based on results obtained in experimental animals. In order to use these results, a number of assumptions must be made about their relevance to potential human health risk. Firstly, it is assumed that experimental animals respond to the agent of interest in a pharmacological and toxicological manner similar to that anticipated in humans (i.e. the test animals and humans metabolize the chemical similarly and have identical responses and toxicity at the target organ). Secondly, the immune system of the experimental species must be very similar to that of humans: the vertebrate immune system is highly conserved among higher vertebrate species, and the immune components and their interactions in mice, rats, and humans are closely similar. Thus, if toxicokinetic properties are similar, it is reasonable to test for potential adverse effects in humans using laboratory rodents.

As immunosuppressive agents cannot ethically be administered to humans, quantitative comparisons of dose–responses in humans and experimental animals are limited (although it is possible to do so in hypersensitivity tests). Nonetheless, controlled human exposures have been studied and the results compared with the immune effects observed in animals. As an example, the immune effects of cyclosporin A in various species are compiled in Table 12, which shows that the mouse is much less sensitive to cyclosporin A than other species, in particular the rat, and that humans are slightly more sensitive than other species (Dean & Thurmond, 1987); however, for the most part, there was good qualitative and quantitative agreement between the species examined. Selgrade et al. (1995) compared phagocytosis by human and murine

Table 12. Comparison of doses of cyclosporin A that suppress the immune response in various species

Species	Response	Cyclosporin A (mg/kg body weight)
Mouse	Antibody production	50–300
	Cell-mediated immunity (delayed-type hypersensitivity)	100–300
	Graft-versus-host reaction	50–250
Rat	Antibody production	20–50
	Graft-versus-host reaction	10–60
Guinea-pig	Cell-mediated immunity (delayed-type hypersensitivity)	10–100
Dog	Cell-mediated immunity (delayed-type hypersensitivity)	15–30
Rhesus monkey	Antibody production	50–250
Human	Cell-mediated immunity	10–20

Adapted from Dean & Thurmond (1987); White et al. (1994)

alveolar macrophages after exposure to ozone *in vitro* and *in vivo* (Table 13): The effects of ozone on alveolar macrophage function in murine species are predictive of effects that occur in humans, and the effects on macrophage phagocytosis seen *in vitro* are predictive of those that occur *in vivo*. Quantitative comparisons have also been made in mice and humans for the ability of UVR to inhibit the contact hypersensitivity response (Table 14). As described in section 2.2.11, exposure to UVB inhibits delayed-type hypersensitivity (Kripke et al., 1979). Noonan & Hoffman (1994) described three strains of mice with low, intermediate, and high susceptibility to UVR-induced immunosuppression, and Oberhelman et al. (1992) reported that suppression of the hypersensitivity response also occurs in humans and that the dose of radiation required to induce 50% suppression in fair- and dark-skinned individuals is similar to that required to inhibit the response in mice with high and intermediate susceptibility, respectively.

Data from immunotoxicology also differ from those for carcinogenicity and possibly other non-cancer end-points because the immune system contains components with overlapping functions. For example, when an individual is exposed to an infectious agent, multiple immune components may work either independently or in concert to help defend the host; i.e. there is redundancy between immune functions.

Table 13. Effect of exposure to ozone on phagocytosis by alveolar macrophages

Treatment	Phagocytic index (no. fluorescent particles ingested/100 macrophages)			
	Mice		Humans	
	Mean	SE	Mean	SE
Air *in vitro*	369.2 ($n = 6$)	26.4	386.7 ($n = 6$)	50.5
Ozone *in vitro*	291.7 ($n = 6$)	17.4*	275.0 ($n = 6$)	45.1*
Suppression	21%		29%	
Air *in vivo*	330.6 ($n = 4$)	10.4	714.9 ($n = 10$)	46.1
Ozone *in vivo*[a]	194.0 ($n = 4$)	19.7*	539.2 ($n = 10$)	22.3*
Suppression	42%		25%	
Suppression corrected for dosimetric difference[b]	28%		25%	

Adapted from Selgrade et al. (1995)

* Significantly different from air control ($P < 0.05$; Student's t test)

[a] Mice were exposed to 0.8 ppm for 3 h; humans were exposed to 0.08 ppm for 6.6 h while undergoing intermittent exercise.

[b] On the basis of studies using ^{18}O, alveolar macrophages of mice exposed to 0.8 ppm ozone for 3 h receive roughly 1.5 times more ozone than those of humans exposed to 0.08 ppm ozone for 6.6 h while exercising moderately.

Furthermore, while a significant change in any immune function can be considered potentially deleterious, in that it may increase the risk of developing clinical disease, a change in immune function does not necessarily precipitate a disease or clinical health affect. That is, immunocompromised individuals function normally in the absence of infectious agents. Thus, immune function reserve and redundancy are relative terms, depending on the dose of infectious agent. This complicates dose–response assessment, and models should be developed that incorporate available information on the quantitative relationship between immune function and clinical disease and potential redundancy.

Table 14. Comparison of doses of ultraviolet radiation that cause 50% suppression of contact sensitivity in mice and humans

Mouse[a]			Human[b]	
Sensitivity (phenotype)	kJ/m²	mJ/cm²	Skin type	mJ/cm²
High (C57Bl)	0.7-2.3	70-230	Fair	100
Intermediate (C3H)	4.7-6.9	470-690	Dark	225
Low (BALB/c)	9.6-12.3	960-1230		

Adapted from Selgrade et al. (1995)
[a] Data from Noonan & Hoffman (1994)
[b] Data from Oberhelman et al. (1992)

The increasing evidence that environmental contaminants affect wildlife populations has led to risk assessment at the level of the ecosystem; however, the limited evidence for immunotoxic effects in wildlife precludes an adequate understanding of the risks posed by current levels of environmental pollution. The demonstra-tion of immunosuppression in harbour seals fed herring from the contaminated Baltic Sea in a semi-field experiment (De Swart et al., 1994; Ross et al., 1995) provided a first indication that ambient levels of contaminants in certain areas present an immunotoxic risk to mammalian wildlife occupying a high trophic level. These results may partially explain the severity of a series of unrelated epizootic viral episodes in various marine mammalian populations in coastal areas of Europe and North America (Dietz et al., 1989; Van Bressem et al., 1991). In similar semi-field experiments in a bottom-dwelling fish species, flounder exposed to contaminated sediments had more viral lymphocytic infection and liver tumours than controls (Vethaak & Wester, 1993). While the difficulties in conducting adequate immunological studies with wildlife may preclude an approach as comprehensive as that in humans, such semi-field strategies may provide the best available direction.

Should the application of field studies be possible, correlative approaches to immunotoxicology may substantiate an effect on the ecosystem. Such an approach was used to establish a correlation between the induction of mixed-function oxidases and pollutant burden (as measured by the toxic equivalence of organochlorine chemicals) in cormorant (*Phalocrocorax carbo*) chicks collected from various sites in the Netherlands (van den Berg et al., 1994). A combination of laboratory experiments under controlled conditions, semi-field experiments under controlled conditions with exposure to environmental mixtures of

pollutants, and correlative field studies is necessary to understand immunotoxicity in wildlife populations.

6.2 Complements to extrapolating experimental data

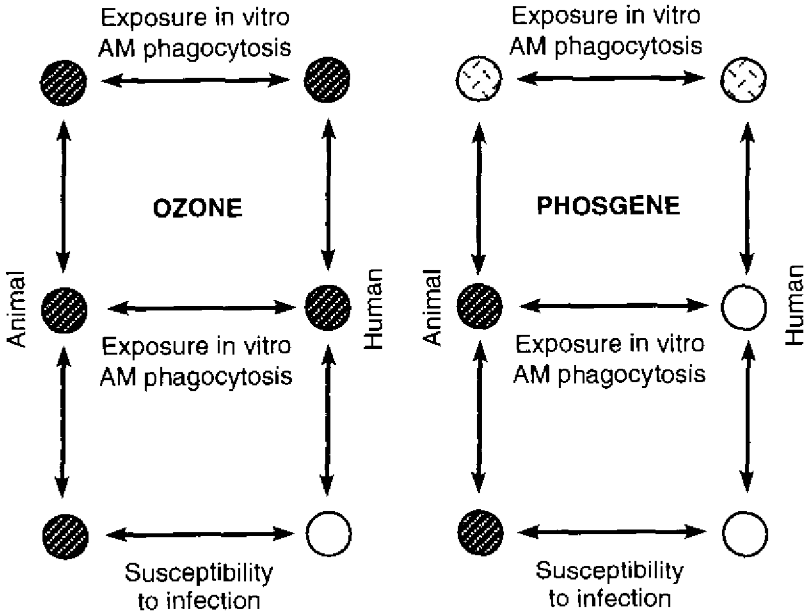
6.2.1 *In-vitro approaches*

The complexities of the immune system and the requirement of many agents for metabolism and distribution in order to produce an immunotoxic response have resulted in the almost exclusive use of animal models *in vivo* for assessing immunotoxicity. Culture systems have been used extensively, however, to study the mechanisms by which agents induce immunosuppression. In-vitro test systems with immune cells of human origin are particularly attractive, given the uncertainties in extrapolating the results of studies in experimental animals to humans and the accessibility of human peripheral blood cells. Although many of the immune cells obtained from human blood are immature forms, the large numbers and diverse populations (i.e. polymorphonuclear granulocytes, monocytes, NK cells, T cells, and B cells) that can be obtained and the ease of conducting challenge assays *in vitro* provide an attractive alternative (or, preferably, adjunct) to more conventional studies in animals. Surprisingly few laboratories have conducted studies with immunosuppressive agents in which immune function responses in human immune cells are compared with those in rodents *in vivo* (Cornacoff et al., 1988; Luo et al., 1992; Wood et al., 1992; Lang et al., 1993). Although studies in human cells *in vitro* have been hampered by a lack of assays to assess primary antigen-specific immune responses, a relatively good interspecies correlation has been observed in the limited responses examined. Furthermore, some of these assays have been successfully modified to include metabolic fractions of liver homogenates (Shand, 1975) or co-culture with primary hepatocytes (Kim et al., 1987) to allow for chemical metabolism.

6.2.2 *Parallelograms*

Interpretation of studies in experimental animals or *in vitro* can be improved when even limited data on human exposure *in vivo* are available, using a 'parallelogram' approach. In general, if a parallelogram can be constructed in which data are available for five of the six angles (Figure 37), it may be easier to predict the outcome at the remaining angle, at least qualitatively. For example, cytokine and phagocytic responses of alveolar macrophages or pulmonary epithelial cells after exposure to ozone *in vitro* can be compared with the responses to ozone after exposure of humans and animals *in vivo*. If the in-vitro data prove

Figure 37. Ladder (parallelogram) model designed for the use of data on ozone in order to interpret results of exposure *in vitro* to more toxic air pollutants, to which humans cannot be exposed experimentally *in vivo*.



From Selgrade et al. (1995)
Filled circles, data obtained experimentally, showing either suppression of alveolar macrophage function or enhancement of infection after exposure to an air pollutant. Open circles, data that could be obtained experimentally but are not currently available. Hatched circles, data that cannot be obtained experimentally because humans cannot be exposed to phosgene or a bacterial challenge; the results must therefore be extrapolated from other points on the ladder. The data on ozone suggest that the effects of human exposure to phosgene *in vivo* on phagocytosis may be predicted on the basis of the effects of exposure *in vitro* and animal data *in vivo*.

to be predictive indicators of the in-vivo effects in humans, more weight can be given to in-vitro studies with similar agents or with compounds that are too toxic to be assessed in clinical studies but for which data are available on both animals *in vivo* and animals and humans *in vitro*. A similar approach can be used to establish the relationship between

acute and subchronic effects as a means of extrapolating from acute effects in humans to chronic effects, for which few data are usually available. Another situation in which this approach may be applicable is in extrapolating deficits in immune function to increased susceptibility to disease in animal models, as a means of interpreting the risk of disease in humans, for whom data on immune function but not infectious disease may be available.

6.2.3 *Severe combined immunodeficient mice*

Another approach, which warrants further exploration, is the use of SCID mice grafted with human immune cells. This model is described in section 4.5.3. In short, SCID mice have been successfully grafted with human fetal lymphoid tissue in order to study human haematopoiesis (McCune et al., 1988) or with human peripheral blood lymphocytes, which allow production of human immunoglobulins, to study secondary antibody responses (Mosier, 1990). Reconstituted mice have been used to study autoimmunity and the efficacy of antiviral therapeutic agents. There are still limitations to the use of these animals for immunotoxicology (Pollock et al., 1994).

6.3 Host resistance and clinical disease

A major limitation in assessing the risk of immunotoxicity is the difficulty in establishing quantitative relationships between immunosuppression and clinical diseases. The diseases are usually manifested as increases in the frequency, duration, or severity of infections, increased incidences of certain cancers, such as Kaposi's sarcoma and non-Hodgkin's lymphoma (malignancies often observed in immunosuppressed individuals), or an increased incidence of autoimmune diseases.

Despite overwhelming experimental and clinical evidence that increases in the incidences of neoplastic and/or infectious diseases occur in animals and individuals with secondary immunodeficiency (Austin et al., 1989; Ehrke et al., 1989), neither the most appropriate immune end-points for predicting clinical disease nor the quantitative relationship between changes in immune function and impairment of host defence are clearly defined. For example, it would be useful to determine whether certain immune end-points (or quantity of changes) predict certain outcomes (e.g. increased susceptibility to influenza and decreased antibody responses). A better understanding of these relationships would be particularly beneficial for risk assessment, since changes in immune function are more readily quantifiable in populations at risk than are changes in the frequency or severity of infections. A

particularly relevant question for risk estimation is whether increases in host susceptibility to challenge agents follow linear or 'threshold-like' models as a function of increased immunosuppression. While terms such as 'immune reserve' and 'immunological redundancy' are applicable for individual responses, it is unclear how they would be applied to large populations. Since the potential outcomes of immunosuppression are increases in infections or neoplastic diseases and there is already a background incidence of these diseases in the population (Centers for Disease Control, 1991), it would be helpful to determine the additional frequency of disease that is associated with increased loss of immune function. Qualitative relationships are well established, but the quantitative relationship between immune function and clinical disease in humans has proved difficult to explore, owing, in part, to the complexity of the immune system, overlapping (i.e. redundant) immune responses, and variability in the virulence of infectious agents. Nonetheless, several studies have shown quantitative relationships in humans. For example, in longitudinal studies of a relatively large population, asymptomatic individuals with low NK cell activity were found to be at risk for developing upper respiratory infections (Levy et al., 1991). Larger population studies have been conducted in AIDS patients, as the depletion of $CD4^+$ cells following HIV-1 infection is a clinical hallmark of the disease. The normal human range of $CD4^+$ cells is 800–1200 cells/µl, but this level generally declines to less than 500 cells/µl within three to four years after HIV-1 infection and to 200 cells/µl before overt opportunistic infections are seen (Masur et al., 1989; Phair et al., 1990). It has also been shown in seropositive individuals that a drop in $CD4^+$ cells by 7% or more in a year increases the relative risk of developing AIDS (Burcham et al., 1991).

Because of the uncertainties about the quantitative relationship between immune function and disease, there has been continuing interest in developing sensitive, reproducible experimental models of host resistance to define altered immune function after exposure to environmental agents. Most of these models were developed in mice and, to a lesser extent, in rats; they include bacterial, viral, protozoan, fungal, and syngeneic or semisyngeneic transplantable tumour cell models. Although the target organs and general host defence activities have been defined for most of these models, multiple immune and nonimmune, mechanisms are involved in resistance, making it difficult to determine the exact defect without assessing immune function responses to the challenge agent. For example, defence against extracellular organisms involves the interactions of T lymphocytes, B lymphocytes, macrophages, and polymorphonuclear granulocytes, in

addition to a variety of cell-secreted products, whereas resistance to generalized infection from intracellular pathogens and neoplastic diseases is likely to involve macrophages, NK cells, and T cell-mediated immune processes.

Although many host resistance assays are relatively simple to perform, they normally require large numbers of animals and appear to be less sensitive than immune function tests (Luster et al., 1993). Other studies have shown that host resistance assays are more sensitive than immune function tests (Vos et al., 1991; Burleson et al., in press). The dose of challenge agent used in experimental studies is important, since too low or too high a dose will not allow detection of changes in immunocompromised groups in comparison with controls (Selgrade et al., 1982; Luster et al., 1993). The sensitivity of a host resistance assay also depends on the end-point measured. For example, tests involving survival or tumour models (i.e. dichotomous) are by nature less sensitive than those with end-points that provide continuous data, such as enumeration of tumours, bacteria, or soluble immune activation markers, and several end-points in one model of infection, such as *T. spiralis*. This is attributable partly to differences in the types of statistical analyses used to establish group differences. With dichotomous data, two approaches can be used. Some laboratories use a challenge dose that produces a response in 10–30% of animals in the control group. An alternative is to use a dose slightly below that which would induce the desired response in any of the animals in the control group. The latter design increases the statistical power of data analysis. In most cases, extreme accuracy is needed in the delivery of the agent, to ensure that the administered dose of agent is only slightly smaller than that which will give the desired response. In either approach, statistical significance is heavily dependent on the dose of challenge agent and the number of animals in each treatment group: Table 15 shows that doubling the number of animals in a study greatly increases the ability to detect a significant change. Even more obvious is the increased ability to detect significant differences when the dose of the agent in the control group is lowered to a subclinical concentration. These hypothetical values demonstrate how statistically significant changes can be obtained in susceptibility assays by modifying the experimental design. In the first design, an effective dose of 30% is used in the control group (i.e. a concentration of agent that produces a response in 30% of normal animals) with 15 animals per treatment group. In the second design, increasing the group size to 30 allows for even greater statistical significance. In the third design, a challenge inoculation is given which produces no effect in the control group (effective dose, 0), allowing for greater statistical significance.

Table 15. Chi-squared values (hypothetical)

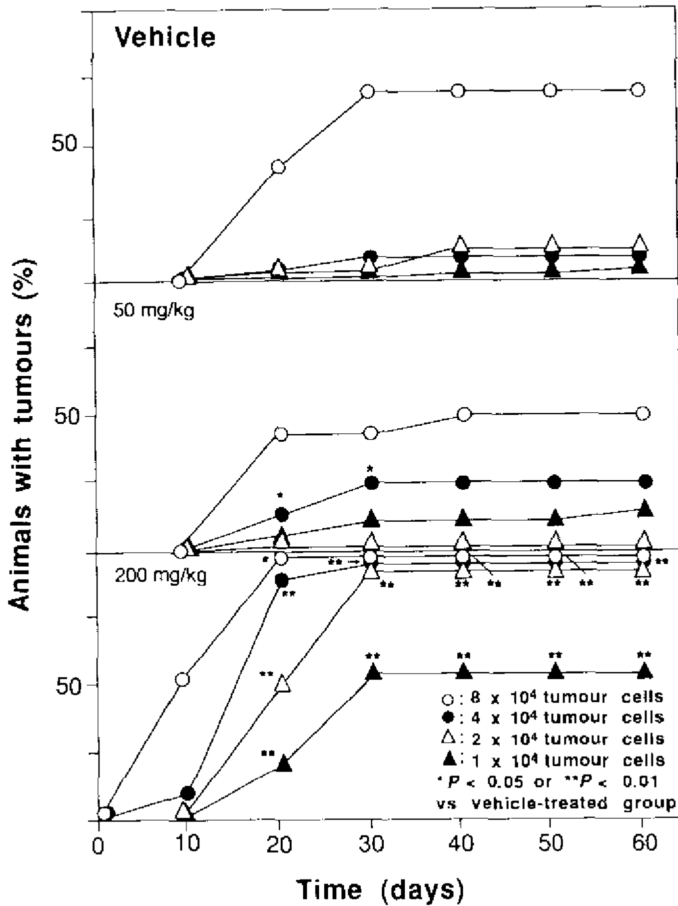
Treatment	Design 1		Design 2		Design 3	
	No. affected/ no. tested	One-tailed *P* value	No. affected/ no. tested	One-tailed *P* value	No. affected/ no. tested	One-tailed *P* value
Control	3/15	–	6/30	–	0/15	–
Dose 1	4//15	0.532	8/30	0.428	1/15	0.516
2	5/15	0.411	10/30	0.273	2/15	0.274
3	6/15	0.312	12/30	0.165	3/15	0.150
4	7/15	0.233	14/30	0.096	4/15	0.084
5	8/15	0.173	16/30	0.055	5/15	0.048*
6	9/15	0.128	18/30	0.031*	6/15	0.028*
7	10/15	0.094	20/30	0.017*	7/15	0.017*
8	11/15	0.069	22/30	0.009*	8/15	0.010*
9	12/15	0.051	24/30	0.005*	9/15	0.006*
10	13/15	0.038*	26/30	0.003*	10/15	0.004*
11	14/15	0.028*	28/30	0.002*	11/15	0.002*
12	15/15	0.021*	30/30	0.001*	12/15	0.002*

Two variables that influence the quantitative relationship between immune function and disease are the virulence and amount of the infectious agent. These remain a constant in most experimental studies but may vary between experiments as well as in the human population. For example, in the general population one can assume that an infectious disease such as influenza can develop in any individual, independently of their immune capacity or prior immunization, provided that the virulence or quantity of the challenging agent is sufficient to overwhelm the individual's defensive capacities. In Figure 38, groups of mice pretreated with either vehicle (saline), 50 mg/kg cyclophosphamide (causing minimal immunosuppression), or 200 mg/kg cyclophosphamide (causing severe immunosuppression) were administered various numbers of PYB6 tumour cells. Even vehicle-treated mice developed a high frequency of tumours, provided that the challenge was sufficiently high (i.e. 8×10^4 tumour cells). In contrast, severely immunosuppressed mice (high dose of cyclophosphamide) developed an increased tumour frequency at all challenge levels of PYB6 tumour cells. The groups treated with the low dose of cyclophosphamide were of particular interest, since evidence of increased susceptibility appeared but only as a function of the tumour cell concentration. Assuming that these observations are applicable to human populations, even small changes in immune function could increase the likelihood of disease.

As indicated earlier, while experimental data have been used occasionally in risk assessment, most immunotoxicological data have focused on hazard identification. Although comparative data on the effects of specific immunotoxic agents in humans and animals are limited, other factors contribute to the minimal use of these data in risk assessment, including the concern that immunotoxicity testing has often been conducted without full knowledge of its predictive value in humans or its quantitative relationship to immune-mediated diseases.

Luster et al. (1988) reported on the design and content of a screening battery involving a tier approach for detecting potential immunosuppressive compounds in mice. This battery has been used to examine a variety of compounds, and the database, generated on over 50 compounds, has been analysed in an attempt to improve the accuracy and efficiency of tests for screening chemicals for immunosuppression and to identify better those tests that predict experimentally induced immune-mediated diseases (Luster et al., 1992, 1993). Specifically, attempts have been made to develop a 'streamlined' test configuration for accurately predicting immunotoxic agents and to establish models that could be used to provide insight into the qualitative and quantitative relationships between the immune and host resistance assays commonly used to examine potential immunotoxic chemicals in experimental

Figure 38. Effects of cyclophosphamide on PYB6 tumour formation as a function of tumour cell challenge



From Luster et al. (1993)

Groups of mice were administered a single intraperitoneal injection of saline or 50 or 200 mg/kg body weight cyclophosphamide, followed 48 h later by subcutaneous injection of either 1 x 10^4 (filled triangles), 2 x 10^4 (open triangles), 4 x 10^4 (filled circles), or 8 x 10^4 (open circles) PYB6 tumour cells. The data are presented as the percentage of animals that developed palpable tumours within 60 days. Each point represents 20 mice. *, $P < 0.05$; **, $P < 0.01$ in comparison with the vehicle-treated group by Fisher's exact test at the same time, given equal numbers of tumour cells

animals. While the analyses had a number of limitations, several conclusions can be drawn from the results:

(1) With this particular testing configuration, examination of only two or three immune parameters was needed in order to identify potential immunotoxicants. Lymphocyte enumeration and quantification of the T cell-dependent antibody response appeared to be particularly useful. Furthermore, some commonly employed measures (e.g. leukocyte counts, lymphoid organ weights), while probably good predictors of immunotoxicity, are apparently not as sensitive as the other tests. Obviously, inclusion of additional tests that are not part of the original battery may improve the prediction of immunotoxicity.

(2) A good correlation was found between changes in immune tests and altered host resistance, in that there was no instance in which host resistance was altered without a significant change in the immune test(s). In many instances, however, immune changes were observed in the absence of detectable changes in host resistance (Table 16), indicating that immune tests are generally more sensitive than host resistance assays.

(3) No single immune test could be considered highly predictive for altered host resistance; however, many of the tests were good indicators, while others, such as leukocyte counts and proliferative response to lipopolysaccharide, were relatively poor indicators of a change in host resistance. Some of the tests that showed the highest association with host resistance were those described previously as the best indicators of immunotoxicity, such as the plaque-forming assay and surface markers, but also included tests such as delayed-type hypersensitivity and thymic weight.

(4) Regression modelling, using a large data set on one chemical agent, indicated that most, but not all, of the immune function–host resistance relationships follow a linear model. It was not possible, however, to establish linear or threshold models for most of the chemicals studied when the data from all 50 chemicals were combined; thus, a more mechanistically based mathematical model will have to be developed. A similar conclusion was drawn on the basis of a limited data set collected by the Environmental Protection Agency (Selgrade et al., 1992), in which changes in NK cell activity were correlated with changes in susceptibility to cytomegalovirus in a murine model. It is impossible, at present, to determine how applicable these analyses will be for immunotoxic compounds with different immune profiles; however, as more analyses become available, the ability to estimate potential clinical effects accurately from the results of immunological tests should increase.

Table 16. Association between the results of host resistance models and immune tests

Challenge agent	No. of tests	Frequency[a]		
		Specificity (–/–)	Sensitivity (+/+)	Concordance (Total)
Listeria monocytogenes	34	100	52	65**
PYB6 tumour	24	100	39	54
Streptococcus pneumoniae	19	100	38	58
B16F10 melanoma	19	100	40	68
Plasmodium yoelii	11	100	38	55
Influenza	9	100	17	44
Any of the above[b]	46	100	68	78*

From Luster et al. (1993)

* Agreement statistically significant at $P < 0.05$

[a] Frequency is defined as: specificity, the percentage of non-immunotoxic chemicals with no effect on the host resistance models; sensitivity, the percentage of potentially immunotoxic chemicals causing a change in a host resistance model; concordance, percentage of qualitative agreement

[b] Frequency calculated on the basis of all of the host resistance models used to study an agent

7. SOME TERMS USED IN IMMUNOTOXICOLOGY

Accessory cell. Passenger cell (leukocyte, mainly monocytes) or stationary cell (reticulum cell, epithelial cell, endothelial cell) that aids T or B lymphocytes in inducing immunological reactions, either by direct contact or by releasing factors; normally expresses MHC class II molecules

Acquired immunity. A state of protection against pathogen-induced injury, with rapid immune elimination of pathogenic invaders; due to previous immunization or vaccination

Activation. The process of going from a resting or inactive state to a functionally active state, of leukocytes (lymphocytes, monocytes) or proteins (complement, coagulation)

Acute-phase protein. Non-antibody humoral factor that emerges in increasing amounts in the circulation shortly after induction of an inflammatory response; e.g. α2-macroglobulin, C-reactive protein, fibrinogen, α1-antitrypsin, and complement components

Adaptive immunity. A state of specific acquired protection against pathogenic invaders, induced by immunization or vaccination

Addressin. Receptor for lymphocytes on endothelial cells of venules, involved in homing of cells in lymphoid tissue; belongs to the immunoglobulin gene superfamily, integrins, and selectins

Adenoid. See *Tonsil*

Adhesion receptors Molecule involved in cellular adhesion between passenger cells and the extracellular stationary matrix (endothelium, connective tissue); comprises three main families; member of the immunoglobulin gene superfamily, integrins, and selectins

Adjuvant. Material that enhances an immune response

Adoptive immunity or tolerance. Transfer of a state of immunity or tolerance via cells or serum from an immune or tolerant individual to a naive individual

Affinity. Binding strength of an antibody-combining site to an antigenic determinant (epitope); expressed as an association constant (Ka)

Agglutination. Process of aggregation of visible antigenic particles (e.g. erythrocytes) mediated by antibodies directed towards the particles

Allele. One or more genes at the same chromosomal locus which control alternative forms (phenotypes) of a particular inherited characteristic

Allergen. Antigen that induces an allergic or hypersensitivity reaction, resulting in immune-mediated or nonimmune-mediated tissue damage; restricted mainly to immediate hypersensitivity or anaphylactic reactions

Allergy. State of altered immunity, resulting in hypersensitivity reaction on contact with antigen or allergen; often restricted to immediate hypersensitivity or anaphylaxis

Alloantigen. Antigen that differs between different (not inbred) individuals within one species

Allogeneic. Genetically different phenotypes in different (not inbred) individuals within one species; opposite of isogeneic, or xenogeneic

Allotype. Genetically different antigenic determinants on protein of (not inbred) individuals within one species

α **Chain**. First chain of a multimeric receptor molecule: in immunoglobulin molecules, the α heavy chain forming the IgA class; in T-cell receptor molecules, one of the chains forming the dimeric α–β receptor molecule; in MHC class I molecules, the main polypeptide chain associated with the β2-microglobulin molecule; in MHC class II molecules, one of the chains in the dimeric molecule

Alternative pathway of complement activation. Activation of complement pathway by substances other than antigen–antibody complexes; involves factor B, properdin, and complement component C3

Anaphylatoxin. Activated components of complement components C3 and C5 (C3a and C5a, respectively), which induce anaphylactic reactions by activating mast cells and basophilic granulocytes

Anaphylaxis or anaphylactic reaction. Local or systemic immediate hypersensitivity reaction initiated by mediators released after immunological stimulation; symptoms can be a drop in blood pressure related to vascular permeability and vascular dilatation, and obstruction of airways related to smooth muscle contraction or bronchoconstriction

Anergy. State of unresponsiveness to antigenic stimulation, due to the absence of responding elements or the loss of capacity of existing elements to mount a reaction; synonym for tolerance

Antibody. Immunoglobulin molecule produced in response to immunization or sensitization, which specifically reacts with antigen

Antibody-dependent cell-mediated cytotoxicity. Cytotoxic reaction in which an antibody forms the bridge between the cytotoxic cell (lymphocyte, macrophage) and the target cell

Antigen. Any substance that induces a specific immunological response

Antigen-binding site (paratope). Part on an antibody molecule that binds antigen (antigenic determinant, or epitope); part of the T-cell receptor that binds the complex of antigen and MHC molecule

Antigenic determinant (epitope). Part of an antigen that binds to antibody or T-cell receptor (the latter in combination with MHC molecule)

Antigenicity. Capacity to react with components of the specific immune system (antibody, receptors on lymphocytes)

Antigen presentation. Process of enabling lymphocytes to recognize antigen on a specific receptor on the cell surface. For presentation to T lymphocytes, includes intracellular processing and complexing of processed peptides with MHC molecule on the cell membrane of the antigen-presenting cell. For presentation to B lymphocytes, can include formation of immune complexes (in germinal centres)

Antigen-presenting cell. Cell that presents antigen to lymphocytes, making possible specific recognition by receptors on the cell surface. In a more restricted way, used to describe MHC class II-positive (accessory) cells which can present (processed) antigenic peptides complexed with MHC class II molecules to T helper–inducer lymphocytes. Includes macrophage populations (in particular, Langerhans cells and dendritic or interdigitating cells), B lymphocytes, activated T lymphocytes, certain epithelia (after MHC class II antigen induction by e.g. interferon γ); others are follicular dendritic cells, not of bone-marrow origin, which present antigen in the form of immune complexes to B cells in germinal centres of peripheral lymphoid tissue; marginal zone macrophages in splenic marginal zone, which present antigen, without contact with T helper cells, to B cells at this location (T cell-independent response, e.g. to bacterial polysaccharide). In rodent skin epidermis, a dendritic epidermal cell, of T-cell origin, has an antigen-presenting function.

Antigen receptor. Multichain molecule on lymphocytes, to which antigens bind. For B lymphocytes, an immunoglobulin molecule that recognizes nominal antigen; for T lymphocytes, a T-cell receptor molecule that recognizes antigenic peptide in combination with the polymorphic determinant of an MHC molecule

Antinuclear antibody. Antibody directed to nuclear antigen; can have various specificities (e.g. to single- or double-stranded DNA or histone proteins); frequently observed in patients with rheumatoid arthritis, scleroderma, Sjögren's syndrome, systemic lupus erythematosus, and mixed connective tissue disease. Also called antinuclear factor

Antiserum. Serum from an individual that contains antibodies to a given antigen

Aplasia. Absence of tissue structure or cellular component, either congenital or acquired

Apoptosis (programmed cell death). Process whereby the cell kills itself after activation, by Ca^{2+}-dependent endonuclease-induced chromosomal fragmentation into fragments of about 200 base-pairs

Appendix. Lymphoid organ in the gastrointestinal tract, at the junction of ileum and caecum; forms part of gut-associated lymphoid tissue

Arthus reaction. Inflammatory response, generally in skin, induced by immune complexes formed after injection of antigen into an individual that contains antibodies

Asthma. Chronic inflammatory disease characterized by bronchial hyperresponsiveness to various stimuli

Atopy. In general terms, 'unwanted reactivity'; used mostly to describe the state of general systemic or local hypersensitivity reactions related to genetic predisposition

Auto-antibody. Antibody to component in the individual itself

Auto-antigen. Antigen to which an autoimmune reaction is directed

Autoimmunity. A state of immune reactivity towards self

Autologous. Derived from self; components of an immunological reaction (e.g. antibody, lymphocytes, grafted tissue) from the same individual; opposite of heterologous

Avidity. Binding strength between antibody and antigen, or receptor and ligand; for antibody, represents the product of more than one interaction between antigen-binding site and antigenic determinant

Bacteraemia. Presence of bacteria in blood

Basophilic granulocyte. Polymorphonuclear leukocyte that contains granules with acid glycoproteins stained by basic (blue) dyes; after release, glycoproteins induce anaphylactic reactions

B-Cell growth and differentiation factor, B-cell growth factor, and B-cell stimulating factor. See *Interleukin-4 and -5*

β Chain. In T-cell receptor molecules, one of the chains forming the dimeric α–β receptor molecule; in MHC class II molecules, one of the chains in the dimeric molecule

β2-Microglobulin. A peptide of 12 kDa, which forms part of MHC class I molecule

B Lymphocyte or cell. Lymphocytes that recognize nominal antigen by immunoglobulin (antibody) surface receptor (on virgin B cell, IgM and IgD) and, after activation, proliferate and differentiate into antibody-producing plasma cells. During a T-dependent process, there is immunoglobulin class switch (IgM into IgG, IgA, IgD, or IgE), with maintenance of the antigen-combining structure. For T-independent antigens, cells differentiate only in IgM-producing plasma cells. B Lymphocytes originate from precursor cells in bone marrow; in avian species, they undergo maturation in the bursa of Fabricius (B, bursa-dependent); in mammals, in the bone marrow

B Lymphocyte area. That part of an lymphoid organ or tissue that is occupied by B lymphocytes, e.g. follicles in peripheral lymphoid tissue, marginal zone in spleen

Birbeck granule. Rod-shaped structure with rounded end, approximately 6 nm thick, found in the cytoplasm of Langerhans cells in the epidermis, and interdigitating dendritic cells in T-lymphocyte area of lymphoid tissues

Blast cell. Large cell (about 15 μm or more) with dispersed nuclear chromatin and cytoplasm rich in ribosomes; in an active stage of the cell cycle before mitosis

Blast transformation. Process of activation of lymphocytes into cell cycle and to form blastoid cells before mitosis

Blocking antibody. Antibody that can interfere with another antibody or with reactive cells in binding antigen, thereby preventing effector reactions (often used in association with an allergic reaction or tissue damage)

Bone marrow. Soft tissue in hollow bones, containing haematopoietic stem cells and precursor cells of all blood cell subpopulations (primary lymphoid organ); major site of plasma cell and antibody production (secondary lymphoid organ)

Booster. Dose of antigen given after immunization or sensitization to evoke a secondary response

Bradykinin. Peptide of nine amino acids split from α2-macroglobulin by the enzyme kallikrein; causes contraction of smooth muscle

Bronchus-associated lymphoid tissue. Lymphoid tissue located along the bronchi, considered to represent the location of presentation of antigens entering the airways; contributes to mucosa-associated lymphoid tissue

Bursa equivalent. Site where B-cell precursors undergo maturation into immunocompetent cells in non-avian species; bone marrow in adult mammals

Bursa of Fabricius. Primary lymphoid organ in avian species, located in cloaca, with an epithelial reticulum, where precursors of B lymphocytes from the bone marrow undergo maturation into immunocompetent cells and then move to peripheral lymphoid organs

C (constant) gene. Gene that encodes the constant part of immunoglobulin chains or T-cell receptor chains (e.g. C_μ, C_δ for immunoglobulin heavy chains, C_κ for immunoglobulin κ light chain, C_α for T-cell receptor α chain)

C (constant) region. Region at carboxy terminal of immunoglobulin chains or T-cell receptor chains, identical for a given immunoglobulin class or subclass or for a given T-cell receptor chain; encoded by C genes in DNA

Cachectin. See *Tumour necrosis factor*

CD (cluster of differentiation). Group of (monoclonal mouse) antibodies that react to identical leukocyte surface molecules in humans (but not necessarily to identical epitopes), on the basis of comparative evaluations during international workshops and transferred to other species by analogy. Does not include MHC or immunoglobulin molecules

CD3 molecule. Molecule consisting of at least four invariant polypeptide chains, present on the surface of T lymphocytes associated with the T-cell receptor; mediates transmembrane signalling (tyrosine phosphorylation) after antigen binding to T-cell receptor

CD4 molecule. Glycoprotein of 55 kDa on the surface of T lymphocytes and part of monocytes and macrophages. On mature T cells, restricted to T helper (inducer) cells; has an accessory function to antigen binding by T-cell receptors, by binding to a non-polymorphic determinant of MHC class II molecule

CD8 molecule. Complex of dimers or higher multimers of 32–34 kDa glycosated polypeptides linked by disulfide bridges, on the surface of T lymphocytes. On mature T cells, the presence is restricted to T cytotoxic–suppressor cells; has an accessory function to antigen binding by the T-cell receptor, by binding to a non-polymorphic determinant of MHC class I molecule

Cell-mediated immunity. Immunological reactivity mediated by T lymphocytes

Central (primary) lymphoid organ. Lymphoid organ in which precursor lymphocytes differentiate and proliferate in close contact with the microenvironment, to form immunocompetent cells; not antigen-driven but can be influenced by mediators produced as a result of antigen stimulation

Centroblast. Intermediately differentiated B lymphocyte present in germinal centres of follicles in lymphoid tissue; a medium to large, 12–18-μm cell, with a round to ovoid nucleus that has moderately condensed heterochromatin and medium-sized nucleoli close to the nuclear membrane, medium-sized to broad cytoplasm containing many polyribosomes and a variable amount of rough endoplasmic reticulum

Centrocyte. Intermediately differentiated B lymphocyte present in germinal centres of follicles in lymphoid tissue; medium-sized, 8–12-μm lymphoid cell with irregular nucleus with condensed heterochromatin; small cytoplasm containing a few organelles

CH50. The amount of serum (or dilution of serum) that is required to lyse 50% of erythrocytes in a standard haemolytic complement assay

Chemiluminescence. Luminescence produced by direct transformation of chemical energy into light energy

Chemotactic factor. Substance that attracts cells to inflammatory lesions

Chemotaxis. Process of attracting cells to a given location, where they contribute to an inflammatory lesion

Class I MHC molecule. Molecule coded by the A, B, or C locus in the HLA complex, the K and D locus in the mouse H2 complex, and less well defined MHC I gene loci in other species, in association with the β2-microglobulin molecule. Two-chain molecule occurring on all nucleated cells, without allelic exclusion

Class II MHC molecule. Molecule coded by the D (DR, DP, DQ) locus in the HLA complex, the I–A and I–E locus in the mouse H2 complex, and less well defined MHC II gene loci in other species, comprising an α and a β chain (intracellular, associated with an 'invariant' chain). Two-chain molecule occurring, without allelic exclusion, on B lymphocytes, activated T lymphocytes, monocytes–macrophages, interdigitating dendritic cells, some epithelial and endothelial cells (variable, dependent on species and state of activation); antigen-presenting cells

Class switch. See *Immunoglobulin class switch*

Classical pathway of complement activation. Activation of complement pathway by antigen–antibody complexes, starting with complement component C1 and ending with complement component C3

Clone. Population of cells that emerge from a single precursor cell; within T or B lymphocytes, cells with a fixed rearrangement of genes coding for T-cell receptor or immunoglobulin

Clonal expansion. Proliferation of cells that have a genetically identical constitution; when uncontrolled, may result in tumour formation

Colony-stimulating factor. Substance that supports clonal cell growth of haematopoietic cells

Complement. Group of about 20 proteinase precursors that activate and split each other, in sequential order. The various components are present in inactive (precursor) form, except for C3, which in a normal state shows a low turnover (major split products C3a and C3b). The split products are either bound to the activating substance (immune complex or antibody-coated cell) or are released as active mediators. The classical cascade starts by activation of component C1, subsequently C4, C2, and C3, and is initiated by (IgG/IgM) immune complexes. The

alternative cascade starts by activation of C3b and factor B, subsequently factors D and C3, and is initiated by nonimmune specific activators like microbial polysaccharides and some 'allergens'. C3 split products (C3b) activate the amplification loop, in which factors D and B are also used, activate C5, and thereafter the terminal cascade C6, C7, C8, and C9, which attack the cell membrane and kill the cell (microorganism). The cascade is under the control of various inhibitors. Major effects of complement split products are adherence to receptors on phagocytes (C3b, C3d); mediator activity, like chemoattraction of inflammatory cells, vasodilatation, increased vascular permeability, and smooth muscle contraction (C3a, C5a); cell lysis by membrane lesions (C6–C9)

Complement fixation. Binding and consumption of complement by antigen–antibody complexes; often used in association with assays for complement activity

Complement receptor (CR). Cell surface molecule that can bind activated complement components in e.g. antigen–antibody complexes; CR1 (CD35) is a receptor for C3b, present on B lymphocytes, monocytes and macrophages, granulocytes, and erythrocytes; CR2 (CD21) is a receptor for C3d, present on mature B lymphocytes; CR3 (CD11b/CD18) is a receptor for C3b present on macrophages, granulocytes, natural killer cells, and a subset of $CD5^+$ B lymphocytes; CR4 (CD11c/CD18) is a receptor for C3b present on monocytes, macrophages, granulocytes, and natural killer cells

Contact sensitivity. Hypersensitivity reaction evoked in skin by placing sensitizing agents or substances on skin

Cords of Billroth. Medullary cords in spleen

Corona. See *Mantle*

Cortex. Outer parenchymal layer of organs

Cross-reactivity. Reactivity of antigen-specific elements (T lymphocytes sensitized by T-cell receptor, B lymphocytes by antibody; antibody molecules) towards antigens other than those used in original sensitization, owing to shared antigenic epitopes on different antigenic molecules; also used to describe reactions towards antigenic determinants other than those originally used in sensitization, due to similarities in structure

Cytokine. Biologically active peptide, synthesized mainly by lymphocytes (lymphokines) or monocytes and macrophages

(monokines); modulates the function of cells in immunological reactions; include interleukins. Some (pleotrophic cytokines) have a broad spectrum of biological action, including neuromodulation, growth factor activity, and proinflammatory activity

Cytokine receptor. Ligand for cytokine on target cell, acts in signal transduction through the cell membrane; many are multichain molecules belonging to different receptor families

Cytolytic antibody. Antibody that can mediate lysis of the target to which it is directed, either in combination with complement or as bridge between cytotoxic cell and target

Cytotoxic cell (killer cell). Effector T cell, natural killer cell, or activated macrophage; kills target cells and tissue extracellularly after binding; mediated by release of substances from cytolytic granules (including serine esterase, cytolysin, and perforins)

Cytotoxic reaction. Effector reaction of antibody or cells, resulting in lysis of target cell or tissue

Cytotoxic T lymphocyte. Subpopulation of T lymphocytes with CD8 phenotype; after recognition of antigen in an MHC class I-restricted manner, differentiates from precursor to effector cytotoxic cell and subsequently kills target cells

Degranulation. Process of fusion of cytoplasmic granules with cell membrane, whereby the content of the granules is released into the extracellular space; mainly used in association with immediate hypersensitivity reactions

Delayed-type hypersensitivity. Inflammatory lesion mediated by effector T lymphocytes or their products, with attraction mainly of macrophages towards the inflammatory lesion. Term originates from the classical skin reaction after challenge of a sensitized individual; maximal (wheal and flare) response reached within 24–72 h

Delayed-type hypersensitivity T cell. Subpopulation of T lymphocytes with CD4 phenotype; after recognition of antigen in an MHC class II-restricted manner, secretes mediators involved in inflammatory responses, e.g. INFγ and tumour necrosis factor

δ Chain. In immunoglobulin molecules, δ heavy chain forming the IgD immunoglobulin class; in T-cell receptor molecules, one of the chains forming the dimeric γ–δ receptor molecule; one of the chains in the CD3 molecule associated with the T-cell receptor

Dendritic cell. Cell in tissue that shows elongations or protrusions of cytoplasm into the parenchyma. Often used in a restricted manner to designate a type of antigen-presenting cell, of which two categories exist: one of bone-marrow origin belonging to the macrophage lineage, including Langerhans cells in skin and interdigitating dendritic cells in T-cell areas of lymphoid tissue and a very small leukocyte population in blood; the second of tissue parenchymal origin (presumably pericytes around blood vessels), the follicular dendritic cells in B-cell areas (follicles) of lymphoid tissue

Dendritic epidermal cell. T Cell in the epidermis that has dendritic morphology; has antigen-presenting function but is not a (macrophage-related) Langerhans cell. Occurs in rodents but not in humans; contributes to skin immune system

Dermal immune system. See *Skin immune system*

Desensitization. Induction of anergy or tolerance to allergic substances by active intervention in immune reactivity (exhaustion of reactive elements or induction of blocking phenomena)

Diapedesis. Passage of cells through blood vessel walls into tissue parenchyma, mediated by constriction of endothelial cells

D (diversity) gene. Gene that encodes the variable part of immunoglobulin heavy chains, or T-cell receptor α, β, or γ chain (e.g. D_{H1}–D_{Hn} for immunoglobulin heavy chains, $D_{\delta 1}$–$V_{\delta n}$ for T-cell receptor α or δ chain)

Domain. Part of polypeptide chain folded to a relatively rigid globular tertiary structure fixed by disulfide bonds. Molecules of the immunoglobulin gene superfamily have a tertiary domain-like structure: each domain is about 110 amino acids long and arranged in a sandwich of two sheets of anti-parallel β strands. See also *Homologous, Homology*

Ectoderm. Outermost of the three cellular layers of the embryo; produces the epidermis and neuronal tissue

Eczema. Superficial inflammation in skin, involving primarily the epidermis; characterized by redness, itching, minute papules and vesicles, weeping, oozing, and crusting. Histological changes include microvesiculation and oedema of the epidermis and an infiltrate of lymphocytes and macrophages in the dermis

Effector cell. General term to describe a cell that mediates a function after a stage of activation, differentiation, and proliferation

Endocytosis. Process of uptake of material by a cell; special forms are phagocytosis and pinocytosis

Endoderm. Innermost of the three cellular layers of the embryo; produces the gastrointestinal lining and some internal organs, such as liver and pancreas

Endoplasmic reticulum. Membrane-like structure in cell cytoplasm; site of protein synthesis

Endothelium. Cells that line blood vessels; exert a major function in traffic of leukocytes from blood into tissue, by altered expression of adhesion molecules (modulation of numbers of receptors; maturation and activation resulting in altered glycosylation, expression of new ligands or altered ligand binding affinity; change in cytoskeleton organization). A special endothelial cell type occurs in T-lymphocyte areas of lymphoid tissue in the high endothelial (postcapillary) venule.

Endotoxin. Lipopolysaccharide from the cell wall of Gram-negative bacteria; has toxic, pyrogenic, and immunoactivating effects

Enzyme-linked immunosorbent assay. Immunoenzymetric assay based on the use of antigens or antibodies labelled with a specific enzyme; combines the virtues of solid-phase technology and enzyme-labelled immunoreagents. The antigen–antibody complex is determined by an enzyme assay involving the incubation of the complex with an appropriate substrate of the enzyme.

Eosinophil chemotactic factor. Acidic tetrapeptide of 0.5 kDa produced by mast cells (preformed mediator); attracts eosinophils to the site of inflammation

Eosinophilia. State of increased proportions of eosinophilic granulocytes in blood

Eosinophilic granulocyte. Polymorphonuclear leukocyte that contains granules with basic proteins stained by acidophilic (red) dyes; after release, the proteins modulate inflammatory reactions

Epithelium. Cells covering the surface of the body and forming the first line of defence against pathogenic invaders. Reticular epithelium forming the stroma of tissue occurs in thymus (in avian species in the bursa of Fabricius); these cells have a major function in processing precursor cells to immunocompetent lymphocytes

Epithelioid cell. Cell of macrophage origin in chronic inflammatory lesions, which resembles an epithelial cell morphologically

Epitope (antigenic determinant). Part of antigen that binds to antibody or T-cell receptors (the latter in combination with MHC molecule)

ε Chain (see also *α Chain*). In immunoglobulin molecules, the ε heavy chain forming the IgE immunoglobulin class; one of the chains in the CD3 molecule associated with the T-cell receptor

Erythema. Redness of skin produced by congestion of blood capillaries due to dermal arterial vasodilatation

Erythrocyte. Red blood cell; a bone marrow-derived blood cell component involved in oxygen transport to tissue; contains a nucleus in distinct avian species like chickens but does not have a nucleus in mammals

Exudation. Inflammation in tissue; contains blood cells and fluid comprising serum proteins of high relative molecular mass

Ex vivo/in vitro. An assay method in which the effects of a xenobiotic are evaluated *in vitro* in cells isolated from an animal or human exposed to the compound of interest

Fab fragment. Part of an antibody molecule in which monovalent binding of an antigenic determinant occurs; formed by the three-dimensional structure of variable parts (domain) of one heavy and one light chain and the adjacent part of the constant part (constant domain); ab, antigen binding

$F(ab')_2$ fragment. Part of an antibody molecule in which divalent binding of antigenic determinants occurs; formed by the three-dimensional structure of both Fab fragments

Fc fragment. Part of an antibody molecule formed by the three-dimensional structure of the constant part (constant domains) of the heavy chains (except that adjacent to the variable domain), involved in antibody effector functions; c, crystallizable

Fc receptor. Structure on leukocytes (lymphocytes, monocytes, macrophages, granulocytes) that mediates binding of immunoglobulin or antibody, alone or after forming aggregates in antigen–antibody complexes. Receptors for IgE (Fcε) occur on mast cells and basophilic granulocytes and are involved in immediate hypersensitivity reactions; receptors for IgG are of three classes: low-affinity FcR III, CD16, on

natural killer cells, monocytes, macrophages, and granulocytes; low-affinity FcR II, CD32, on B cells, myeloid cells, Langerhans cells, and interdigitating dendritic cells; high-affinity FcR III, CD64, on monocytes and macrophages

Follicle. Round to oval structure in lymphoid tissue, where B cells are lodged. Primary follicles contain only small resting B cells; secondary follicles comprise a pale-stained germinal centre, with centrocytes and centroblast, and contain B lymphocytes in a state of activation or proliferation and macrophages, the stroma consisting of follicular dendritic cells. The germinal centre is surrounded by a mantle with small B lymphocytes

Follicular dendritic cell. Cell forming the stationary microenvironment of germinal centres of follicles in lymphoid tissue; elongated, often binucleated cell with long branches extending between germinal centre cells and forming a labyrinth-like structure; linked by desmosomes. Of local parenchymal origin, presumably from pericytes surrounding blood vessels. Its main function is presentation of antigen, trapped as immune complex in the labyrinth, to B lymphocytes.

γ **Chain** (see also *α Chain*). In immunoglobulin molecules, the γ heavy chain forming the IgG immunoglobulin class; in T-cell receptor molecules, one of the chains forming the dimeric γ–δ receptor molecule; one of the chains in the CD3 molecule associated with the T-cell receptor

γ–δ **T cell**. T Lymphocyte with an antigen receptor composed of a γ and a δ chain associated with CD3 transmembrane molecule; develops in part inside the thymus (including intrathymic selection), in part outside the thymus. Has a major role as a cytotoxic cell in the first phase of the (innate) immune response, e.g. in rodents in the mucosal epithelium

Gammaglobulin. Part of serum proteins that move towards the negative electrode (gamma fraction) upon electrophoresis; contains immunoglobulins

Gene rearrangement. For immunoglobulin and T-cell receptor, the process whereby the germline chromosomal genomic structure of variable (diversity), joining, and constant segments recombine to form a specific V–(D–)J–C combination, enabling transcription into mRNA and translation into protein. The V–(D–)J combination of different chains determines the specificity of the receptor (immunoglobulin or T-cell receptor).

Germinal centre. The pale-staining centre in follicles of lymphoid tissue, where B lymphocytes are activated by antigen in a T lymphocyte-

dependent manner and subsequently go into proliferation and differentiation, acquiring the morphology of centroblasts, centrocytes, and plasma cells. Has a special microenvironment made up of follicular dendritic cells and large macrophages (tingible body or starry-sky macrophages)

Glomerulonephritis. Inflammation of glomeruli in kidney, often associated with deposition of immune complexes along the glomerular basement membrane or in the mesangium, and influx of polymorphonuclear granulocytes

Golgi apparatus. Tubular structures in cytoplasm, involved in secretion of synthesized proteins

Granulocyte colony-stimulating factor. Synthesized by T lymphocytes and macrophages, epithelial cells, fibroblasts, and endothelial cells; supports growth of granulocyte progenitors, in synergism with IL-3 and granulocyte–macrophage colony-stimulating factor of monocyte–macrophage progenitors

Granulocyte–macrophage colony-stimulating factor. Produced by T lymphocytes, endothelial cells, macrophages, and lung cells; supports growth and differentiation of macrophages and granulocyte progenitors; activates macrophages and polymorphonuclear macrophages to become tumoricidal and produce superoxide anion

Granuloma. Chronic inflammatory reaction in tissue comprising macrophages (epitheloid cells), lymphocytes, and fibroblasts; formed in a cell-mediated response towards poorly degradable material, in immunological reactions as part of a delayed-type hypersensitivity reaction

Gut-associated lymphoid tissue. Lymphoid organs and tissue located along the gastrointestinal tract, presumed to be a first location of presentation of antigens entering through the digestive tract; comprises Peyer's patches, appendix, in part mesenteric lymph nodes, adenoids, and tonsils; contributes to the mucosa-associated lymphoid tissue

H-2. Major histocompatibility complex of mouse

Haemagglutinin. Antibody or substance that induces agglutination of erythrocytes

Haematopoiesis. Production of cells of blood; subdivided into erythropoiesis, lymphopoiesis, and myelopoiesis

Haematopoietic malignancy. Malignancy of blood-forming cells

Haemolysis. Process of lysis of erythrocytes, with release of haemoglobin

Haemolytic agent (haemolysin). Antibody or substance that induces lysis of erythrocytes

Haemopoietin. Growth factor that induces production of distinct types of blood cells; also enhances the function of the mature cells

Haplotype. Phenotype of inherited characteristic on closely linked genes on one chromosome

Hapten. Structure around one antigenic determinant, which itself does not evoke an immune response unless coupled to a carrier substance but can react with the products (antibodies, cells) of an immune response

Hassall's corpuscle. Epithelial aggregate in onion-like structure, often with debris of other cells; in the medulla of the thymus, surrounded by large epithelial cells secreting thymic hormones; does not occur in rodent thymus

Heat-shock protein. Family of proteins (60–90 kDa) with conserved sequence in evolution; play a prime role in regulation and transport of intracellular proteins. Expression is upregulated when cells are under 'stress' (originally induced by heating), such as inflammatory conditions, and may act as autoantigen in triggering and perpetuating an autoimmune response

H (heavy) chain. One of the 45-kDa polypeptide chains in immunoglobulin molecules, consisting of a variable domain and three constant domains (four constant domains in the 55-kDa μ chain). The combination of the constant part of two heavy chains (α, β, ε, γ, or μ) forms the immunoglobulin class-associated part of the molecule (IgA, IgD, IgE, IgG, or IgM class)

Helper (inducer) T cell. Cell in a subpopulation of T lymphocytes, with CD4 phenotype; after recognizing antigen in an MHC class II-restricted manner, induces immunological reactions, secretes interleukins, and cooperates (supports) B lymphocytes, cytotoxic T-cell precursors, and macrophages

Helper T cell subpopulations. Th1 and Th2: Th1 cells produce interleukin-2 and interleukin-3, interferon γ, tumour necrosis factor α and β, and granulocyte-macrophage colony stimulating factor, and function in induction of delayed-type hypersensitivity, macrophage activation, and IgG2a synthesis. Th2 cells produce interleukin-3,

interleukin-4, and interleukin-5, tumour necrosis factor α and granulocyte–macrophage colony stimulating factor, and function in induction of IgG1, IgA, and IgE synthesis and induction of eosinophilic granulocytes

Heterologous. Derived from foreign source or species; components of an immunological reaction (e.g. antibody, lymphocytes, grafted tissue) derived from another individual of the same species or another species; opposite of autologous

Heterophilic antigen. Antigen in unrelated species; can be directed towards xenogeneic immune reactivity; often has carbohydrate structure; opposite of homocytotropic antibody

High endothelial (postcapillary) venule. Specialized blood vessels in T-lymphocyte area of lymphoid tissue, through which circulating lymphocytes pass into the parenchyma

Hinge region. Stretch in immunoglobulin molecule between Fab and Fc fragments (first constant domain and other constant domains of the heavy chain), where the quaternary structure of the molecule is not rigid but flexible; bending of the hinge region after antigen binding serves as a signal transduction, resulting in effector reactions

Histamine. β-Imidazolylethylamine; component of granules in mast cells and basophilic granulocytes that is released upon activation and induces immediate hypersensitivity reaction, e.g. vasodilatation, vascular permeability, smooth muscle contraction, and broncho-constriction

Histiocyte (histiocytic reticulum cell). Monocyte in tissue. See *Macrophage*

Histiocytosis. Increase in proportion of macrophages in tissue

HLA, human leukocyte antigen. Major histocompatibility complex of humans

Homocytotropic antibody. Antibody that binds preferentially to cells from the same species rather than to cells from other species; opposite of heterologous antibody

Homologous, Homology. Similarity in primary structure between substances; homology region is a synonym for domain

Host defence. Ability of an individual or species to protect itself against opportunistic agents and to eliminate certain tumours and exogenous agents such as (micro)organisms, viruses, and particles that can cause disease

Hot spot. See *Hypervariable region*

Humoral immunity. Immunological reactivity mediated by antibody

Hybridoma. Transformed cell line or cell clone formed by fusion of two different parental cell lines or clones

Hyperplasia. Reversible increase in cell number, usually as the result of a physiological stimulus or persistent cell injury due to irritating compounds

Hypersensitivity. Increased reactivity or sensitivity; in immunological reactions, often associated with tissue destruction

Hypervariable region. Amino acid sequences in the variable regions of antibody molecules or T-cell receptor chains where variability is highest and which together form the antigen-binding site. Synonym for hot spot

Hypoplasia. Reversible decrease in cell number, usually as a result of a physiological stimulus

Ia antigen. MHC class II cell surface molecule

Idiotype. Antigenic determinant of variable domain of immunoglobulin molecules or T-cell receptor

Immediate hypersensitivity. Inflammatory response that occurs within minutes after exposure to allergen; caused by physical or immunological stimulus, with vascular dilatation, increased vascular permeability, and oedema as the main effects. Term originates from the classical skin reaction after challenge of a sensitized individual in skin, which takes 20–30 min to reach maximal (wheal and flare) response and is mimicked by injection of mediator only (histamine)

Immune adherence. Binding of antigen–antibody complexes (antibody-coated particles) to erythrocytes, platelets, or leukocytes; mediated by activation of complement C3

Immune complex. Complex between antigen and antibody

Immune elimination. Rapid clearance of pathogen from the circulation by components of the immune system; often used in association with antibody molecules (removal by immune complex formation and phagocytosis)

Immune exclusion. Process whereby entry of pathogens at mucosal surfaces is prevented by the action of specific (secretory IgA) antibody

Immune interferon. Former name for interferon γ

Immune surveillance. Function (still hypothetical) of the immune system in preventing or eliminating cells after malignant transformation to a neoplastic process

Immunity. State of protection against pathogen-induced injury, with fast immune elimination of pathogenic invaders due to previous antigen contact or a special acquired state of responsiveness

Immunization (vaccination). Active intervention resulting in immunity; used mainly in the context of presentation of (inactivated or attenuated, nonpathogenic) substance to induce immunological memory. Passive immunization is the adoptive transfer of immune system components after previous contact with the pathogen and is performed mainly with antibodies

Immunoblast. Intermediately differentiated B lymphocyte in lymphoid tissue; a large, 15–20 μm, round-to-spherical cell with a rounded euchromatic nucleus. The abundant cytoplasm contains many ribosomes, well-developed rough endoplasmic reticulum and Golgi complex

Immunocompetence. Capacity of B or T lymphocytes to specifically recognize antigen, resulting in a specific immunological reaction

Immunodeficiency. Defects in the immune system resulting in decreased or absent reactivity to pathogens. Primary immunodeficiency is mainly intrinsic defects in the differentiation of T or B lymphocytes and can be congenital or acquired. Secondary immunodeficiency is defects of which the cause is outside the immune system (malnutrition; stress; protein loss after burns, nephrotic syndrome, or intestinal bleeding; viral infection; therapy with immunosuppressive or cytostatic drugs; irradiation).

Immunogen. A substance that can induce an immunological reaction

Immunogenicity. Capacity to evoke an immune response

Immunoglobulin. Formerly the electrophoretically-defined gamma-globulin (in blood) but is also present in the β fraction; synthesized by plasma cells. The basic subunit consists of two identical heavy chains (about 500 amino acid residues, organized into four homologous domains; for μ chain in IgM, about 600 amino acid residues, organized into five homologous domains) and two identical light chains (about 250 amino acid residues organized into two homologous domains), each consisting of a variable domain and one to three constant domains (in the μ chain, four constant domains). The antigen-binding fragment (Fab) consists of variable domains of heavy and light chains (two per basic subunit). Five classes of immunoglobulins exist, which differ according to heavy chain type (constant domains): IgG (major immunoglobulin in blood), IgM (pentamer, consisting of five basic units), IgA (major immunoglobulin in secretions; present mainly as a dimeric molecule), IgD (major function, receptor on B lymphocytes), and IgE. Effector functions after antigen binding are mediated by constant domains of the heavy chain (Fc part of the molecule) and include complement activation (IgG, IgM), binding to phagocytic cells (IgG), sensitization and antibody-dependent cell-mediated cytotoxicity (IgG), adherence to platelets (IgG), sensitization and degranulation of mast cells and basophils (IgE). IgA lacks these effector functions and acts mainly in immune exclusion (prevention of entry in the body) at secretory surfaces ('antiseptic paint').

Immunoglobulin class. Subfamily of immunoglobulins, based on difference in heavy chain. Five classes exist: IgA, secretory immunoglobulin, dimeric; IgD, immunoglobulin on B cells that acts as antigen receptor; IgE, immunoglobulin fixed to mast cells and basophilic granulocytes, involved in immediate hypersensitivity reactions; IgG, main immunoglobulin in circulation; IgM, pentameric immunoglobulin with optimal agglutinating capacity, produced on first antigen contact

Immunoglobulin class switch. Process whereby synthesis of IgM antibody changes into synthesis of antibody of another immunoglobulin class, with maintenance of the same variable part of the immunoglobulin molecule. At the genomic level, this includes gene rearrangement, with an exchange of a constant gene segment to a fixed V–D–J gene segment combination. This switch is thought to occur in germinal centres of follicles in lymphoid tissue, during the change of a primary into a secondary response, and is under the control of cytokines (switch factors)

Immunoglobulin gene superfamily. Group of molecules including immunoglobulins, T-cell receptors, MHC molecules, and others, like

the lymphocyte function-related antigens LFA-2 (CD2) and LFA-3 (CD58), the intercellular adhesion molecules ICAM-1 (CD54) and ICAM-2, the vascular cell adhesion molecule VCAM-1, the neural cell adhesion molecule NCAM (CD56), and the CD4 and CD8 molecules, which have a similar tertiary basic domain-like structure, in which each domain is about 110 amino acids long and stabilized by a disulfide bridge. These molecules are known to be important for specific recognition and adhesion functions

Immunoglobulin light chain type. Defines the light chain in the immunoglobulin unit, either κ or λ, each defined at the germline DNA level by individual constant (C), joining (J), and variable (V) gene segments

Immunoglobulin subclass. Subfamily within a distinct immunoglobulin class, based on subtle differences in heavy chain. For instance, in humans there are two IgA subclasses, IgA1 (α1 heavy chain) and IgA2 (α2 heavy chain), and four IgG subclasses, IgG1–IgG4 (γ1–γ4 heavy chain). In rodents, these are designated IgG1 (γ1), IgG2a (γ2a), IgG2b (γ2b), and IgG3 (γ3)

Immunological memory. Acquired state of the immune system after first contact with antigen, whereby the reaction upon subsequent contact is faster, more intense, and of higher affinity. For antibody response, associated with an immunoglobulin class switch and 'affinity maturation' (by somatic mutation)

Immunosuppression. Prevention or diminution of the immune response by administration of antineoplastic or antimetabolic drugs, antilymphocyte serum, or exposure to e.g. environmental chemicals or microorganisms (viruses)

Immunotoxicant. Drug, chemical, or other agent that is toxic to cells or other components of the immune system

Inducer (helper) T cell. See *Helper (inducer) T cell*

Inflammation. Process whereby blood proteins or leukocytes enter tissue in response to or in association with infection or tissue injury

Inflammatory cell. General description of cells in an inflammatory infiltrate; in acute inflammation, mainly polymorphonuclear leukocytes; in chronic inflammation, mainly lymphocytes and macrophages

Innate immunity. State of protection against pathogen-induced injury; does not require previous immunization or vaccination

Innocent bystander. Cell or tissue component that is destroyed by an immunological reaction specifically directed against a unrelated antigen

Integrin. Family of heterodimeric molecules sharing a β chain (β1, β2, β3, about 750 amino acids long), each with a different α chain (about 1100 amino acids long), with a major function in cell adhesion and migration. Form a protein family rather than a superfamily on the basis of strong structural and functional similarities. Examples: leukocyte function-related antigen LFA-1 (α L/β1, CD11a/CD18; receptor for intercellular adhesion molecules ICAM-1, ICAM-2, and ICAM-3); Mac-1 (αM/β2, CD11b/CD18; complement C3 receptor, CR3); p150,95 (αX/β2, CD11c/CD18); very late antigens VLA-1 (α1/β1, CD49a/CD29; laminin, collagen receptor), VLA-2 (α2/β1, CD49b/CD29; laminin, collagen receptor), VLA-3 (α3/β1, CD49c/CD29; laminin, collagen, fibronectin receptor), VLA-4/LPAM-1 (α4/β1, CD49d/CD29; receptor for fibronectin and VCAM-1), VLA-5 (α5/β1, CD49e/CD29, fibronectin receptor), and VLA-6 (α6/β1, CD49f/CD29; laminin receptor, and αV/β1, CD51/CD29; vitronectin receptor); LPAM-2 (α4/βp, CD49d/.., or α4/β7)

Interdigitating dendritic cell. Leukocyte belonging to the monocyte–macrophage cell lineage, present in T-cell areas in lymphoid organs; has a major function in presentation of antigen (MHC class II-restricted) to helper-inducer T lymphocytes. Cytoplasm contains characteristic rod-like structures called Birbeck granules. Its equivalent in epidermis is the Langerhans cell, and that in lymph, the veiled macrophage.

Interferon. Low-relative-molecular-mass substance produced mainly during viral infection by leukocytes (IFNα), fibroblasts (IFNβ), and lymphocytes (IFNγ); has a major function in interfering with viral replication

Interferon-α. Produced by leukocytes; stimulates B cells to proliferate and differentiate; stimulates natural killer cells and increases cytotoxic T cell generation, but blocks T-cell proliferation and lymphokine-activated killer activity; stimulates macrophage accessory activity and enhances Fc receptor expression and MHC class I and II expression on various cell types; induces antiviral state in cells and is cytostatic for tumour cells, inhibits fibroblast and adipocyte differentiation, and enhances promyelocytic and monoblastic cell differentiation

Interferon-β. Produced by fibroblasts and epithelia; activity similar to that of IFNα

Interferon-γ. Produced by T cells; induces antiviral state and is cytostatic for tumour cells; enhances MHC class I and II expression on various cell types, is antagonistic to interleukin-4 in IgE/IgG1 synthesis, and stimulates IgG2a synthesis; activates macrophages to become cytolytic and enhances natural killer and lymphokine-activated killer activity.

Interfollicular area. Area between follicles in lymphoid tissue, where mainly small T lymphocytes are lodged; recognized by presence of high endothelial venules. In lymph nodes, located in the outer cortex and continuous with the paracortex

Interleukin. Immunoregulatory protein, also known as lymphokine, monokine, interferon, or cytokine. Generally, low relative molecular mass (< 80 kDa) and frequent glycosylation; regulates immune cell function and inflammation by binding to specific cell surface receptors; transient and local production; acts in paracrine or autocrine manner, with stimulatory or blocking effect on growth and differentiation; very potent, functions at picomolar concentrations. Represents an extensive series of mediators (interleukins 1–12), with a wide range of overlapping functions. Other mediators in this series are c-kit ligand, interferon, tumour necrosis factor, and transforming growth factor

Interleukin 1. Comprises two forms, IL-1α and IL-1β; produced mainly by cells of the mononuclear phagocytic system (macrophages), astrocytes, endothelium, and some epithelia, following stimulation by e.g. microorganisms, immune complexes, or particulate compounds. IL-1α is mainly cell-associated; IL-1β is released. IL-1 has (together with IL-6 and tumour necrosis factor) multiple effects in the systemic acute-phase response and in local acute and chronic inflammation: it stimulates T (helper) cells to synthesize IL-2 and IL-2 receptors, interferon γ, and other lymphokines, B cells (proliferation and differentiation), neutrophils, and natural killer cells; stimulates monocytes and macrophages to produce IL-1, IL-6, and tumour necrosis factor; acts in the acute-phase response by inducing synthesis of acute-phase proteins in liver and reducing cytochrome P450 synthesis; induces natriuresis in kidney, insulin production in pancreas β cells, muscular proteolysis ('easy' energy generation) in muscle cells, slow-wave sleep in cerebral cortex; raises the temperature set-point (fever) in hypothalamus; stimulates haematopoiesis and prostaglandin synthesis by various cell types (fibroblasts, macrophages, endothelium); inhibits gastric motility *in vitro*; induces collagenase production by synovial cells and osteoclasts, and antiviral state; inhibits gastric smooth muscle *in vitro*; is cytostatic for tumour cells and activates endothelium

Interleukin 2. Synthesized by T helper cells after activation; stimulates (autocrine) T cells to divide and release mediators, B cells to proliferate and differentiate; activates monocytes and natural killer cells; stimulates lymphokine-activated killer cells; promotes generation of T helper 1 cells.

Interleukin 3. Formerly called multi-colony-stimulating factor; synthesized by T helper cells; promotes growth of pluripotent haematopoietic progenitor cells to granulocytes (eosinophilic, basophilic, neutrophilic), mast cells, macrophages, megakaryocytes, and, together with erythropoietin, to normoblasts and erythrocytes; activates eosinophils and mast cells; stimulates haematopoiesis and B-cell differentiation; blocks lymphokine-activated killer cells

Interleukin 4. Formerly called B-cell growth factor or B-cell stimulating factor; synthesized by T helper and B cells; stimulates IgE and IgG1 production by B cells and enhances MHC class II and IgE receptor expression on B cells; acts in synergism with IL-2 in killer cell generation, is mitogenic for T cells, and activates macrophages. It is the dominant interleukin in generating T helper 2 cells, with a negative feedback on the generation of T helper 1 cells.

Interleukin 5. Formerly called T-cell replacing factor or B-cell growth and differentiation factor II; synthesized by T helper cells; activates B cells and eosinophils, and stimulates IgA production by B cells

Interleukin 6. Formerly called interferon β2; synthesized by T cells, monocytes, endothelial cells, fibroblasts, and smooth muscle cells, among others, during inflammatory reactions; stimulates T and B cells to proliferate and differentiate; has properties similar to IL-1 and acts synergistically with it in the acute-phase response (fever, synthesis of acute-phase proteins); synergizes with IL-3 in promoting haematopoietic progenitor cell proliferation; inhibits production of IL-1 and tumour necrosis factor by monocytes

Interleukin 7. Formerly called lymphopoietin; synthesized by bone-marrow stroma; induces growth of immature T and B lymphocytes

Interleukin 8. Formerly called neutrophil-activating protein; synthesized by monocytes and various tissue cells in response to inflammatory stimuli; performs chemotaxis of neutrophilic granulocytes and subsequent granule exocytosis and respiratory burst; induces increased expression of adhesion molecules CD11b/CD18 (complement C3 receptor CR3) and promotes vascular leakage. Endothelium-derived IL-8 inhibits adhesion of neutrophilic granulocytes induced by IL-1

Interleukin 9. Synthesized by T lymphocytes; stimulates growth of erythroid and megakaryocyte precursors and promotes (mucosal) mast-cell growth; acts synergistically with IL-4 in modulating IgE and IgG production

Interleukin 10. Synthesized by T and B lymphocytes; inhibits mediator synthesis (IL-2, IL-3, tumour necrosis factor, interferon γ, granulocyte–macrophage colony-stimulating factor) by T helper 1 cells, inhibits mediator synthesis (IL-1α, IL-1β, IL-6, IL-8, and tumour necrosis factor α) by monocytes; stimulates IL-2-dependent growth and cytotoxicity of cytotoxic T cells and stimulates mast cell growth together with IL-2 or IL-3 and IL-4; induces MHC class II antigen expression on B cells, but down-regulates MHC class II on monocytes; promotes generation of T helper 2 cells

Interleukin 11. Synthesized by fibroblasts and bone-marrow stromal cells; resembles IL-6 in function: stimulates haematopoietic cell growth and differentiation (myeloid, erythroid, megakaryocyte lineage); enhances T-cell-dependent antibody response; and suppresses adipocyte differentiation and lipoprotein lipase production

Interleukin 12. Also called natural killer cell stimulatory factor; synthesized by monocytes–macrophages, B cells, and accessory cells, in response to bacteria or parasites; stimulates T-lymphocyte proliferation, activates natural killer cells, and stimulates lymphokine-activated killer activity; synergizes with IL-2 in activation of cytotoxic lymphocytes; induces production of interferon γ and other cytokines by lymphocytes. It is the dominant interleukin in generating T helper 1 cells and has a negative feedback on the generation of T helper 2 cells.

In vitro. In the context of this book, exposure of cells or cell systems to the immunotoxic agent *in vitro*. If the donors of cells or cell systems are exposed but these are analysed *in vitro*, the term *ex vivo/in vitro* is used

Isohaemagglutinin. Antibodies mainly of the IgM class that react with (carbohydrate) antigens on erythrocytes from individuals of the same species, resulting in agglutination *in vitro*

Isologous. Synonym for isogeneic

Isotype. Antigenic determinant that defines class or subclass of immunoglobulin molecules

J (joining) chain. A 15-kDa polypeptide chain that acts intracellularly to combine (identical) IgA or IgM immunoglobulin units, consisting of

two heavy and two light chains, to form a dimeric IgA or a pentameric IgM molecule

J (joining) genes. Genes that encode the variable part of immunoglobulin or T-cell receptor chains (e.g. J_{H1}–J_{Hn} for immunoglobulin heavy chains, $J_{\kappa1}$–$J_{\kappa n}$ for immunoglobulin κ light chain, $J_{\alpha1}$–$J_{\alpha n}$ for T-cell receptor α chain)

Kallikrein (kininogenase). Arises in tissue fluids after cleavage of prekallikrein; acts on kininogen to produce kinins, resulting in immediate hypersensitivity reaction, e.g. vasodilatation and oedema. It is a preformed mediator present in mast cell granules

κ Chain. In immunoglobulin molecules, the κ light chain forms the light chain type of the molecule

Keratinocyte. Epithelial cell in the epidermis; in some circumstances, can manifest antigen presentation and produce immunoregulatory cytokines; hence belongs to the skin immune system

Killer cell (K cell). See *Cytotoxic cell*

Kinin system. Humoral amplification system involved in inflammation, whereby substrate proteins become active after enzymatic cleavage; cause vasodilatation, increased vascular permeability, hypotension, and contraction of smooth muscle

c-Kit ligand. Also called stem cell growth factor or mast cell growth factor; synthesized by various stromal cells, fibroblasts, and liver cells; stimulates growth of early pluripotent progenitor cells and that of myeloid, erythroid, and lymphoid progenitors in synergy with interleukin-1, -3, -6, -7, and granulocyte–macrophage colony-stimulating factor; promotes growth of mast cells

Kupffer cells. Macrophages on or between endothelial cells lining the sinusoids of the liver

λ Chain. In immunoglobulin molecules, the λ light chain forms the light chain type of the molecule

Lamina propria. Thin layer of connective tissue under the villous epithelium of the gastrointestinal tract; site of plasma cells, producing mainly dimeric IgA, including J chain

Langerhans cell. Leukocyte belonging to the monocyte–macrophage cell lineage, present in skin epidermis; has a major function in uptake

and processing of antigen, followed by presentation (MHC class II restricted) to T helper lymphocytes. Cytoplasm contains characteristic rod-like structures, Birbeck granules. Its equivalent in lymphoid tissue is the interdigitating dendritic cell, and that in lymph, veiled macrophage; forms part of the skin immune system

Large granular lymphocyte. Intermediate-sized, 10–12-μm lymphocyte with a kidney-shaped nucleus and prominent, large, azurophilic granules in the cytoplasm; occurs in the circulation and in tissue and has a major function as a natural killer cell; forms a heterogeneous population with either T markers or monocyte–macrophage markers.

Lectin. Plant-derived substance that binds to lymphocytes and can induce cell proliferation; some also bind to other haematopoietic cells

Leukaemia. Neoplasia of lymphoid cells in blood or bone marrow

Leukocyte. Bone marrow-derived white blood cell, including cells in the lymphoid, myeloid, and monocyte lineages; sometimes used to describe only granulocytes

Leukocytosis. Increased proportion of leukocytes in blood

Leukopenia, leukocytopenia. Reduced proportion of leukocytes in blood

Leukotriene. Formerly called slow-reacting substance of anaphylaxis; products of arachidonic acid metabolism following the lipoxygenase pathway, which act as mediators in the immediate hypersensitivity reaction, mainly as chemoattractants for granulocytes and monocytes, and in smooth muscle contraction; newly synthesized by mast cells upon activation

L (light) chain. One of the 22-kDa polypeptide chains in immunoglobulin molecules, consisting of a variable domain and a constant domain. The light chain, either κ or λ, determines the light chain type of the immunoglobulin molecule

Ly antigen. T Lymphocyte antigen in mice

Lymph. Fluid in lymphatic vessels

Lymph node. Secondary (peripheral) lymphoid organ, the main function of which is to filter lymphatic vessels. Present throughout the body, at connecting places of lymphatics and blood vessels; forms a major site of encounter between pathogenic substances in the lymph and

lymphocytes entering from blood vessels, and subsequent initiation of antigen-specific immunological reactions

Lymphatic. Vessel that collects fluid from interstitial spaces and goes via lymph nodes (filtering) to the thoracic duct and blood

Lymphocyte. Cell belonging to the lymphoid lineage of bone marrow-derived haematopoietic cells. In a restricted way, the designation of a small, resting or recirculating mononuclear cell in blood or lymphoid tissue, which measures about 7–8 µm, has a round nucleus containing densely aggregated chromatin, and little cytoplasm. Plays a key role in immune reactions by specific recognition of antigens

Lymphocytosis. Increased proportions of lymphocytes in blood

Lymphoid organ. Tissue in the body where cells of the immune system, mainly lymphocytes, are lodged in an organized microenvironment, either in a resting state or in a state of activation, differentiation, or proliferation. Includes bone marrow, thymus, lymph nodes, spleen, and mucosa-associated lymphoid tissue

Lymphokine. Hormonal substance synthesized by lymphocytes, which modulates the function of cells in immunological reactions

Lymphoma. Neoplasia of lymphoid cells in tissue

Lymphopenia, lymphocytopenia. Reduced proportions of lymphocytes in blood

Lymphotoxin. Former name for tumour necrosis factor β; lymphokine synthesized by T lymphocytes, which kills selected target cells

Lysosome. Granule present in many cell types that contain hydrolytic enzymes; also performs intracellular degradation of pathogens after phagocytosis

Lysozyme (muramidase). A low-relative-molecular-mass, cationic enzyme present in tissue fluids and secretions, which degrades mucopeptides of bacterial cell walls

Macroglobulin. Glycoprotein of relative molecular mass > 200 kDa

Macrophage. Large, 12–20-µm bone marrow-derived mononuclear cell in the monocyte–macrophage lineage, present in tissue, and forming the mononuclear phagocytic system. Its reniform nucleus usually has pronounced peripheral condensation of nuclear chromatin; its cytoplasm

contains a great variety of cell organelles, including rough endoplasmic reticulum, mitochondria, ribosomes, lysosomes, and Golgi complex. Has a major function in (chronic) inflammatory reactions, by virtue of its phagocytic capacity, with immunoglobulin Fc and complement C3 receptors which bind to immune complexes. Macrophages develop into killer cells after activation by e.g. T-cell factors and can mediate antibody-dependent cell-mediated cytotoxicity; also functions as an accessory cell in induction of immune responses (antigen presentation, mediator secretion). Macrophages in blood are called monocytes. Subtypes with special functions are interdigitating dendritic cells: T-cell area of lymphoid tissue, Langerhans cell (skin), Kupffer cells (liver), metallophilic macrophages (spleen), microglia (brain), osteoclasts (bone), tingible body macrophage (starry-sky macrophage), veiled macrophage (lymph).

Macrophage colony-stimulating factor. Synthesized mainly by endothelial cells and fibroblasts, and possibly macrophages; supports growth of monocyte–macrophage progenitors.

Major histocompatibility complex (MHC). Set of genes that codes for tissue compatibility markers, which are targets in allograft rejection and hence determine the fate of allografts; plays a central role in control of cellular interactions during immunological reactions. Tissue compatibility is coded by classes I and II loci (see *Class I* and *Class II MHC molecule*). Genes within or closely linked to MHC control certain complement components (MHC class III genes). The MHC complex of humans is HLA, that of mice H-2, and that of rats, RT-1.

Mantle (corona). Zone in secondary follicles surrounding the central germinal centre, densely packed with small resting B lymphocytes

Marginal zone. Outer layer of white pulp in spleen, surrounding follicles, and periarteriolar lymphocyte sheath; separated from these by the marginal sinus; populated by intermediate-sized, slightly pyroninophilic B cells which have a major function in the T cell-independent antibody response. The microenvironment manifests a special type of macrophage, the marginal metallophilic macrophage

Margination. Adherence of blood leukocytes to endothelium during inflammatory reactions

Mast cell. A bone marrow-derived polymorphonuclear leukocyte present in tissue; has a major function in immediate hypersensitivity reactions; has a round or oval nucleus and abundant cytoplasm with basophilic (blue) granules stained by metachromatic dyes; granules

contain mediators of immediate hypersensitivity reactions, e.g. heparin, histamine, serotonin, tryptase, kallikrein, and chemoattractants for neutrophilic and eosinophilic granulocytes; has a high-affinity receptor for IgE. After activation (physical stimuli, cross-linking via allergen–IgE–IgE receptor), there is immediate granule release and synthesis of other mediators, including prostaglandins, thromboxanes, leukotrienes, and platelet-activating factor. The cell can exert modulatory activity by secreting cytokines such as IL-3, IL-6, and tumour necrosis factor. Two subtypes exist, in the mucosa and in connective tissue; the equivalent in the circulation is the basophilic granulocyte

Mast cell growth factor. See *Interleukin 9*

Medulla. Inner parenchymal layer of organs

Medullary cord. Parenchyma in medulla of lymph nodes separating lymphatic sinusoids

Megakaryocyte. Large, multinucleated giant cell, precursor of blood platelets, formed by separation of portions of membrane-bound cytoplasm; occurs in haematopoietic tissue, including bone marrow

Memory. See *Immunological memory*

Mesoderm. Middle of the three cellular layers of the embryo; produces connective tissue and blood cells

Metallophilic macrophage. Subtype of macrophage identified by silver impregnation, present at the inner border of the marginal zone in spleen

MHC restriction. Immunological reactions can occur only in associated recognition with the polymorphic determinant of a given MHC molecule and not with that of another MHC molecule. Applies to T lymphocytes with an α–β T-cell receptor, which recognizes antigenic peptides in combination with the polymorphic determinant of MHC molecules, and part of the T cell population with the γ–δ T-cell receptor.

Microglia. Macrophages in central nervous system

Migration inhibitory factor. A lymphokine that inhibits the movement of macrophages

Milky spot. Aggregate of lymphoid cells in omentum, macroscopically visible as a small white spot; not organized tissue but rather the product of immune stimulation in that area of the body

Minor histocompatibility antigen. Ill-defined histocompatibility marker not encoded by the MHC, which is a target in allograft reactions (apart from products of the MHC)

Mitogen. Substance that activates resting cells to transform and proliferate

Monoclonal. Derived from a single clone. For T and B lymphocytes, a cell population in which all cells have a distinct V–D–J gene rearrangement product (as seen in lymphoma and leukaemia). Monoclonal antibodies are products of hybridomas prepared after fusion of antibody-producing cells and a transformed (non-producing) plasmacytoid cell line.

Monocyte. Large, 10–15-μm bone marrow-derived mononuclear cell in the monocyte–macrophage lineage, present in the blood and in lymphatic vessels

Monokine. Hormonal substance synthesized by monocytes–macrophages; modulates the function of cells in immunological reactions

Mononuclear cell. Leukocyte with a single rounded nucleus, e.g. lymphocytes and monocytes–macrophages

Mononuclear phagocytic system. Formerly called reticuloendothelial system; composite of phagocytic cells in the body, including monocytes and tissue macrophages. Main populations are Kupffer cells in liver, microglia in the central nervous system, macrophages in red pulp of spleen, alveolar macrophages in lung, and, after induction, peritoneal macrophages in the peritoneal cavity; others are mesangial macrophages in kidney and osteoclasts in bone

μ Chain. In immunoglobulin molecules, the μ heavy chain forming the IgM immunoglobulin class

Mucosa-associated lymphoid tissue. Lymphoid tissue or organs in immediate contact with the mucous-secreting mucosal layer in nasal cavity and nasopharynx (nasal-associated lymphoid tissue), airways (bronchus-associated lymphoid tissue), and intestinal tract (gut-associated lymphoid tissue). Serves as the immunological defence at secretory surfaces, to some extent independent of the systemic (internal) response; includes IgA synthesis by plasma cells in the lamina propria and excretion into the lumen

Multiple myeloma. Tumour of plasma cells in bone marrow

Muramidase. See *Lysozyme*

Myeloblast. Immature precursor cell in the lineage of polymorphonuclear cells (granulocytes, mast cells)

Nasal-associated lymphoid tissue. Lymphoid organs or tissue located in the nasal cavity and nasopharynx, presumed to be a first location for presentation of antigens entering through the nose; contributes to the mucosa-associated lymphoid tissue

Natural antibody. Antibody in serum of individuals with no previous exposure to the corresponding antigen; often generated by contact with cross-reacting agents, e.g. bacterial products; often restricted to antibodies that react to xenogeneic antigens

Natural killer cell. Leukocyte with a limited repertoire to recognize antigen; can kill target cells without prior sensitization; can be of lymphoid or monocyte–macrophage origin. Large granular lymphocytes are the main population

Necrosis. Death of tissue and cells

Neoantigen. New antigen appearing on cells or tissue during malignant transformation or (viral) infection

Neoplasia. Uncontrolled malignant transformation of cells resulting in tumour formation

Neutralization. Process whereby a pathogenic substance becomes inactivated by effector components (antibodies) of the immunological reaction

Neutropenia. Reduced proportions of neutrophilic granulocytes in blood

Neutrophil chemotactic factor. Preformed mediator with a relative molecular mass > 750 kDa, present in granules of mast cells and basophilic granulocytes; released after activation and attracts neutrophilic granulocytes to the site of inflammation or hypersensitivity

Neutrophilic granulocyte. Polymorphonuclear leukocyte that contains granules stained by neither acidophilic nor basophilic dyes; can phagocytose immune complexes by receptor-mediated endocytosis, followed by intracellular degradation in lysosomes. Degranulation releases catepsins and lysosomal enzymes, resulting in tissue damage.

Nonspecific immunity. Immunity induced by non-immunological mechanisms, for instance by action of complement, lysozyme, phagocytosis, or interferon

Oedema. Swelling of tissue due to extravasation of fluid from the intravascular space following increase in vascular permeability

Ontogeny. Life cycle of an organism; in immunological terms, often used to describe the process whereby the immune system develops immunocompetence

Opsonization. Adherence of pathogen to phagocytic cell due to action of antibody or activated complement

Osteoclast. Macrophage in bony tissue involved in bone resorption

Paracortex. Area in the inner cortex of lymph node where T lymphocytes are lodged; recognized by the presence of high endothelial venules; continuous with interfollicular areas in the outer cortex on one side and with the medullary cords on the other

Paratope (antigen-binding site). Part of antibody that binds antigen (antigenic determinant, or epitope); part of T-cell receptor that binds the complex of antigen and MHC molecule

Periarteriolar lymphocyte sheath. Area in white pulp of spleen surrounding the central artery, populated mainly by small T lymphocytes

Peripheral (secondary) lymphoid organ. Lymphoid organ in which immunocompetent lymphocytes recognize antigen, subsequently initiate immunological reactions, and produce effector elements of these reactions

Peyer's patch. Lymphoid tissue in wall of small intestine, particularly ileum, separated from the gut lumen by a domed area and an epithelial layer ('dome' epithelium); forms part of gut-associated lymphoid tissue; main function is initiation of immunological reactions towards pathogens entering through dome epithelium

Phagocytosis. Uptake of material > 1 µm by cells, by receptor-mediated endocytosis, by cells of the mononuclear phagocytic system; requires Fc receptors, with accessory help of complement receptors; is blocked by cytochalasins. Occurs via a 'zipper' mechanism, in which the opsonized particle (coated with antibody or complement) becomes enclosed by the cell membrane of the phagocyte; a second mechanism

involves oxidative burst, with formation of superoxide anion, peroxide anion, and hydroxyl radicals, which kill or degrade the phagocytosed particle

Phagolysosome. Membrane-bound cytoplasmic vesicle formed by fusion of a phagosome and a lysosome

Phagosome. Membrane-bound vesicle in phagocytic cells containing phagocytosed material

Pharmacokinetics. Fate of drugs or chemicals in the body over time, including the processes of absorption and distribution in tissues, biotransformation, and excretion

Phenotype. Characteristic of a distinct cell or individual, reflecting the expression of a genetically determined property

Phenotypic marker. Expressed characteristic(s), for instance an antigenic determinant, of a given cell or molecule, associated with function or specificity

Phylogeny. Evolutionary history of a particular species

Pinocytosis. Uptake of material < 1 μm by cells; often restricted to a receptor-mediated process in leukocytes of the monocyte–macrophage series, e.g. uptake of lipoproteins and viruses into clathrin-coated vesicles

Plasma. Fluid of uncoagulated blood after removal of cells

Plasma cell. Terminally differentiated B lymphocyte that synthesizes and secretes immunoglobulin; these medium-sized, 10–15-μm cells have a small excentric nucleus, with heterochromatin organized in a 'cartwheel'-like structure, and abundant cytoplasm filled with rough endoplasmic reticulum.

Platelet. Small bone marrow-derived cytoplasmic fragment in blood responsible for coagulation; main role is to block damaged vessel walls and prevent haemorrhage, by clumping and aggregation; contains heparin and serotonin, which contribute after release to the acute vascular response in hypersensitivity reactions and produce oxygen radicals

Platelet-activating factor. Low-relative-molecular-mass phospholipid generated from alkyl phospholipids in mast cells, basophilic and neutrophilic granulocytes, and monocytes–macrophages, which

mediates microthrombus formation of platelets in hypersensitivity reactions

Polyclonal. Derived from many different clones; for T and B lymphocytes, cell populations in which the cells have different V–D–J gene rearrangement products. Polyclonal activation is stimulation of multiple lymphocyte clones, resulting in a heterogeneous response

Polymorphonuclear granulocyte. Leukocyte of bone-marrow origin, with a lobulated nucleus, involved in acute inflammatory reactions. Main subsets are basophilic, eosinophilic, and neutrophilic granulocytes (different cytoplasmic granule colours after haematological staining). Contributes to (acute) inflammatory reactions after attraction by specific (immune complex-mediated) or nonspecific stimuli (including complement components); after activation, releases granules containing various hydrolytic enzymes

Postcapillary venule. Small blood vessel though which blood flows after leaving the capillaries before reaching veins; often the site where inflammatory cells leave the circulation and enter the tissue

Primary (central) lymphoid organ. Lymphoid organ where precursor lymphocytes differentiate and proliferate in close contact with the microenvironment to form immunocompetent cells; not antigen-driven but can be influenced by mediators produced as a result of antigen stimulation

Primary follicle. See *Follicle*

Primary response. Immunological reaction after first contact with antigen, resulting in generation of immunological memory

Programmed cell death. See *Apoptosis*

Prostaglandin. Aliphatic acid produced by arachidonic acid metabolism following the cyclo-oxygenase pathway; synthesized by mast cells after activation; mediates immediate hypersensitivity reactions, mainly smooth muscle contraction or bronchoconstriction; also decreases the threshold for pain

Prothymocyte. Precursor of T lymphocytes in bone marrow before moving to the thymus, or present in the thymus just before intrathymic processing

Pyrogen. Substance that increases the temperature in the central nervous system, resulting in fever; examples are bacterial endotoxin and IL-1

Reactive oxygen intermediate. Reactive species of oxygen produced e.g. by phagocytes (granulocytes and monocyte–macrophages) in response to phagocytic stimuli like bacteria

Reagin. Former designation of IgE class antibody

Recall antigen. Antigen used to elicit a response from an individual already sensitized to that antigen; may be one that the host has knowingly been sensitized to or, in humans, one that it is assumed that most individuals have been sensitized to

Red pulp. Area in spleen comprising venous sinuses filled with blood and splenic cords; venous sinuses mainly contain erythrocytes surrounded by endothelial cells; cords comprise macrophages, lymphocytes, and occasionally megakaryocytes, but other types of blood cells can also be present. Main function is phagocytosis of particulate material and removal of old erythrocytes from blood. In rodents, the red pulp can also be a site of haematopoiesis.

Reticuloendothelial system. See *Mononuclear phagocytic system*

Repertoire. All specific antigen-recognizing capacities (diversity) within a population of T or B lymphocytes

RT-1. The major histocompatibility complex of rats

Secretory immunoglobulin. Immunoglobulin encountered in secretions like tears, saliva, and jejunal juice; concerns mainly secretory IgA, a dimer of the basic four-chain immunoglobulin structure, linked by a J chain and surrounded by a secretory piece molecule.

Secretory piece. A 70-kDa molecule produced by epithelial cells covering mucosa-associated lymphoid tissue; functions as a receptor for IgA or IgM, thereby facilitating intercellular transport of these molecules into the lumen. During this process, the secretory piece becomes associated with the immunoglobulin, thereby enhancing its stability in nonphysiological conditions of secretory fluid

Secondary follicle. See *Follicle*

Secondary (peripheral) lymphoid organ. Lymphoid organ in which immunocompetent lymphocytes recognize antigen, subsequently initiate immunological reactions, and produce effector elements of those reactions

Secondary response. Response after first contact (immunization, primary response) with an antigen, based on the presence of

immunological memory; characterized as faster, more intense, and of higher affinity; for the antibody response, associated with an immunoglobulin class switch

Selectin. Cell surface glycoprotein that has a prominent function in the interaction between lymphocytes, monocytes, neutrophilic granulocytes, and endothelium. They share an N-terminal domain of approximately 120 amino acids that is homologous to many Ca^{2+}-dependent animal lectins and binds to carbohydrates. Examples are L-selectin (MEL-14, LAM-1, present on leukocytes; adherence of endothelial cells, role in lymphocyte recirculation and neutrophil and leukocyte inflammation); E-selectin (ELAM-1, present on endothelium; adherence of monocytes, neutrophils, and T cells; role in inflammation); P-selectin (PADGEM, GMP-140, CD62, present on platelets and endothelium; adherence of monocytes, neutrophils, and T cells; role in inflammation).

Self-MHC restriction. See *MHC restriction*; applies to MHC molecules of the individual

Sensitization. Induction of specialized immunological memory in an individual by exposure to antigen

Serotonin. 5-Hydroxytryptamine; catecholamine with relative molecular mass of 176 Da; preformed mediator of immediate hypersensitivity reactions, present in granules of mast cells and in platelets. After activation, is released and mediates vasodilatation and increased vascular permeability

Serum. Fluid of blood after coagulation (removal of fibrinogen) and removal of cells

Serum sickness. Systemic vasculitis, glomerulonephritis, or arthritis due to immune complex formation after the reaction between antibody and injected foreign antigen (serum)

Skin-associated lymphoid tissue. See *Skin immune system*

Skin immune system. Combination of immune system components and their function, present in skin; antigen presentation by Langerhans cells, by dendritic epidermal cells, and in some conditions by keratinocytes; immunoregulation by e.g. keratinocyte-derived cytokines, and distinct dermatotropic T-cell populations

Slow-reacting substance of anaphylaxis. See *Leukotriene*

Somatic mutation. Small changes in genes resulting in alterations in amino acids built into protein chains. For immunoglobulin molecules, changes in diversity of antigen-binding site (variable region)

Spleen. Lymphoid organ in the left abdominal cavity, for filtering blood; main function is phagocytosis of particles from blood, removal of old erythrocytes in red pulp, and initiation of immunological reactions in white pulp. The marginal zone of the white pulp serves as the main site of T cell-independent antibody formation.

Starry-sky macrophage. See *Tingible body macrophage*

Stem cell. Multipotential, self-renewing precursor cell of all haematopoietic cell lineages, present in bone marrow

Stem-cell growth factor (synonym for *c-Kit ligand*). An interleukin that supports continuous growth of mast cells and augments the response of progenitor cells to stem growth factors; interacts via *c-kit* proto-oncogene

Subcapsular sinus. Area in lymph node just under the capsule and surrounding the cortex, which is connected with afferent lymphatics, and through cortical (peritrabecular) sinuses with medullary sinuses; contains dendritic macrophages

Superantigen. Antigenic moiety that, in MHC-restricted presentation to T lymphocytes, is not present in the 'groove' made by the quaternary structure of the MHC molecule but is complexed with the MHC molecule. Examples are the endogenous viral-encoded Mlsa (minor lymphocyte stimulatory) antigen, which is present in certain mouse strains, and *Staphylococcus* enterotoxin A

Suppressor T cell. Subpopulation of T lymphocytes with CD8 phenotype; after recognition of antigen in an MHC class I-restricted manner, suppresses immunological reactions, in part by cytotoxic activity

Systemic lupus erythematosus. Chronic, remitting, relapsing, inflammatory, and often febrile multisystemic disorder of connective tissues, with possible involvement of the central nervous system, skin, joints, kidneys, and serosal membranes; can be acute or insidious in onset. The etiology is unknown but is thought to follow a failure of the regulatory mechanisms of the immune system that sustain self-tolerance. Many drugs and chemicals can induce lupus-like symptoms (drug-induced lupus erythematosus)

T-Cell receptor. Heterodimeric molecule on the surface of the T lymphocyte that recognizes antigen. The polypeptide chains have a variable and a constant part, and can be α, β, γ, or δ. The α–β T-cell receptor occurs on most T cells and recognizes antigenic peptides in combination with the polymorphic determinant of MHC molecules (self-MHC restricted). The γ–δ T-cell receptor occurs on a small subpopulation, e.g. in mucosal epithelium, and can recognize antigen in a non-MHC restricted manner. The T-cell receptor occurs exclusively with the CD3 molecule, which is thought to mediate transmembrane signalling.

T-Dependent antigen. Antigen for which antibody formation requires T cells.

Terminal pathway of complement activation. Activation of complement components C6–C9, with formation of the membrane attack complex and subsequent lysis of the cell

Tingible body macrophage (starry-sky macrophage). Large macrophage in cortex of thymus and germinal centres of follicles in lymphoid tissue, filled with condensed nuclear material with high affinity for dyes; has a major function in phagocytosis, presumably of apoptotic cells

T Lymphocyte or cell. Lymphocyte that induces, regulates, and effects specific immunological reactions after stimulation by antigen, mostly in the form of processed antigen complexed with MHC product on an antigen-presenting cell. They originate from precursors in the bone marrow and undergo maturation in the thymus (T, thymus-dependent). Most T lymphocytes recognize antigen by a heterodimeric α–β surface receptor molecule associated with the CD3 molecule, which mediates transmembrane signalling. Subsets include helper–inducer and suppressor–cytotoxic cells.

T-Lymphocyte area. That part of a lymphoid organ or tissue that is occupied by T lymphocytes, e.g. paracortex or interfollicular area in lymph node, periarteriolar lymphocyte sheath in spleen

Thrombocyte. See *Platelet*

Thrombocytopenia. Reduced proportion of platelets in blood

Thromboxane. Product of arachidonic acid following the cyclo-oxygenase pathway; synthesized by mast cells after activation and mediates immediate hypersensitivity reactions, mainly smooth muscle contraction, bronchoconstriction, and platelet aggregation

Thymocyte. Lymphocyte in the thymus

Thymoma. Tumour of the thymus; neoplastic cell is an epithelial cell

Thymus. Central lymphoid organ located dorsal to the cranial part of the sternum in the thorax, comprising two lobes, each consisting of many lobules. Its main function is generation of immunocompetent T lymphocytes from prothymocytes from the bone marrow

Tolerance. State of unresponsiveness to antigenic stimulation, due to the absence of responding elements or loss of capacity of existing elements to mount a reaction. Synonym for anergy

Tolerogen. Antigen that evokes tolerance

Tonsil. Organized mucosa-associated lymphoid tissue in oronasopharynx. Adenoids *strictu sensu* are also tonsils. The main function is initiation of immunological reactions towards pathogens entering through the mouth. Contributes in part to the gut-associated lymphoid tissue. Together with lymphoid aggregates in oronasopharynx, these tissues form the ring of Waldeyer

Transforming growth factor β. Mediator synthesized by lymphocytes or macrophages, with a function in down-regulation of immune reactions; suppresses T- and B-lymphocyte growth, IgM and IgG production, and down-regulates MHC class II expression; interferes with production of tumour necrosis factor and adhesion of granulocytes to endothelial cells; is chemotactic for monocytes and induces interleukin-1 and interleukin-6 expression

Transudation. Transfer of fluid and low-relative-molecular-mass proteins from intravascular to extravascular tissue during inflammatory processes

Tryptase. Proteolytic enzyme present in granules of mast cells; released after activation and activates complement component C3, with formation of the anaphylatoxin C3a

Tumour necrosis factor. General mediator of inflammation and septic shock; formerly named cachectin and lymphotoxin. Two forms: α and β, both produced by monocytes–macrophages, TNF-β also by T lymphocytes and natural killer cells. Has activity similar to interleukin-1 and acts synergistically with it; promotes antiviral state and is cytotoxic for tumour cells; stimulates granulocytes and eosinophils, activates macrophages to interleukin-1 synthesis, stimulates B cells to

proliferate and differentiate, T cells to proliferate, interleukin-2 receptor synthesis, and interferon γ synthesis; induces fibroblasts to synthesize prostaglandin and proliferate; induces fever and synthesis of acute-phase proteins; reduces cytochrome P450 synthesis; activates endothelium and promotes adherence of neutrophilic granulocytes to endothelium; induces cell adhesion molecules like lymphocyte function-associated antigens LFA-1 and LFA-3, ICAM-1, and ELAM-1; inhibits gastric motility *in vitro*; reduces lipoprotein lipase synthesis by adipocytes; and activates osteoclasts to bone resorption

Urticaria. Transient eruption of skin characterized by erythematous or oedematous swelling (wheal) of the dermis or subcutaneous tissue

Vaccination. See *Immunization*

Valency. Number of antigenic determinants or ligands that can bind to one antibody molecule or receptor

V (variable) gene. Gene that encodes the variable part of immunoglobulin or T-cell receptor chains (e.g. V_{H1}–V_{Hn} for immunoglobulin heavy chains, $V_{\kappa 1}$–$V_{\kappa n}$ for immunoglobulin κ light chain, $V_{\alpha 1}$–$V_{\alpha n}$ for T-cell receptor α chain)

Variable gene family. Groups of germline V genes (which encode immunoglobulin chains or T-cell receptor genes) that have more than about 80% nucleotide sequence identity

V (variable) region. Region at the amino terminal of immunoglobulin or T-cell receptor chains, which contributes to the antigen-binding site of the molecule. Encoded by V (variable), D (diversity), and J (joining) genes in DNA

Vasoconstriction. Contraction of capillary venules, resulting in decreased blood flow

Vasodilatation. Dilatation of capillary venules, resulting in increased blood flow through capillaries and lowering of local blood pressure

Veiled macrophage. Leukocyte belonging to the monocyte–macrophage lineage, present in lymph; has a major function in uptake and processing of antigen, followed by presentation (MHC class II-restricted) to helper–inducer T lymphocytes. Cytoplasm contains characteristic rod-like structures, Birbeck granules. Its equivalent in lymphoid tissue is the interdigitating dendritic cell, and that in skin is the Langerhans cell

Waldeyer's ring. Lymphoid tissue of tonsils and adenoids located around the junction of the pharynx and oral cavity in humans and domestic animals. Main function is initiation of immunological reactions towards pathogens entering through the mouth. Contributes to the gut-associated lymphoid tissue

White blood cell. Polymorphonuclear leukocyte, lymphocyte, or monocyte in peripheral blood

White pulp. Area in spleen around central arterioles where lymphoid cells reside. Comprises three major compartments: the periarteriolar lymphocyte sheath, follicles, and marginal zone

Xenobiotic. Chemical or substance that is foreign to the biological system

Xenogeneic. Genetically different phenotypes in individuals of different species; opposite of allogeneic, or isogeneic

ζ Chain (see *ε Chain*). One of the chains in the CD3 molecule associated with the T-cell receptor

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RESUME

1. Le système immunitaire a évolué de manière à contrer les atteintes que peuvent porter à l'intégrité du soi des microorganismes ou des cellules ayant échappé au contrôle de l'organisme. Une intrusion xénobiotique peut perturber le fonctionnement du système immunitaire et c'est la reconnaissance de cet état de choses qui a permis les progrès de ces vingt dernières années. Toute une méthodologie expérimentale a été développée et validée (essentiellement sur des rongeurs) par des études impliquant une multitude de laboratoires. La présente monographie examine les fonctions et l'histophysiologie du système immunitaire et présente les données nécessaires à la compréhension et à l'interprétation des modifications pathologiques provoquées par les agressions immmunotoxiques. L'accent est mis sur le système immunitaire de l'homme et des rongeurs, mais sans négliger d' autres espèces, les poissons en particulier, auxquelles des études immunotoxicologiques ont été consacrées. Il importe, pour comprendre l'impact des effets immunotoxiques, de connaître la pathophysiologie du système immunitaire, et notamment la sensibilité variable de ses constituants, les modifications subies par les organes lymphoïdes et la réversibilité de ces modifications.

2. L'immunosuppression et l'immunostimulation ont toutes deux des conséquences sur le plan clinique. On a établi une corrélation entre les états d'immunodéficience ou d'immunodépression grave que l'on observe par exemple chez des patients greffés ou sous traitement cytostatique, et l'accroissement de l'incidence des maladies infectieuses (opportunistes en particulier) et du cancer. L'exposition à des substances chimiques immunotoxiques présentes dans l'environnement peut toutefois donner lieu à des formes plus subtiles d'immunodépression, difficiles à déceler, qui entraînent une augmentation de l'incidence de maladies infectieuses comme la grippe ou le rhume. Des travaux effectués sur des animaux de laboratoire et sur l'homme montrent que de nombreux produits chimiques présents dans l'environnement dépriment la réponse immunitaire. Ces substances xénobiotiques immunotoxiques ne se limitent pas à untype particulier de composé chimique ou d'agent physique. Il y a, parmi les médicaments, les pesticides, les solvants, les hydrocarbures halogénés, les hydrocarbures aromatiques et les métaux, des substances susceptibles d'altérer le système immunitaire et le rayonnement ultra-violet a également cette propriété. L'administration, à des fins thérapeutiques, d'agents immunostimulants, peut avoir des effets nocifs et certaines substances (béryllium, silice, hexachlorobenzène) présentes dans l'environnement,

ont des propriétés immunostimulantes qui peuvent se traduire par des effets cliniques.

3. Du fait de sa complexité, le système immunitaire offre une multiplicité de cibles potentielles à l'action de ces agents avec toutes sortes de séquelles pathologiques. Les premières stratégies élaborées par les immunotoxicologues ont consisté à choisir et à mettre en oeuvre une batterie de tests multiphasiques sur animaux de laboratoire afin d'identifier les agents immunosuppresseurs ou immunostimulants. Ces batteries de tests peuvent varier selon l'organisme ou le laboratoire qui les mettent en oeuvre ou encore selon l'espèce animale utilisée, mais elles comportent toutes un ou plusieurs des éléments suivants : modification du poids ou de l'histologie des organes lymphoïdes ; examen des leucocytes du sang périphérique, des cellules du tissu lymphoïde ou de la moelle osseuse à la recherche de modifications ; intégrité de la fonction des cellules effectrices et régulatrices et modification éventuelle de la sensibilité à une exposition à des agents infectieux ou à des cellules tumorales.

La directive expérimentale originale No 407 de l'Organisation de coopération et de développement économiques, publiée en 1981, n'avait pas pour but la mise en évidence d'une immunotoxicité potentielle, aussi a-t-elle été modifiée pour la rendre plus adaptée à l'identification des substances immunotoxiques. Des systèmes d'épreuve multiphasiques permettant une investigation plus large de l'immunotoxicité ont été conçus par l'US National Toxicology Program, l'Institut Néerlandais de la Santé Publique et de la Protection de l'Environnement, l'US Environmental Protection Agency (Office of Pesticides), ainsi que par le Center for Food Safety andApplied Nutrition de l'US Food and Drug Administration.

Des études ont été effectuées sur des souris et, à un moindre degré, sur des rats afin d'évaluer la spécificité, la précision (reproductibilité), la sensibilité, l'exactitude et la pertinence, pour l'appréciation du risque sanitaire, de diverses mesures de l'état immunitaire. On a entrepris la validation interlaboratoires, au niveau international, de toutes ces méthodes, dans le cadre de l'Etude collective internationale sur l'immunotoxicité organisée par le PISC, l'Union européenne et le Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin. Des études analogues ont été menées sur des rats Fischer 344 à propos de la cyclosporine A.

4. Les épreuves utilisées dans le système multiphasique sont décrites à la section 3 avec les raisons qui ont guidé ce choix et un exposé des difficultés que l'on peut rencontrer dans leur mise en oeuvre. Bien que

conçus à l'origine pour des études sur des rats et des souris, certains de ces protocoles ont pu être utilisés avec fruit pour des travaux d'immunotoxicologie portant sur d'autres espèces, notamment des primates non humains, des mammifères marins, des chiens, des oiseaux et des poissons.

Lorsqu'on se propose de déterminer dans quelle mesure un agent environnemental ou un médicament est susceptible d'avoir une influence nocive sur le système immunitaire, il faut prendre en considération un certain nombre de facteurs. Il s'agit notamment du choix d'un modèle animal et de variables d'exposition appropriés, de la prise en compte des paramètres toxicologiques généraux, de la pertinence biologique des points d'aboutissement retenus, de la mesure de grandeurs dûment validées et de la mise en oeuvre d'un système d'assurance de la qualité. Les conditions expérimentales doivent tenir compte des modalités de l'exposition humaine (voie de pénétration et concentration ou intensité) et de toute information disponible d'ordre toxicodynamique ou toxicocinétique. Les doses et la taille des échantillons doivent être choisies de manière à permettre l'obtention de bonnes courbes dose-réponse et la détermination de la dose sans effet (nocif ou non) observable. Ces stratégies sont améliorées en permanence afin de permettre une meilleure prévision des situations susceptibles d'entraîner une pathologie. En outre, on devrait pouvoir disposer de techniques qui faciliteraient l'étude du mode d'action des agents en cause. Il pourrait s'agir de méthodes *in vitro*, de l'étude des réponses immunitaires locales (par exemple au niveau de la peau, des poumons et de l'intestin), de techniques de biologie moléculaire et de l'utilisation d'animaux génétiquement modifiés.

5. Mettre en évidence des modifications d'ordre immunologique après une exposition à des composés potentiellement immunotoxiques est plus complexe chez l'homme que chez l'animal de laboratoire. Les possibilités d'expérimentation sont limitées, il est difficile d'établir le niveau d'exposition à l'agent en cause (c'est-à-dire la dose) et de plus, l'état immunitaire des populations est extrêmement hétérogène. Cette hétérogénéité trouve son origine dans un certain nombre de facteurs: âge, sexe, race, gravidité, stress et aptitude à y faire face, pathologies et états infectieux cocomitants, état nutritionnel, tabagisme et prise de certains médicaments.

L'intérêt de telle ou telle étude pour l'évaluation du risque est conditionné par un élément important, à savoir le concept épidémiologique qui la sous-tend. La plupart du temps on procède à une étude transversale, qui consiste à déterminer l'exposition et la morbidité à un moment donné ou sur une courte période. On compare ensuite la

fonction immunitaire des sujets exposés à celle d'un groupe analogue de sujets non exposés. Ce genre d'étude peut receler un certain nombre de pièges.

Nombre des altérations que produit l'exposition à une substance chimique ne se manifestent chez l'homme que de manière subtile et sporadique, aussi faut-il étudier des populations récemment exposées et utiliser des épreuves sensibles.

La plupart des épreuves concernant l'immunité spécifique (à médiation cellulaire ou humorale), l'immunité non spécifique et les processus inflammatoires ont été conçues pour rechercher des anomalies chez des patients atteints d'une déficience immunitaire et ne sont pas forcément capables de déceler les modifications subtiles provoquées par les substances chimiques. Le PISC, les Centers for Disease Control et l'Académie des Sciences des Etats-Unis ont, chacun de leur côté, défini une méthodologie pour évaluer les modifications du système immunitaire quipourraient résulter d'une exposition à des produits immunotoxiques mais les épreuves proposées demandent encore à être validées.

6. L'évaluation du risque consiste à analyser les données pertinentes sur un agent donné (effets biologiques, relations dose-réponse et exposition) pour tenter d'obtenir une estimation qualitative et quantitative des diverses conséquences nocives de la présence de cet agent. Elle se caractérise par quatre phases principales : reconnaître le danger, établir la relation dose-réponse, évaluer l'exposition et caractériser le risque. Jusqu'ici, l'immunotoxicologie s'est essentiellement attachée à reconnaître le danger et, dans une certaine mesure, à établir des relations dose–réponse, mais très peu d'études ont été consacrées à l'évaluation du risque ou à sa caractérisation.

Comme dans d'autres domaines de la toxicologie, des incertitudes demeurent qui sont susceptibles de gêner l'interprétation des données immunotoxicologiques dans l'optique du risque pour la santé humaine. Les deux questions qui sont actuellement les plus problématiques—l'extrapolation à l'organe dans son ensemble des effets constatés sur la cellule et l'extrapolation à l'homme des données obtenues sur l'animal—valent pour la plupart des points d'aboutissement des effets, cancer excepté. Le premier problème tient aux incertitudes que comporte l'établissement d'une relation quatitative entre les modifications subies par la fonction immunitaire d'un individu et l'altération de sa résistance aux maladies infectieuses et néoplasiques. Le deuxième est lié à l'incertitude qui entache l'évaluation du risque pour la santé humaine à partir des résultats obtenus sur des animaux de laboratoire.

L'évaluation du risque a pour finalité de protéger la santé humaine et l'environnement. Il faut donc que le choix des modéles expérimentaux soit judicieux. La toxicocinétique de la substance étudiée et la réponse immunitaire suscitée dans le modèle doivent pouvoir être comparées à celles qu'on observerait chez l'homme.

Pour tirer une limite d'exposition des résultats expérimentaux, il est convenu d'appliquer un facteur d'incertitude aux données d'évaluation du risque. Cette convention ne tient pas compte de la réserve fonctionnelle ou de la redondance du système immunitaire. Une méthode plus récente d'évaluation du risque consiste à ultiliser des modèles *in vitro* en complément aux études sur animaux de laboratoire. Cette méthode a l'avantage de permettre une extrapolation plus fidèle de l'animal à l'homme et de ne nécessiter qu'un minimum d'animaux. Elle permet également de pallier l'absence de données dans les cas où des considérations d'éthique limitent l'expérimentation sur l'homme. Le Chapitre 6 donne deux exemples de situation où les données obtenues *in vitro* permettent de lever en partie les incertitudes dans l'évaluation du risque dû à l'exposition à l'ozone et au rayonnement ultra-violet. L'utilisation des données immunotoxicologiques dans l'évaluation du risque reste limitée par la difficulté d'établir des relations quantitatives entre l'immunodépression et les manifestations cliniques d'une pathologie donnée.

RESUMEN

1. El sistema inmunitario ha evolucionado para hacer frente a las amenazas a la integridad del organismo vivo provenientes de microorganismos o de células que han escapado a los mecanismos de control del organismo. El reconocimiento de que las sustancias xenobióticas pueden trastornar el funcionamiento del sistema inmunitario ha llevado a avances en el campo de la inmunotoxicología durante los dos últimos decenios. Se han formulado métodos experimentales (empleando principalmente especies de roedores), que han sido validados en estudios multilaboratorio. En esta monografía se examinan la función y la histofisiología del sistema inmunitario, presentándose la información necesaria para la comprensión e interpretación de los cambios patológicos causados por las agresiones inmunotóxicas. Si bien se hace hincapié en los sistemas inmunitarios del ser humano y de las especies de roedores, se hace referencia a otras especies, incluidos los peces, que han sido objeto de estudios inmunotoxicológicos. La fisiopatología del sistema inmunitario, incluidas la sensibilidad variable de sus componentes, las alteraciones de los órganos linfoides y la reversibilidad de los cambios, es importante para comprender las repercusiones de la inmunotoxicidad.

2. Tanto la inmunosupresión como la inmunoestimulación tienen consecuencias clínicas. Se ha observado que los estados de inmunodeficiencia y de inmunosupresión grave, como los que se pueden presentar en casos de trasplante y de terapia citostática, van acompañados de mayor incidencia de enfermedades infecciosas (especialmente las oportunistas) y de cáncer. Con todo, cabe prever que la exposición a los productos químicos inmunotóxicos enel medio ambiente dará origen a formas más sutiles de inmunosupresión cuya detección podría resultar difícil, lo que se traduciría en una mayor incidencia de infecciones tales como la gripe y el resfriado común. Estudios realizados con animales de laboratorio y seres humanos han mostrado que muchos de los productos químicos presentes en el medio ambiente provocan la supresión de la respuesta inmunitaria. Las sustancias xenobióticas inmunotóxicas no se limitan a una clase determinada de productos químicos. Entre los compuestos que tienen efectos nocivos para el sistema inmunitario se cuentan fármacos, plaguicidas, disolventes, hidrocarburos halogenados y aromáticos y metales; la radiación ultravioleta puede resultar también inmunotóxica. La administración terapéutica de agentes inmunoestimulantes puede provocar reacciones adversas; asimismo, algunos de los productos químicos presentes en el medio ambiente que poseen propiedades

inmunoestimulantes (berilio, sílice, hexaclorobenceno) pueden tener consecuencias clínicas.

3. La complejidad del sistema inmunitario lleva aparejadas multiplicidad de posibles puntos vulnerables y secuelas patológicas. Los métodos iniciales ideados por los inmunotoxicólogos que realizan investigaciones sobre toxicología y evaluación de la inocuidad consistían en seleccionar y aplicar una serie de valoraciones escalonadas para identificar los agentes inmunosupresores e inmunoestimulantes en animales de laboratorio. Si bien la configuración de esas series de análisis puede variar según la institución o el laboratorio en que se llevan a cabo, así como según las especies de animales empleadas, en todas se mide uno o más de los siguientes parámetros: alteraciones del peso y de la histología de los órganos linfoides; cambios en la celularidad del tejido linfoide, de los leucocitos en la sangre periférica y/o de la médula ósea; trastornos de la función celular a nivel de los efectores o de la regulación, y alteración de la sensibilidad a la amenaza que presentan los agentes infecciosos o las células tumorales.

La Directriz de pruebas inicial N° 407 de la Organización de Cooperación y Desarrollo Económicos, publicada en 1981, no preveía la detección de los riesgos de inmunotoxicidad, y se han propuesto modificaciones destinadas a aumentar la utilidad de esa Directriz para la identificación de las sustancias inmunotóxicas. El Programa Nacional de Toxicología de los Estados Unidos, el Instituto Nacional de Salud Pública y Protección del Medio Ambiente de los Países Bajos, la Oficina de Plaguicidas de la Agencia para la Protección del Medio Ambiente de los Estados Unidos y el Centro de Seguridad de los Alimentos y Nutrición Aplicada de la Administración de Alimentos y Medicamentos de los Estados Unidos han elaborado sistemas de pruebas escalonadas para la investigación en mayor escala de los riesgos de inmunotoxicidad.

Se han realizado estudios con ratones, y en menor medida con ratas, de diferentes indicadores del estado inmunológico con el fin de determinar su especificidad, precisión (reproducibilidad), sensibilidad, exactitud y pertinencia para la evaluación de los riesgos para la salud humana. Los métodos han sido objeto de validaciones internacionales interlaboratorios en el marco del Estudio Internacional en Colaboración sobre Inmunotoxicidad del IPCS, la Unión Europea y el *Bundesinstitut für Gesundheitlichen Verbraucherschutz und Veterinärmedizin*, y en estudios sobre la ciclosporina A en ratas Fisher 344.

4. Las pruebas empleadas en los programas de verificaciones escalonadas se describen en la Sección 3, en la que se indican la razón de ser de su selección y las complejidades que entraña su realización. Si

bien esos protocolos fueron diseñados para estudios realizados con ratas y ratones, algunos de ellos se han aplicado con buenos resultados al estudio de la inmunotoxicidad en otras especies animales, incluidos primates no humanos, mamíferos marinos, perros, aves y peces.

A la hora de evaluar las posibles repercusiones negativas de un agente ambiental o fármaco sobre el sistema inmunitario de los animales experimentales deberán considerarse diversos factores. Entre ellos cabe señalar: la selección de los modelos y las variables de exposición apropiados para los animales, la inclusión de parámetros toxicológicos generales, la comprensión de la importancia de los parámetros objeto de medición, el empleo de medidas validadas y el control de la calidad. En las condiciones experimentales deberán tenerse en cuenta las vías y el nivel posibles de exposición del ser humano, así como toda la información disponible sobre toxicodinámica y toxicocinética. Las dosis y el tamaño de las muestras deberán seleccionarse de manera que se puedan obtener curvas de dosis-respuesta bien definidas, además del nivel sin efectos adversos observados y del nivel sin efectos observados. Los métodos se perfeccionan continuamente para poder predecir mejor las condiciones que podrían ser causantes de enfermedades. Además, deberán elaborarse técnicas que contribuyan a la identificación de mecanismos de acción; éstos podrían incluir los métodos *in vitro*, el examen de las respuestas inmunitarias locales (por ejemplo, en la piel, los pulmones y los intestinos), y el empleo de las técnicas de la biología molecular y de animales modificados genéticamente.

5. La detección de los cambios inmunológicos ocurridos tras la exposición a compuestos que podrían ser inmunotóxicos resulta más complicada en el ser humano que en los animales de laboratorio. Las posibilidades de realizar pruebas son limitadas; los niveles de exposición al agente (es decir, la dosis) son difíciles de establecer y el estado inmunitario de las poblaciones es sumamente heterogéneo. La edad, la raza, el sexo, la gestación, el estrés agudo y la capacidad para hacerle frente, las enfermedades e infecciones coexistentes, el estado nutricional, el humo del tabaco y algunos medicamentos se cuentan entre los factores que contribuyen a esa heterogeneidad.

El diseño de los estudios epidemiológicos es un factor importante para determinar la utilidad de un estudio determinado para la evaluación de riesgos. El tipo de diseño empleado más frecuentemente en materia de inmunotoxicidad son los estudios transversales, en los que se miden las condiciones de exposición y el estado de la enfermedad en un momento dado, o durante un periodo breve. A continuación se compara la función inmunitaria de los sujetos «expuestos» con la de un grupo

comparable de individuos «no expuestos». Ese tipo de diseño lleva aparejados posibles escollos.

Como muchos de los cambios observados en la respuesta inmunitaria de los seres humanos tras su exposición a un productoquímico podrían ser esporádicos y sutiles, habrá que estudiar las poblaciones que han estado expuestas recientemente empleando pruebas de gran sensibilidad para la evaluación del sistema inmunitario. Las conclusiones sobre los efectos inmunotóxicos deberán estar basadas en las variaciones detectadas no en un parámetro aislado sino en el perfil inmunológico del individuo o de la población.

La mayoría de las pruebas existentes para determinar la inmunidad específica (de base celular y humoral), la inmunidad no específica y la inflamación han sido concebidas para detectar las alteraciones inmunitarias en pacientes que padecen de inmunodeficiencia y no siempre resultan adecuadas para detectar las alteraciones sutiles provocadas por los productos químicos presentes en el medio ambiente. El IPCS, los Centros de Control de Enfermedades y la Academia de Ciencias de los Estados Unidos han descrito procedimientos para evaluar los cambios que ocurren en el sistema inmunitario del ser humano como consecuencia de la exposición a sustancias inmunotóxicas; con todo, las pruebas descritas deberán ser evaluadas con este fin.

6. La evaluación de riesgos es un proceso en el que se analizan la información pertinente sobre los efectos biológicos, las relaciones dosis-respuesta y la exposición a un agente determinado con miras a establecer estimaciones cualitativas y cuantitativas de los resultados adversos. Por regla general, la evaluación de los riesgos supone cuatro pasos fundamentales: identificación de los riesgos; evaluación de la relación dosis-respuesta; evaluación de la exposición y caracterización de los riesgos. Hasta ahora, lainmunotoxicología se ha centrado principalmente en la identificación de los riesgos y, en cierta medida, en la evaluación de las relaciones dosis-respuesta; muy contados han sido los estudios que han incluido la evaluación de la exposición o la caracterización de los riesgos.

Al igual que ocurre en otros campos de la toxicología, existen incertidumbres que podrían afectar a la interpretación de los datos sobre inmunotoxicidad en cuanto a los riesgos para la salud humana. Las dos cuestiones más problemáticas - la extrapolación de los efectos observados en células individuales a todo un órgano, o a niveles superiores, y la extrapolación al ser humano de los resultados obtenidos en experimentos con animales - son comunes a la mayoría de los parámetros de valoración no relacionados con el cáncer. La primera obedece a las incertidumbres vinculadas al establecimiento de una

relación cuantitativa entre los cambios observados en la función inmunitaria del individuo y la perturbación de la resistencia a las infecciones y enfermedades neoplásicas. La segunda cuestión es consecuencia de las incertidumbres que lleva aparejadas la evaluación de los riesgos para la salud humana basándose en los estudios realizados con animales de laboratorio.

El objetivo fundamental de la evaluación de los riesgos es la protección de la salud de los seres humanos y del medio ambiente. Por lo tanto, deberán seleccionarse sistemas modelo idóneos. La toxicocinética del material de prueba y la índole y magnitud de la respuesta inmunitaria generada en el modelo deberán ser comparables a la de los seres humanos.

Habitualmente, en la evaluación de los riesgos se emplean factores empíricos de incertidumbre para determinar el límite de exposición aceptable a partir de los resultados experimentales. Ese procedimiento no toma en cuenta la reserva funcional ni la redundancia del sistema inmunitario. Un adelanto más reciente en materia de evaluación de riesgos es el empleo de modelos *in vitro* como complemento de los estudios realizados con animales de laboratorio. Ese procedimiento tiene la ventaja de que permite aumentar la exactitud de la extrapolación al ser humano de los resultados obtenidos en los experimentos realizados con animales, reduciendo al mínimo el número de animales necesarios; asimismo, permite colmar la brecha entre ambos tipos de información, sobre todo en los casos en que los experimentos con seres humanos se ven limitados por consideraciones de índole ética. En el capítulo 6 se presentan dos ejemplos de cómo la información *in vitro* permite reducir las incertidumbres en materia de evaluación de riesgos relacionadas con la exposición al ozono y a la radiación ultravioleta. La dificultad para establecer relaciones cuantitativas entre la inmunosupresión y las enfermedades clínicas ha limitado el empleo de los datos inmunotoxicológicos en la evaluación de los riesgos.

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