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IPCS Harmonization Project

Guidance for Immunotoxicity Risk Assessment for Chemicals

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

World Health Organization

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization.

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GUIDANCE FOR IMMUNOTOXICITY RISK ASSESSMENT FOR CHEMICALS

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FOREWORD

Harmonization Project Documents are a family of publications by the World Health Organization (WHO)/International Programme on Chemical Safety (IPCS). Harmonization Project Documents complement the Environmental Health Criteria (EHC) methodology (yellow cover) series of documents as authoritative documents on methods for the risk assessment of chemicals.

The main impetus for the current coordinated international, regional and national efforts on the assessment and management of hazardous chemicals arose from the 1992 United Nations Conference on Environment and Development (UNCED). UNCED Agenda 21, Chapter 19, provides the "blueprint" for the environmentally sound management of toxic chemicals. This commitment by governments was reconfirmed at the 2002 World Summit on Sustainable Development and in 2006 in the Strategic Approach to International Chemicals Management (SAICM). The IPCS project on the Harmonization of Approaches to the Assessment of Risk from Exposure to Chemicals (Harmonization Project) is conducted under Agenda 21, Chapter 19, and contributes to the implementation of SAICM. In particular, the project addresses the SAICM objective on Risk Reduction and the SAICM Global Plan of Action activity to "Develop and use new and harmonized methods for risk assessment".

The IPCS Harmonization Project goal is *to improve chemical risk assessment globally, through the pursuit of common principles and approaches, and, hence, strengthen national and international management practices that deliver better protection of human health and the environment within the framework of sustainability.* The Harmonization Project aims to harmonize global approaches to chemical risk assessment, including by developing international guidance documents on specific issues. The guidance is intended for adoption and use in countries and by international bodies in the performance of chemical risk assessments. The guidance is developed by engaging experts worldwide. The project has been implemented using a stepwise approach, first sharing information and increasing understanding of methods and practices used by various countries, identifying areas where convergence of different approaches would be beneficial and then developing guidance that enables implementation of harmonized approaches. The project uses a building block approach, focusing at any one time on the aspects of risk assessment that are particularly important for harmonization.

The project enables risk assessments (or components thereof) to be performed using internationally accepted methods, and these assessments can then be shared to avoid duplication and optimize use of valuable resources for risk management. It also promotes sound science as a basis for risk management decisions, promotes transparency in risk assessment and reduces unnecessary testing of chemicals. Advances in scientific knowledge can be translated into new harmonized methods.

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This Harmonization Project Document was planned at a World Health Organization (WHO)/ International Programme on Chemical Safety (IPCS) Scoping Meeting and prepared by the WHO/IPCS Drafting Group on Immunotoxicity, both under the chairmanship of Professor Henk Van Loveren. Additional experts contributed through the preparation of case-studies and provision of comments during the public review period and at an International Workshop. Meetings associated with document development were hosted by the WHO Collaborating Centre for Immunotoxicology and Allergic Hypersensitivity at the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.

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LIST OF ACRONYMS AND ABBREVIATIONS

AA	adjuvant arthritis
ADI	acceptable daily intake
ADME	absorption, distribution, metabolism and elimination
AEL	acceptable exposure level
AFA	antifibrillarin autoantibody
AIDS	acquired immunodeficiency syndrome
AO Mix	antioxidant mix of 0.3% butylated hydroxytoluene/tocopherol/eugenol
ARfD	acute reference dose
AUC	area under the concentration versus time curve
BLL	blood lead level
BMC	benchmark concentration
BMD	benchmark dose
BrdU	5-bromo-2′-deoxyuridine
$BW^{3/4}$	body weight raised to the ¾ power
CAS	Chemical Abstracts Service
CDC	Centers for Disease Control and Prevention (USA)
CEL	consumer exposure level
CET	closed epicutaneous test
CI	confidence interval
CMV	cytomegalovirus
CSAF	chemical-specific adjustment factor
CSF	colony stimulating factor (e.g. CSF-1)
CTL	cytotoxic T lymphocyte
CYP	cytochrome P450
DCAC	dichloroacetyl chloride
DDE	dichlorodiphenyldichloroethylene
DDT	dichlorodiphenyltrichloroethane
DEP	diethyl phthalate
DEREK	Deductive Estimation of Risk from Existing Knowledge
DES	diethylstilbestrol
DNA	deoxyribonucleic acid
DNEL	derived no-effect level
DTH	delayed-type hypersensitivity
EAE	experimental allergic encephalomyelitis
EBV	Epstein-Barr virus
EC_3	effective concentration of a chemical required to produce a 3-fold increase in proliferation of lymph node cells
EHC	Environmental Health Criteria
ELISA	enzyme-linked immunosorbent assay
EtOH	ethanol
EU	European Union
FCA	Freund's complete adjuvant
FCAT	Freund's complete adjuvant test
GD	gestational day
GM-CSF	granulocyte macrophage colony stimulating factor
GPMT	guinea-pig maximization test
GRAS	Generally Recognized as Safe

HCB	hexachlorobenzene
HgIA	mercury-induced autoimmune disease
HHV	human herpes virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMT	human maximization test
HRIPT	human repeated insult patch test
HSV	herpes simplex virus
IC_{50}	median inhibitory concentration
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IFN	interferon (e.g. IFN-α, IFN-γ)
IFRA	International Fragrance Association
Ig	immunoglobulin (e.g. IgA, IgE, IgG, IgM)
IL	interleukin (e.g. IL-4, IL-6, IL-10, IL-12)
IPCS	International Programme on Chemical Safety
KLH	keyhole limpet haemocyanin
K_{ow}	octanol/water partition coefficient
LD_{50}	median lethal dose
LLNA	local lymph node assay
LOAEL	lowest-observed-adverse-effect level
LOEL	lowest-observed-effect level
LPS	lipopolysaccharide
MDI	diphenylmethane diisocyanate
MET	minimum elicitation threshold
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
MOA	mode of action
mRNA	messenger ribonucleic acid
ND	not determined
NESIL	no expected sensitization induction level
NK	natural killer
NOAEL	no-observed-adverse-effect level
NOD	non-obese diabetic
NOEC	no-observed-effect concentration
NOEL	no-observed-effect level
NR	not relevant
NTP	National Toxicology Program (USA)
OECD	Organisation for Economic Co-operation and Development
OET	open epicutaneous test
OPPTS	Office of Prevention, Pesticides, and Toxic Substances (USEPA)
OR	odds ratio
PBB	polychlorinated biphenyl
PBPK	physiologically based pharmacokinetic
PBTK	physiologically based toxicokinetic
PCB	polychlorinated biphenyl
PEG	polyethylene glycol
PFC	plaque-forming cell
PHA	phytohaemagglutinin
PLNA	popliteal lymph node assay

PMNL	polymorphonuclear leukocyte
PND	postnatal day
POD	point of departure
POP	persistent organic pollutant
PWM	pokeweed mitogen
QRA	quantitative risk assessment
QSAR	quantitative structure–activity relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemical Substances
RfC	reference concentration
RfD	reference dose
RIFM	Research Institute for Fragrance Materials
RIVM	National Institute for Public Health and the Environment (the Netherlands)
RNA	ribonucleic acid
ROAT	repeated open application test
RR	relative risk
SAF	sensitization assessment factor
SAR	structure–activity relationship
SCCNFP	Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers
SD	standard deviation
SE	standard error
SI	stimulation index
SIAT	single injection adjuvant test
SPT	skin prick test
SRBC	sheep red blood cell
TCA	trichloroacetic acid
TCAH	trichloroacetaldehyde hydrate
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCE	trichloroethylene
TDI	tolerable daily intake
TGF	transforming growth factor (e.g. TGF-β)
Th	T helper cell (e.g. Th1, Th2)
TNF	tumour necrosis factor (e.g. TNF-α)
TNP	trinitrophenyl
Toc	α-tocopherol
TrlC	Trolox C
TTC	threshold of toxicological concern
TWA	time-weighted average
U	unit
USA	United States of America
USEPA	United States Environmental Protection Agency
USFDA	United States Food and Drug Administration
VNR	vehicle not reported
WBC	white blood cell
WHO	World Health Organization

EXECUTIVE SUMMARY

A well-functioning immune system is essential in maintaining the integrity of the organism, and malfunction may have severe health consequences. These consequences range from reduced resistance to infection and neoplasia to allergic and autoimmune conditions. Chemicals may act directly on components of the immune system, leading to immunosuppression and resulting in reduced resistance to infections and tumours. Direct toxicity may also lead to dysregulation of homeostasis, resulting in exaggerated immune responses, which may facilitate allergic or autoimmune phenomena. Alternatively, chemicals may be recognized by the immune system as foreign or alter host tissues in such a way that they are recognized as foreign, resulting in allergy or autoimmunity, respectively. Whereas infectious and neoplastic diseases remain a significant burden on public health, allergic and autoimmune diseases have risen over the last decades. The role of chemical exposures in changes to these immune-related health outcomes over time remains an open question. A proper risk assessment of chemicals in terms of immunotoxicity is therefore warranted.

Immunotoxicity is a non-cancer end-point comparable to other types of potential systemic toxicity considered in a general risk assessment. As with other types of systemic toxicity, assessment of immunotoxicological risk relies on a variety of end-points that reflect immune system health and, in aggregate, are predictive of disease states. In general, the differences in the approaches to assess the risk of immunotoxicity and other forms of toxicity are minimal, and apparent differences likely stem from a lack of familiarity with immune system end-points that link cellular toxicity with downstream disease outcomes.

As is the case with the reproductive and central nervous systems, the immune system is particularly vulnerable to chemical exposure during development, and function declines with age, resulting in increased risk of adverse health outcomes from chemical exposure at the extremes of age. A special case does exist for dose–response relationships related to allergy, because the dose required to induce allergic hypersensitivity in a naive individual is typically higher than the dose required to elicit symptoms in sensitized individuals. As such, dose–response relationships for allergic hypersensitivity are typically more complex than for other immune system end-points.

The *Guidance for Immunotoxicity Risk Assessment for Chemicals* provides background information on the immune system and its perturbation by xenobiotics and clear step-by-step weight of evidence approaches to assess the risk for immunotoxicity from chemical exposures. The guidance is organized in separate chapters that represent different types of immunotoxicity that may follow exposure to chemicals. A table of entry points for the risk assessment process will help the risk assessor to identify the type(s) of immunotoxicity suggested by the data and decide which of the risk assessment schemes should be followed. Nevertheless, it is critical to realize that the same chemical or chemical class may exert different or sometimes partly overlapping types of immunotoxicity, and a comprehensive risk assessment demands that all types of immunotoxicity be addressed.

The ultimate goal of the risk assessment process is to integrate hazard identification, hazard characterization and exposure assessment into a plain language risk characterization aimed at estimating the likelihood that identified adverse effects will occur in exposed people and presenting immunotoxicity information and reference values that are useful to the risk manager. The assessment should include a critical review of the quality of the assessment,

including uncertainties and confidence in conclusions, which includes a weight of evidence approach.

Whereas in general terms human data may be preferable to animal data for risk assessment, both types of data have limitations. Epidemiological studies often lack precise information on exposure and may not control for important confounding variables. Laboratory animal data typically do not suffer the same shortcomings, but extrapolation to likely human effects may be problematic. For both approaches, dose–response relationships, biological plausibility and mode of action are critical aspects that need to be considered and from which uncertainty factors may be determined and used to arrive at reliable reference values.

Whereas several guidelines for immunotoxicity testing are in place, deficiencies are also evident that have so far inhibited full implementation of immunotoxicity risk evaluation. Guidelines for mouse or guinea-pig skin tests are available for identifying skin sensitizers, but no such guidelines exist for respiratory sensitizers. For direct immunotoxicity testing, guidelines for (sub)chronic or reproductive toxicity testing in rodents include a range of immune parameters. Guidance is available for assessing autoimmunity (IPCS, 2006a), although none of the animal models of autoimmunity have been validated for use in regulatory decision-making. For human testing of immunotoxicity, no specific gudelines are in place. Hence, the risk assessor is often faced with incomplete information on which the risk assessment needs to be based. Depending on how complete or incomplete the entry points for assessing the risk of immunotoxicity may be, the risk assessor may want to seek advice from someone with expert knowledge of immunotoxicity to aid in the interpretation of the data.

The internationally developed guidance presented in this document aims to improve immunotoxicity risk assessment of chemicals to reduce or prevent human exposure to immunotoxic concentrations of chemicals and thereby protect public health. The guidance also aims to facilitate harmonization of immunotoxicity risk assessment and to promote transparency, mutual understanding and sharing of the chemical risk assessments produced, to avoid duplication of effort. This guidance is intended for adoption and use by regulatory authorities and other risk assessment bodies, industry, research institutions and others involved in chemical risk assessment.

The guidance includes six case-studies of selected immunotoxic chemicals provided to illustrate how the risk assessment guidance can be used for assessing the risk of immunotoxicity.[1]

[1] The case-studies were developed for illustrative purposes only and are not to be interpreted as comprehensive risk assessments or final regulatory positions of any agency or government.

1. INTRODUCTION TO THE GUIDANCE DOCUMENT

1.1 Purpose of this document

This Harmonization Project Document provides guidance for immunotoxicity risk assessment for chemicals. Immunotoxicity can be defined as any adverse effect on the immune system that can result from exposure to a range of environmental agents, including chemicals. It encompasses studies of various immune pathologies, including allergy, immune dysregulation (suppression or enhancement), autoimmunity and chronic inflammation.

The document is intended for adoption and use by regulatory authorities and other risk assessment bodies, industry, research institutions and others involved in chemical risk assessment. The guidance has been prepared with the generalist risk assessor in mind, providing triggers for when specialist immunotoxicology advice might be needed.

A range of national and European requirements and guidelines on the subject of immunotoxicity risk assessment are in existence. A selection of those relating to the European Union (EU) and the United States of America (USA) are outlined in Annex 1. Use of the internationally developed guidance presented in this document aims to facilitate harmonization of immunotoxicity risk assessment. It promotes transparency, mutual understanding and sharing of the chemical risk assessments produced, to avoid duplication of effort. Finally, this document translates current scientific knowledge into guidance for the risk assessment process.

1.2 Scope

The present guidance document focuses on aspects of risk assessment specific to immunotoxicity. It is intended to complement guidance on the process of risk assessment in general.

Problem formulation precedes risk assessment and establishes the goals, scope and focus of the risk assessment, along with policy and regulatory considerations (Figure 1.1). The risk assessment process consists of four main steps: hazard identification, hazard characterization, exposure assessment and risk characterization. The term risk analysis is used to describe the overall procedure comprising all the steps of risk assessment, risk management and risk communication. Risk management can be described as the process of weighing policy alternatives, decision-making and action taking.

The reader is referred to the World Health Organization (WHO)/International Programme on Chemical Safety (IPCS) web site (http://www.who.int/ipcs/methods/en/) for guidance on risk assessment methodology. A selection of relevant documents is listed in Annex 2. Finally, the guidance is complementary to the classification criteria contained in the Globally Harmonized System of Classification and Labelling of Chemicals (http://www.unece.org/trans/danger/publi/ghs/ghs_welcome_e.html).

This guidance builds upon previous WHO/IPCS publications on the subject of immunotoxicity, including:

- Environmental Health Criteria 180: *Principles and methods for assessing direct immunotoxicity associated with exposure to chemicals* (http://www.inchem.org/documents/ehc/ehc/ehc180.htm);

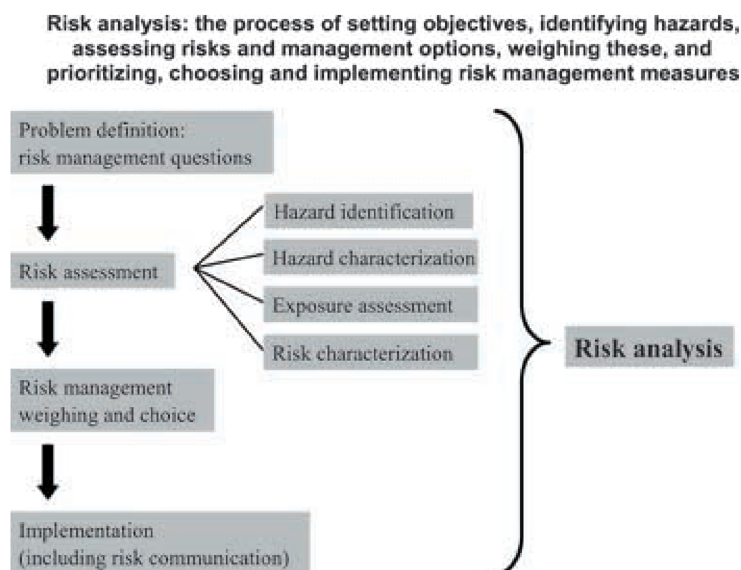

Figure 1.1: The various stages of risk assessment and risk analysis.

- Environmental Health Criteria 212: *Principles and methods for assessing allergic hypersensitization associated with exposure to chemicals* (http://www.inchem.org/documents/ehc/ehc/ehc212.htm);
- Environmental Health Criteria 236: *Principles and methods for assessing autoimmunity associated with chemicals* (http://www.who.int/entity/ipcs/publications/ehc/ehc236.pdf).

This guidance has been developed cognizant of the data available for risk assessment. Relevant Organisation for Economic Co-operation and Development (OECD) test guidelines[1] relating to laboratory animal data include those relating to skin sensitization (Test Guidelines 406 and 429), repeated dose/subchronic toxicity (e.g. Test Guidelines 407, 408, 409 and 413), chronic toxicity (Test Guideline 452) and reproductive toxicity, including developmental immunotoxicity (Test Guideline 443), as well as the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) tripartitite guideline on Immunotoxicity Studies for Human Pharmaceuticals (ICH S8).[2]

1.3 Contents

Chapter 2 outlines special features of the immune system and why it is a special case in risk assessment.

Chapter 3 presents a framework for immunotoxicity risk assessment for chemicals, comprising entry points that help to determine whether or not immunotoxicity needs to be considered and what type of immunotoxicity needs to be evaluated. Chapter 3 also provides information on general, broadly applicable considerations for immunotoxicity risk assessment. As the data for a given chemical may indicate one or more types of immunotoxicity, the risk assessor performing a risk assessment for a given chemical is encouraged to consult

[1] http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788;jsessionid=1960xn9o2ktmr.delta
[2] http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002851.pdf

chapter 3 in conjunction with the guidance for specific areas of immunotoxicity provided in subsequent chapters.

Review and risk assessment guidance for the different types of immunotoxicity are presented in these subsequent chapters, addressing immunosuppression (chapter 4), immunostimulation (chapter 5), sensitization and allergic response (chapter 6) and autoimmunity and autoimmune disease (chapter 7). The application of risk assessment guidance from chapters 3–7 is illustrated by case-studies, which appear at the end of the report. It should be emphasized that these case-studies are provided to illustrate how the risk assessment guidance can be used in the various areas of immunotoxicity, but they do not represent a comprehensive risk assessment of the chemical in question, nor do they represent final regulatory positions.

A glossary of selected terms is also included in the report.

2. BACKGROUND

2.1 The immune system as a special case

The immune system presents a readily accessible toxicological target regardless of the route of chemical exposure, in that, as a fully dispersed system, it is represented in most tissues, organs and peripheral sites (e.g. respiratory, dermal, gastrointestinal, neurological, cardio-vascular, reproductive, hepatic and endocrine). The implication is that xenobiotic exposure via virtually any route will result in exposure of some immune system components. Therefore, from a risk assessment perspective, the issue is not whether immune exposure occurs following a chemical exposure, but whether a given exposure is likely to produce an adverse immunotoxic outcome among susceptible populations.

The immune system is designed both to provide host integrity and to ensure appropriate function of organs and tissues. To accomplish this, it must be able to distinguish self from non-self and be able to assess tissue-specific cell status and function. The immune system protects host integrity via surveillance against a myriad of invading pathogens as well as the transformation of host cells into tumour cells. It also responds to tissue damage and any external stimuli that appear to pose a threat to the host. During the course of a lifetime, an individual will be exposed to pathogens ranging from small intracellular deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses to large extracellular bacteria and parasites. Ideally, immune responses against invading pathogens and emerging tumour cells should be tailored to the task at hand, including the nature of the disease challenge, the temporal nature of the challenge and the specific location of the challenge. It is increasingly recognized that inappropriate immune responses in scope, duration, location or specificity have the potential to be as problematic, in terms of disease risk, as insufficient immune responses. Therefore, immunotoxicity risk assessment has evolved to consider not only whether chemical exposures might compromise the sufficiency of immune responses, but also whether exposure to chemicals can increase health risk by changing the scope, duration, location or specificity of responses.

Table 2.1 provides examples of diseases that have environmental risk factors and are associated with immune dysfunction. Two categories are shown. In the first category, evidence suggests that environmentally induced immune dysfunction contributes directly to the disease or condition. In the second category, patients with the disease have reported immune dysfunction and/or misregulated inflammation, but a cause–effect relationship between immune dysfunction and these diseases has yet to be established. Reduction in the incidence of these diseases is one expected outcome of effective immunotoxicological risk assessment.

A second, often overlooked role of the immune system concerns the homeoregulatory maintenance of organ and tissue function. This role is played by resident myelomonocytic derived cells (e.g. macrophages) that can take numerous forms and are given different tissue-specific names (e.g. microglia, alveolar macrophages, Kupffer cells, Langerhans cells). Because the forms are diverse and tissue specific, the interaction of these cell populations with the environment also has the potential to differ across tissues. Therefore, potential sensitivity of a Kupffer cell to a chemical exposure is likely to differ significantly from that of an alveolar macrophage. The problem from an immunotoxicity perspective is that chemical-induced disruption of the homeoregulatory role of the immune system may take the form of organ function alteration. Unless the mechanism is clear, the role of immune cells as targets leading to the adverse outcome may go unrecognized. Examples of the importance of

Table 2.1: Potential target diseases and disorders for immunotoxicology-driven risk reduction.[a]

Disease or disorder	*Sex predominance, if any*	*References*
Category 1[b]		
Acute otitis media	—	Boyle et al. (2006); Dallaire et al. (2006); Hirano et al. (2007)
Allergic diseases	Age of onset differences	Boyle & Tang (2006); Gao et al. (2007); Dietert & Zelikoff (2008); Guedes & Souza (2009)
Atherosclerosis	Males	Abou-Raya & Abou-Raya (2006); Palinski et al. (2007); Varthaman et al. (2008)
Autoimmune hepatitis	Females	Diamantis & Boumpas (2004); Hegde et al. (2008); Stanca et al. (2008)
Autoimmune thyroid disease (Graves disease and Hashimoto disease)	Females	Villanueva et al. (2000); Caturegli et al. (2007)
Childhood and occupational asthma	Males earlier in onset (childhood)	Yeatts et al. (2006); Tager (2008); Tarlo (2008); Wang & Pinkerton (2008); Malo & Chan-Yeung (2009)
Childhood leukaemia	Males (all)	Greaves (2006); MacArthur et al. (2008); Dietert (2009b)
Coeliac disease	Females (for adult diagnosis)	Peters et al. (2003); Poole et al. (2006); Di Sabatino et al. (2007); Dietert & Zelikoff (2009)
Crohn disease	Slightly higher for females	Lerner (2007); Peyrin-Biroulet & Chamaillard (2007); Saruta et al. (2007)
Inflammatory bowel disease	—	Innis & Jacobson (2007); Neuman (2007); Weng et al. (2007); Rahman et al. (2008)
Influenza (increased susceptibility)	—	Vorderstrasse et al. (2006); Ciencewicki et al. (2007); Hogaboam et al. (2008)
Kawasaki disease	Slightly higher for males	Lee et al. (2007); Yilmaz et al. (2007)
Late-onset neonatal sepsis	Males	Graham et al. (2006); Gille et al. (2008); Molloy et al. (2008)
Multiple sclerosis	Females	Guo et al. (2007); Bar-Or (2008); Ebers (2008)
Rheumatoid arthritis	Females	Verwilghen et al. (1993); Cope et al. (2007)
Sarcoidosis	Females (based on hospitalizations)	Fireman et al. (2006); Kieszko et al. (2007); Allen et al. (2008)
Scleroderma	Females, except for some occupational exposures	Gold et al. (2007); Boin et al. (2008); Tolle (2008)
Sjogren syndrome	Females	Kroneld et al. (1997); Tabbara & Vera-Christo (2000)
Systemic lupus erythematosus	Females	Dahlgren et al. (2007); Pilones et al. (2007)
Type 1 diabetes	Males slightly higher	Kureja & Maclaren (2002); Wen & Wong (2005); Nicolls et al. (2007)
Category 2[c]		
Alzheimer disease	Incidence slightly higher in females; prevalence significantly greater in females	Reitz et al. (2007); Rosenkranz et al. (2007); Skaper (2007)

Table 2.1 (continued)

Disease or disorder	*Sex predominance, if any*	*References*
Autism and autism spectrum disorders	Males	Ashwood et al. (2006); Dietert & Dietert (2008b); Garbett et al. (2008); Pessah et al. (2008); T. Schneider et al. (2008)
Myalgic encephalomyelitis	Females	Klimas & Koneru (2007); Dietert & Dietert (2008a); Nijs & Fremont (2008)
Parkinson disease	Males slightly higher	Barlow et al. (2004); Liu (2006); Wang et al. (2007)
Schizophrenia	Age of onset differences	Meyer et al. (2008); Romero et al. (2010)

[a] Adapted from information in IPCS (2006a); Dietert & Dietert (2007, 2010); Dietert (2008, 2009a); Dietert et al. (2010).
[b] Category 1 diseases and disorders have immune dysfunction as a critical factor in the development of the disease.
[c] Category 2 diseases and disorders have reported immune dysfunction among patients that may or may not be causative for disease development.

specialized macrophage populations in tissue-specific toxicity can be seen with asbestos toxicity in the lung and acetaminophen toxicity in the liver. Table 2.1 also includes diseases that fall into this category of dysfunction.

The immune system has several primary sites important for immune cell maturation (bone marrow, liver, thymus, Peyer's patches), as well as specific secondary lymphoid sites (spleen, lymph nodes, tonsils) that provide opportunities for antigen presentation and the initiation of adaptive immune responses. Not surprisingly, an exposure to an immunotoxicant is likely to affect these various sites of immune maturation and cell cooperation differentially.

Beyond the presence of immune cells in most tissues, there exists a regional organization of immune cell populations that are important in local immunity and are generally referred to as mucosal-associated lymphoid tissue. Two of these regional immune sites are also connected with two important chemical exposure routes: respiratory and oral. These are the bronchus-associated lymphoid tissue and the gut-associated lymphoid tissue, respectively. Additionally, mucosal lymphoid tissue is present in the urogenital tract. Another important route is the skin, which has a specialized immune system based on the Langerhans cell. Comparative immunotoxicity information about these regional lymphoid sites is only beginning to emerge.

2.2 The importance of immune challenge in detecting immunotoxicity

Because the immune system is designed to protect host integrity from foreign challenge and potential insult, a gold standard for measuring immune system status is to evaluate the host response to foreign challenge. This requires challenge with an infectious agent (or tumour cells) or immunization with a foreign antigen. Depending upon the nature of the agent used for challenge or the immunogen, various immune cells will interact and respond with a predictable profile of responses. These responses can be standardized quite exquisitely in terms of kinetics of responses, patterns of cell mobilization and emigration, spectrum of effector functions and magnitude of the response, given the species, age and genetic background employed. Because the goal is to detect chemically induced immune dysfunction

with an impact on health risk, almost by definition, immune function data are needed for the evaluation.

In human and wildlife populations, it may be assumed that some low level of antigenic challenge is occurring at any given time. However, even in those populations, assessment of an immune system that is responding to an overt challenge (immunization, vaccination or challenge with an infectious agent) is beneficial for detecting immunotoxic outcomes. For example, Luster et al. (2005b) advocated the use of childhood vaccination responses, instead of standard blood cell profiles, as the preferable and more sensitive biomarker for childhood immunotoxicity. This was employed by Heilmann et al. (2006) to demonstrate the inverse relationship between cord blood polychlorinated biphenyl (PCB) levels and response of the offspring to childhood vaccinations at age 7. Therefore, opportunities to take advantage of normal immune challenges among human populations should be utilized for the most sensitive indicator of immune status.

In safety testing employing laboratory animals (e.g. rodents), there is the added problem that controlled environments may limit the opportunity for robust antigenic stimulation or infectious agent challenge. As a result, the resting immune system of a laboratory animal in a controlled, relatively pathogen-free environment is an insensitive test system for evaluating chemically induced immune dysfunction. In contrast, if host challenge is performed, the low ambient background stimulation in the laboratory animals becomes an advantage rather than a disadvantage. However, to accommodate developmental/reproductive or other safety testing, prior protocols have excluded immunization or infectious agent challenge using the argument that such animal manipulation would alter the routine toxicological assessment performed on other physiological systems. It is important to recognize that evaluation of a resting immune system is unlikely to generate data that can be extrapolated with confidence to predict the spectrum of chemically induced immune dysfunctions of concern. Using data generated by the United States National Toxicology Program (NTP), Luster et al. (1992) reported eight different combinations of three immune parameters that resulted in 100% predictability for the identification of immunotoxicants. None of these eight combinations lacked a functional assay, and a majority of the combinations included more than one immune function assay. Among the eight combinations, the T helper-1 (Th1)-driven delayed-type hypersensitivity (DTH) assay was included in several combinations, the plaque-forming cell (PFC) assay in four combinations and natural killer (NK) cell activity in three combinations.

If immune responses to antigen challenge are recognized as a sensitive approach for evaluating immune system status and structural evaluations of the resting immune system are considered as less sensitive (as per Luster et al., 2005b), then first-tier immunotoxicity evaluations should include functional responses to antigenic challenge. Testing strategies predicated solely on the detection of structural (e.g. immunopathology) alterations of an unchallenged immune system may be convenient, but the parameters measured are far removed from the adverse effects of greatest concern. Vaccination responses in adults and children have been proposed as the gold standard for identifying human immunotoxicity (Van Loveren et al., 2001; Luster et al., 2005b), and the data support this (Sleijffers et al., 2001; Weisglas-Kuperus et al., 2004; Heilmann et al., 2006).

2.3 Dose–response relationships and thresholds

Comparative sensitivity of immune parameters to xenobiotic modulation can vary widely depending upon the nature of the toxicant, the route of exposure and differential sensitivity of immune cell populations. Across the literature, there are examples in which virtually every well-defined immune cell population has at some point been observed as the most sensitive target in immunotoxicity studies. For this reason, no single immune biomarker identified to date is reliable as an indicator of hazard identification for the immune system. The status of the host at the time of immune parameter measurement can have an influence on sensitivity of the immune parameter as a biomarker of immunotoxicity. For example, the dose–response curve is likely to differ for NK cell activity following exposure to an immunotoxicant, depending upon whether the host has received treatments that would activate NK cells (e.g. a viral infection) compared with NK activity measured from an unchallenged host system. Use of activated instead of resting NK cells is more likely to provide a broader dose–response range, making it potentially easier to detect significant alterations (e.g. suppression) in function. This could influence the lowest-observed-adverse-effect level (LOAEL) for this parameter and therefore might have an impact on the low-dose end of a dose–response curve. Some studies have been published comparing the shape of dose–response curves for immunotoxicants determined under activated versus resting conditions (Daniels et al., 1987). However, further comparisons could prove beneficial for risk assessment considerations.

Additionally, because two immune parameters influenced by xenobiotic exposure may have different dose–response ranges as well as differently shaped dose–response curves, a "safe" dose for one parameter may not be "safe" for the second. It should be noted that the dose–response relationship for a chemical toxicant can be influenced by the immunizing dose of antigen or challenge dose of infectious agent. Furthermore, in the case of chemical sensitizers with biphasic host responses, different dose–response relationships can exist for the sensitization phase of the response compared with the induction phase of the allergic response.

One of the concerns for immunotoxicity risk assessment and for toxicity to other systems as well is the potential for non-monotonic (e.g. U-shaped) dose–response relationships. These types of responses were recently discussed for endocrine disrupting chemicals, such as bisphenol A (Hotchkiss et al., 2008). The most comprehensive review of dose–response curves for immune and immunotoxic responses was performed by Calabrese (2005), including perspectives of this analysis (Dietert, 2005; Hastings, 2005; Holladay et al., 2005; Ladics & Lovelace, 2005). The conclusions were that whereas linear and simple dose–response relationships exist for most xenobiotics over some range of exposures, the entire dose–response curves can often take the shape of "U" or inverted "U" forms. In these cases, very low dose exposures may produce effects that are opposite to those of high-dose exposures, otherwise known as hormesis. U-shaped dose–response curves are also discussed by Portier & Ye (1998), including their observation of the impact of toxicants on host responses to infectious agent challenge (Luster et al., 1993). Holladay et al. (2005) discussed how the U-shaped dose–response curves appear to be common with endocrine disruption of the immune system and are likely to represent different contributing mechanisms, each with its own responding range of concentrations for a given chemical. Hastings (2005) also provided examples of immunotoxicants with more than one mechanistic impact on the immune system, where each mechanism had a potentially different dose–response curve. From a practical discussion of the no-observed-adverse-effect level (NOAEL) extrapolation from experimental animals to humans, Ladics & Lovelace (2005) pointed out the likelihood that most hormetic responses would not be observed in the context of current safety testing,

and they may be even more common than anticipated. However, the authors added that there are significant challenges in applying a potential low-dose beneficial effect seen in a study of an otherwise highly immunotoxic chemical when considering risk for a diverse human population.

One of the factors contributing to immune hormetic responses and usual dose–response curves may be the role of regulatory lymphocyte populations, such as the CD4+CD25+highFoxp3+ regulatory T cells, as targets of immunotoxicity. The cells have the capacity to control tolerance, autoimmunity and tumour immunology (Allan et al., 2008; Apostolou et al., 2008; Kretschmer et al., 2008; Piccirillo et al., 2008; Sakaguchi et al., 2008; Welters et al., 2008). Subtle shifts in the population size or activity of regulatory T cells following chemical exposure could significantly affect the course of antigen-driven immune responses, and regulatory T cells are known to serve as targets of some immunotoxicants (Marshall et al., 2008). Therefore, regulatory T cells may play a role in mediating the opposing immune response outcomes observed at opposite ends of some dose–response curves.

The question of possible thresholds for immunotoxicity (or the lack thereof) remains a controversial issue. Certainly, NOAELs have been found in many immunotoxicity investigations, including those with early-life exposure to chemicals. Kroes et al. (2000) reviewed the opportunity to apply a threshold of toxicological concern (TTC) standard to determine priorities in toxicology testing among chemicals. Their analysis included the NTP database for the immune system. However, other studies have found the latency effect, where supposed subthreshold exposures to xenobiotics seem to rewire the immune system for problematic later-life responses (potentially requiring a second subthreshold exposure to the same chemical). These findings raise problems with the threshold concept. Additionally, hormetic U-shaped dose–response curves suggest that some apparent subthreshold results might be simply the bottom of the "U" on the U-shaped curve. These contradictory findings indicate the importance of defining subthreshold/threshold boundaries in the context of those immune parameters evaluated. A prior WHO document, Environmental Health Criteria (EHC) 170 (IPCS, 1994), considered risk assessment for chemical exposures with non-threshold effects (see section 3.1.1 of EHC 170). Among the approaches utilized were 1) quantitative extrapolation by mathematical modelling of the dose–response curve, 2) relative ranking of potencies in the experimental range and 3) division of effect levels by an uncertainty factor. The same document (see section 3.1.2 of EHC 170) also discussed risk assessment with threshold values as well as the application of uncertainty factors as influenced by such factors as species, sex, strain, age, developmental status of test animals, group size, sensitivity of methodology, duration of exposure and selection of the doses examined.

2.4 Induction of tolerance

Mechanisms of self-tolerance were recently reviewed in section 3.4 of the WHO document, EHC 236 (IPCS, 2006a).

The capacity to distinguish between self and non-self is a critical feature of the immune system and one that operates at self levels in several locations, involving multiple cell types, receptors, cytokines and metabolites. Improper recognition of, and response directed against, autoantigens can result in autoimmune disease. These self-reactions can occur by a variety of mechanisms and cause damage to a variety of tissues and organs classified under a variety of names (e.g. type 1 diabetes, multiple sclerosis, Graves disease, systemic lupus erythematosus,

myasthenia gravis). It should be noted that most autoimmune diseases show sex bias (occurring predominantly in either females or males). Therefore, environmentally mediated risk of specific autoimmune manifestations is significantly different across sexes.

Central immune tolerance is generated via the thymus (for T cells) and bone marrow (for B cells) during development. In the thymus, thymocytes receive positive or negative selection signals from the epithelial stoma. The nature of the signal is dependent upon the binding affinity of a given thymocyte for self major histocompatibility complex (MHC) molecules (Daniels et al., 2006). Autoreactive thymocytes recognize self-MHC peptides with a strong binding affinity and receive a negative selection signal in the thymus that results in programmed cell death or apoptosis. This subsequent deletion of autoreactive thymocytes is directly linked to the level of the T cell receptor affinity binding for self-MHC peptides (Naeher et al., 2007). This negative selection event in early development is critical for reduced risk of autoimmunity (Sohn et al., 2007). Generation of self-recognizing T cells via impaired negative selection in the developing thymus (Parish & Heath, 2008) increases the risk of autoimmunity. B cell tolerance can be achieved by receptor editing, revision of the B cell repertoire, via a negative selection deletion of cells (fetal) in the bone marrow or via T cell anergy in the periphery (Caucheteux et al., 2008).

Peripheral immune tolerance is important, as negative selection in the thymus is not fully efficient, and T cell receptor editing and revision may lead to the emergence of autoreactive T cells in the periphery. Generation of diversity in the periphery can help to maintain resistance to a wide spectrum of pathogens, but the downside is the potential for peripherally generated autoreactive T cells (Wagner, 2007). The balance between having a sufficiently robust T cell receptor repertoire and the potential for autoimmunity is one of the major areas of concern with xenobiotic–immune system interactions.

A variety of cell types and cell-derived factors are important in the control of tolerance (reviewed in section 3.4 of EHC 236; IPCS, 2006a). Production of transforming growth factor–beta (TGF-β) and interleukin-10 (IL-10) is a critical link in tolerance maintenance (Li & Flavell, 2008). Interactions of invariant NK T cells with dendritic cells appear to be important in the tolerogenic network (Yamamura et al., 2007; Tamura et al., 2008). Additionally, classic immunotoxicants can cause persistent antigen-specific tolerance (i.e. immunosuppression) through the inappropriate stimulation of regulatory T cells (Kang et al., 2008). The status of regulatory T cells is an important factor in risk of human disease (Cools et al., 2007a,b). These cells can induce tolerance and suppress autoimmune responses. However, if their activity is inappropriately enhanced, their action can result in immunosuppression. In contrast, if regulatory T cell activity is impaired following chemical exposure, a higher risk of autoimmunity can result. Finally, regulatory B cells as well as specialized populations of myelocytic cells have also been identified as playing a role in tolerance and appear to be mediated via TGF-β and IL-10 production (Mauri & Ehrenstein, 2008).

2.5 The possibilities for adverse outcomes: immune stimulation, suppression and/or misregulation

Because the immune system has numerous effector and regulatory cell functions that operate at local, regional and systemic levels, exposure to xenobiotics has the capability of producing any combination of the following recognized adverse outcomes: 1) focused or more extensive immunosuppression, 2) increased propensity for allergic disease, including atopy, food allergies and asthma, 3) hypersensitivity reactions directed at the chemical itself, 4) increased

risk of autoimmune disease and 5) dysfunctional responses of innate immune cells producing tissue or organ damage or dysfunction. These dysfunctional responses include pseudoallergic responses or exaggerated or inappropriately prolonged inflammatory responses that result in significant organ insult, dysfunction and disease. The innate immune inflammatory dysfunction may also affect the status of adaptive immune function, creating additional health risks.

The present document recognizes that each adverse outcome has potentially serious implications for health risk. Whereas immunosuppression might be expected to increase the risk of infectious and neoplastic diseases, inappropriate stimulation could elevate the risk of allergic and autoimmune diseases. Likewise, misregulation of immune responses can affect the risk of both inflammatory and autoimmune conditions. Therefore, the ramifications of inappropriate immune stimulation, suppression and misregulation are given equal weight in the present document. As discussed below, these adverse outcomes are not always mutually exclusive. Instead, they can co-exist following exposure to certain toxicants.

Numerous examples of combinations of these immunotoxic outcomes exist in the literature. For example, exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) can produce immunosuppression (Smialowicz et al., 2004), increased risk of autoimmunity (Mustafa et al., 2008) and misregulated inflammation (Luebke et al., 2002). In fact, it seems likely that a greater percentage of immunotoxicants than previously recognized do produce a broad spectrum of dysfunction or misregulation rather than only immunosuppression. Additionally, chemicals with little to no systemic immunosuppressive effects at relevant exposure levels are not necessarily free from producing effects locally that result in immunotoxic adverse outcomes. If testing did not include assessment of risk of allergic disease and autoimmunity, the potential risk remains unknown. One reason why these broader possibilities for adverse outcomes were not previously recognized is that prior immunotoxicity testing analyses were focused primarily on immunosuppression or chemical-specific contact hypersensitivity. They were never designed to detect elevated risk of allergic disease, autoimmunity, misdirected inflammation or even limited or local immunosuppression. The categorization of a xenobiotic as an immunosuppressive agent, as an allergy-inducing agent or as posing little immunotoxic risk is only as accurate as the range of testing performed. Therefore, in many cases, assessment has been incomplete, and the categorization should be viewed in this context.

The impact of genotype is important in determining the risk of any potential immunotoxic adverse outcome for an individual. Not only can the toxicokinetics (or pharmacokinetics) of chemical metabolism and distribution differ across populations, but so can the propensity for certain spectra and levels of immune responses. Therefore, low-level exposure of a population to a chemical capable of increasing the risk of allergic disease or reducing childhood vaccine responses would be expected to produce adverse outcomes in only a portion of the exposed population. This means that a significant exposure to a risk factor for increased allergic disease (e.g. diesel exhaust) might shift only a subpopulation of the exposed group into atopy. Nevertheless, the impact of a 10–15% increase in allergic disease or, alternatively, childhood vaccine failures represents a significant public health concern.

2.6 The developing immune system

If the immune system itself is a special toxicological case based on its systemic distribution and multifunctional nature, risk assessment for the developing immune system represents an extra complexity of health concern (IPCS, 2006b). Developmental immunotoxicity

considerations were not included in the prior WHO document, EHC 180, on immunotoxicity associated with exposure to chemicals (IPCS, 1996). However, significant progress has been made in developmental immunotoxicology research and potential developmental immunotoxicity testing in the interim. Therefore, it is appropriate that the developing immune system be included.

Because normal maturation of the immune system is dependent upon specific processes that differ in both time and location within the body, the immune system of the non-adult is truly a moving toxicological target for xenobiotic interactions (Dietert et al., 2000; Holladay & Smialowicz, 2000; Van Loveren & Piersma, 2004; Burns-Naas et al., 2008). Much of immune development in humans occurs during gestation (Holsapple et al., 2003; Leibnitz, 2005), although critical adjustments in the level and spectrum of immune response capacities as well as in the capacity for immunological memory (a hallmark of the immune system) continue to change after birth. Hence, the prenatal, neonatal, juvenile and adolescent immune systems should be viewed as distinct from that of the adult in terms of risk assessment.

Prenatal immune maturational processes are dependent upon reservoirs of immature immune cell types (e.g. fetal liver, bone marrow) as well as specific organs (thymus) or regions (Peyer's patch) in which specific cell maturation and repertoire selection can occur. Both cell trafficking and cell–cell interactions are prerequisites for immune maturation. It is during these early-life processes that critical functions emerge, such as the ability to correctly identify self from non-self and to establish resident immune populations in each organ for homeoregulatory surveillance. In an effort to facilitate examination of the comparative susceptibility of these processes to immunotoxic disruption, workshop participants and others partitioned both systemic and local immune development into critical windows or stages, which would be expected to differ in immunotoxic vulnerability (Dietert et al., 2000; Landreth, 2002; Dietert & Piepenbrink, 2006b; Dietert & Dietert, 2008b). The windows of potential differential vulnerability are defined by functional or active processes involving the immune system rather than more static morphometric alterations. At issue is not simply whether the non-adult differs from the adult in specific immune-based health risks following a given xenobiotic exposure, but also whether different stages of life within the non-adult would exhibit significant differential vulnerabilities.

Research results using age-timed exposures and several categories of xenobiotics suggest that critical windows of immune development do differ in terms of the potential health risk that would result from a given early-life exposure to a toxicant (Bunn et al., 2001a; Hogaboam et al., 2008). For example, early gestational exposure of rats to the heavy metal lead targets macrophage activity, whereas later gestational exposure produces the hallmark shift in Th function towards a Th2 bias (Bunn et al., 2001a).

There are several ways in which the young are at greater health risk from immunotoxicants compared with adults (reviewed in Dietert & Piepenbrink, 2006b). First, the fetus and neonate are sensitive to lower doses of immunotoxicants compared with the adult. Additionally, both the duration (persistence versus transitory adverse outcomes) and the spectrum of effects are usually greater following early-life compared with adult exposure. Finally, early-life, low-level exposure to toxicants can rewire the immune system for unpredictable host responses with later-life, lower-level exposure to the same chemical and/or adult stress. This has been termed latency and is among the outcomes considered in the next section. Obviously, a significantly increased dose sensitivity in early life, potentially permanent effects after chemical exposure has ceased, broader-spectrum adverse effects and fetal rewiring of the

immune system are all special developmental immunotoxic risks that need to be considered in determining safe levels of chemical exposure for the young. An example of the challenge follows.

Dose sensitivity is greater in early life than in adulthood for those immunotoxicants compared across ages. Luebke et al. (2006a) reported that dose sensitivity to lead prenatally was approximately 10-fold higher than that in adults. In contrast, the age-based sensitivity to TCDD differed by approximately 100-fold in rats. This suggests the utility of having direct developmental immunotoxicity data when possible, as application of standard uncertainty factors may be problematic.

The spectrum of adverse outcomes from developmental immunotoxicity testing falls into several subcategories; all of them are encompassed under the umbrella of immune dysfunction and/or misregulation. Immunosuppression can produce an increased risk of infections and cancer (Vorderstrasse et al., 2006; Ng et al., 2006), whereas exaggerated responses (Rowe et al., 2006) are likely to contribute to an increased risk of allergic or autoimmune diseases. However, the same chemical can produce both targeted immune suppression (Gehrs & Smialowicz, 1999) and increased risk of autoimmunity (Mustafa et al., 2008). Because specific immune testing is frequently limited to a few immune parameters, detection of immunosuppression in one series of tests would not preclude the test chemical from also producing enhanced risk of allergy or autoimmunity unless predictive immunotoxicity testing eliminated those additional possibilities. An example of the latter is with diethylstilbestrol (DES), an agent that produces significant thymic atrophy and T cell loss with prenatal exposure (Besteman et al., 2005) while concomitantly increasing the positive selection and persistence of autoreactive T cell clones (Brown et al., 2006). Prenatal exposure to heavy metals such as lead and mercury can suppress certain immune function capacities while simultaneously elevating the risk of allergies and/or autoimmunity (Miller et al., 1998; Pilones et al., 2007). Sex-related effects are common with developmental immunotoxicity (Blyler et al., 1994; Bunn et al., 2001b; Rooney et al., 2003; Guo et al., 2005a); for this reason, differential health risks following chemical exposure of female and male fetuses and neonates should be taken into consideration in risk assessment.

2.7 Early-life exposure/later-life effects

Later-life diseases linked to early-life immune insults were recently considered by several researchers (Bakker et al., 2000; Holladay, 2005; Dietert & Dietert, 2007, 2010; Selgrade, 2007; Dietert, 2008, 2009a,b; Dietert & Zelikoff, 2009; Dietert et al., 2010). These diseases and disorders, as listed in Table 2.1, fall into two major categories: 1) those diseases clearly linked to environmental risk factors and immune dysfunction, where the immune dysfunction appears to be causally related to the disease, and 2) those diseases where immune dysfunction and/or misregulated inflammation are evident, which may represent either a cause of the disease or an associated adverse outcome.

The former group includes enhancement of paediatric diseases such as childhood ear infections, leukaemia, influenza, asthma, type 1 diabetes and allergic diseases (Greaves, 2006; Yeatts et al., 2006; Hirano et al., 2007; Dietert & Zelikoff, 2008; A. Schneider et al., 2008). Additional diseases of paediatric and/or adult onset include multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, coeliac disease, autoimmune hepatitis, autoimmune thyroiditis (Graves and Hashimoto diseases) and atherosclerosis (Villanueva et al., 2000;

Briani et al., 2008; Compston & Coles, 2008; Rahman et al., 2008; Stanca et al., 2008; Hanson, 2009).

Diseases in the second category have possible links to early-life misregulated inflammation and/or immune insult. However, in these cases, cause–effect relationships between the immune problems and the diseases have not been firmly established. These diseases include autism, myalgic encephalomyelitis, schizophrenia, Parkinson disease and Alzheimer disease (Block & Hong, 2007; Klimas & Koneru, 2007; Ashwood et al., 2008; Muller, 2008; Steinman, 2008; Lorusso et al., 2009; Rentzos et al., 2009). It should be noted that some of these diseases may involve prenatal immunotoxicity paired with postnatal events that serve as a disease trigger. For example, a dysfunctional immune response to common childhood infections is now seen as a final step leading to childhood leukaemia (Greaves, 2006). An environmentally induced, prenatal–perinatal immunotoxic event would be needed to produce the required neonatal immune dysfunction. This would precede the infectious agent trigger for the disease (discussed in Dietert, 2009b; Dietert & Dietert, 2010). Similarly, it has been suggested that risk of Parkinson disease may involve a combination of early-life inflammatory insults in the brain and cell loss through normal ageing processes (Block & Hong, 2007; Soreq et al., 2008; Yankner et al., 2008). Early-life immune insult can interact with the normal ageing process such that the adverse outcome of developmental immunotoxicity may not appear until long after the original prenatal–neonatal immune insult and may affect multiple health risks over a life course (Dietert & Zelikoff, 2009). In other cases, a later adult exposure to the same or a similar chemical may be needed for the developmental immunotoxicity–related adverse outcome to become apparent. An example of this has been reported with estrogenic chemicals (Fenaux et al., 2004).

One of the concerns with developmental immunotoxicity is that an early-life insult of the thymus may affect the age-related decline in T cell–dependent immune responses observed in geriatric populations. Mouse models suggest that normal ageing involving the thymus can produce holes in the diversity of the T cell repertoire in concert with otherwise normal levels of immune response (Yager et al., 2008). The heightened disease risk is that these repertoire gaps increase the likelihood of failed responses to specific infectious agents and/or tumour cells. Investigators have observed this as a reduced spectrum of effective host resistance responses as well as the potential for reduced responses to vaccinations (Yager et al., 2008). Some low-level prenatal–neonatal exposures to xenobiotics may result in later adult host resistance gaps that might be seen only among geriatric populations following challenge with an infectious agent or vaccine. This is one potential early-life exposure/later-life disease risk that has yet to be investigated extensively.

2.8 Current practices

At the time of the development of this document, current practices in immunotoxicity testing are varied and employ both unchallenged as well as challenged immune systems. Therefore, testing results in different types of data for use in risk assessment. The basic regulations for immunotoxicity testing of chemicals and drugs are discussed in two reviews (Schulte & Ruehl-Fehlert, 2006; Spanhaak, 2006). Most immunotoxicity testing historically has been organized into tiers that increase in specificity at each subsequent level (Luster et al., 1988, 1992; Hinton, 2000). Use of second or third tiers enables the generation of data of increased quality and specificity. However, this also comes as a result of added time, cost and potential use of laboratory animals. Therefore, the nature of the primary screen (tier 1) is crucial in the type of data usually available to the risk assessor. The organization and composition of each

tier have evolved in recent decades and are under further discussion at this time. The primary issues are as follows:

1) Should histopathology and cell enumeration of an unchallenged immune system be considered sufficient for hazard identification of immunotoxicants?
2) When would immune function assessment be performed in a testing scheme?
3) What is sufficient as a functional assessment of the immune system?
4) How and when would relevant developmental immunotoxicity data be collected?
5) How will testing strategies originally designed to detect immunosuppression be modified for detection of the full spectrum of immune dysfunction (e.g. risk of immunosuppression, allergy, autoimmunity and misregulated inflammation)?

Obviously, the simplest and least expensive practice is for extended histopathology and cell counting data to be collected on existing animals. These animals may also be used in developmental and reproductive protocols for age-based evaluations. However, this information would usually be collected from an unchallenged immune system. The potential pitfalls of this strategy have been previously discussed. If histopathology–haematology of an unimmunized or unchallenged immune system represents the primary screen for immune-related hazard identification (e.g. as used in OECD Test Guidelines 407, 416, 419 and 421, the EU's Registration, Evaluation, Authorisation and Restriction of Chemical Substances [REACH] and the ICH S8 protocols), then immune function data may never be collected. It should be noted that the 1998 immunotoxicity testing guidelines of the Office of Prevention, Pesticides, and Toxic Substances (OPPTS) of the United States Environmental Protection Agency (USEPA) (OPPTS 870.7800: USEPA, 1998) indicate that routine toxicity testing (e.g. histology, organ weights, haematology) alone is not sufficient to predict immunotoxicity. It seems highly questionable whether combined histological analysis, organ weights and cell counting in the absence of robust functional testing would detect unintended immunosuppression as well as unintended immune stimulation. Unintended immune stimulation has significant implications for potential disease (Karrow et al., 2004; Guo et al., 2005a; Ponce, 2008). Additionally, unintended immune stimulation would affect the risk of allergy and autoimmunity, two categories of disease that need to be addressed in immunotoxicity risk assessment (Luster et al., 1999; Smith & Germolec, 1999; Selgrade et al., 2006; Yeatts et al., 2006) (see Table 2.1).

Several recent reviews have discussed these testing issues for the adult and/or developing immune system. Some have emphasized using histopathology, organ weights and cell enumeration without the necessity of functional testing (Snodin, 2004) or placed a special emphasis on the use of pathology (Burns-Naas et al., 2008). In contrast, others have advocated the benefits of extending beyond histopathology to include functional immune assessment of a challenged immune system in primary testing (Luster et al., 2003; Putman et al., 2003; Van Loveren et al., 2003; Germolec et al., 2004a; Van Loveren & Piersma, 2004; Van der Laan & Van Loveren, 2005; Descotes, 2006; Dietert & Holsapple, 2007). At present, following recent (2008) harmonization efforts, there is no requirement for functional testing of a challenged immune system in any primary testing tier (e.g. OECD test guidelines, EU REACH and ICH S8), except for pesticide testing in the USA (OPPTS 870.7800: USEPA, 1998). However, given the recent call for direct immune function data to be collected from children to aid in their protection (Luster et al., 2005b), continued reliance on non-functional data derived from unchallenged animals seems doubtful.

Some strategies to obtain immune function data in the most relevant context for human disease risk have used infectious agent challenges that combine immune function evaluations with host resistance measures (Mitchell & Lawrence, 2003; Vorderstrasse et al., 2006; Burleson & Burleson, 2007). This can offer an extensive challenge of the immune system (potentially beyond that normally obtained with protein antigen immunization) and also permits a direct assessment of cytotoxic T lymphocyte function along with other standard immune function tests. If infectious agent challenge is used to replace immunizations (e.g. with sheep erythrocytes or keyhole limpet haemocyanin [KLH]), then combined immune function and host resistance data may be collected using the same number of animals as required for routine immune function assays (e.g. a T cell–dependent antibody response assay).

2.9 Newer approaches

Although there is incentive to obtain data more directly indicative of immune function status in immunotoxicity screening, there is also a desire to minimize laboratory animal use in safety screening. This combination has led to the pursuit of several new avenues of investigation into screening tools (Chatterjee et al., 2006). The new tools may help to direct which chemicals should be subjected to more detailed immunotoxicity testing. One of the new areas is that of immunotoxicogenomics. Progress in this area was recently reviewed by Luebke et al. (2006b), Vandebriel & Van Loveren (2010) and Vandebriel et al. (2011). The strategy behind this approach is to be able to detect critical changes in the expression of immune function–related genes or pathways that might direct more extensive functional testing among candidate chemicals. In addition to genes, profiling of protein expression (i.e. proteomics) may also be applied for this purpose (Osman et al., 2009, 2010). Because it is not feasible to test all chemicals fully for immunotoxic potential, an immunotoxicogenomics screen could serve to identify the subset of chemicals of greatest health concern requiring further testing. In a similar vein, Baken et al. (2007, 2008), Hochstenbach et al. (2010) and Vandebriel et al. (2010) recently reported the use of overlapping gene expression as a strategy to assess immunotoxicity. While the immunotoxicogenomics and immunoproteomics approaches are new and offer considerable promise, additional work is needed in defining the optimum set of biomarkers reflecting the array of immune function concerns. Currently, such information may aid in the understanding of mechanisms of immunotoxicity, but the application will likely be limited to hazard identification for the immediate future.

In addition to traditional in vivo exposure assessment, in vitro approaches continue to play a role as screening tools (Lankveld et al., 2010; Vandebriel et al., 2010). One of the newer strategies has been the use of a “cell chip” and various immune cell lines (e.g. T cells, mast cells, monocytes) as a reporter system for chemically induced changes in immune-relevant gene expression (e.g. cytokine expression) (Trzaska et al., 2005; Wagner et al., 2006). Large numbers of chemicals can be compared with the in vitro procedure. It remains to be determined precisely how and when this would be integrated into other testing strategies, and currently such information may be used to elucidate mechanisms of immunotoxicity. Yet development of sensitive and predictive in vitro approaches using molecular biomarkers for identifying immunotoxicants moves quickly, and, based on proper phenotypic anchoring, they do hold promise for hazard identification and eventually may aid in qualitative or quantitative risk assessment (Adler et al., 2011).

3. FRAMEWORK FOR IMMUNOTOXICITY RISK ASSESSMENT FOR CHEMICALS

3.1 Risk assessment

The risk assessment process is composed of four main steps: hazard identification, hazard characterization (or dose–response assessment), exposure assessment and risk characterization. IPCS (2004) defined hazard as the inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub)population is exposed to that agent. An adverse effect is defined as any change in the morphology, physiology, growth, development, reproduction or lifespan of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences. Risk is described as the probability of an adverse effect in an organism, system or (sub)population caused under specified circumstances by exposure to an agent. Safety is defined as the practical certainty that adverse effects will not result from exposure to an agent under defined circumstances and is considered the reciprocal of risk. A risk assessment should be preceded by a problem formulation phase, which establishes the goals, scope and focus of the risk assessment. The term risk analysis is used to describe the overall procedure comprising all the steps of risk assessment, risk management and risk communication. Risk management can be described as the process of weighing policy alternatives, decision-making and action taking.

A risk assessment can be performed as a basis for a safety assessment or as an actual risk assessment estimating the likelihood and the nature of adverse health effects in the case of an exposure in excess of a derived safe exposure limit or in the absence of a safe exposure limit (for thresholded toxicity) or in the case of an exposure to a substance for which a threshold for toxicity is not or cannot be assumed to exist. Several reviews (see chapter 1) on safety and risk assessment processes have been published to which the reader is referred for detailed information. The toxicological hazard identification is aimed at identifying the nature of the potential health effects of a chemical substance. Information on toxicological end-points may be derived from laboratory animal studies, but human data should be used whenever available. If the data from humans and animals address comparable exposure scenarios and end-points and are of similar quality, human data should take precedence over extrapolation from laboratory animal data. During the characterization of the hazard database, mode of action (MOA) information can be used to determine the human relevance for immunotoxicity following chemical exposure and can contribute to a weight of evidence approach. A postulated MOA is a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. It describes key cytological and biochemical events—that is, those that are both measurable and necessary to the observed effect—in a logical framework. MOA contrasts with mechanism of action, which generally involves a sufficient understanding of the molecular basis for an effect and its detailed description so that causation can be established in molecular terms (Boobis et al., 2006). Key event approaches have been discussed in general by Julien et al. (2009) and focused on an immune disorder (food allergy) by Taylor et al. (2009). WHO recently published a framework for MOA analysis for non-cancer end-points, including immunotoxicity (Boobis et al., 2008). The hazard characterization investigates and describes the dose–effect relationships for the identified hazards. Where a threshold for toxicity is assumed, as is the case for immunotoxicity, a level of exposure considered to present minimal or no risk for health effects to the general population can be established on the basis of an

overall NOAEL and an applicable uncertainty/safety/assessment factor. The basic method for deriving these health-based guidance values is similar across national and international agencies; however, the definitions and specific methods used to derive these values from toxicity data differ slightly and depend on the agency involved. Commonly derived values include the reference dose/reference concentration (RfD/RfC) used by the USEPA, the minimal risk levels used by the United States Agency for Toxic Substances and Disease Registry and the acceptable daily intake/tolerable daily intake (ADI/TDI) used by WHO (IPCS, 1994, 1999a, 2009). There are also a number of adaptations of these general terms, such as the acceptable exposure level (AEL) commonly referred to in risk assessments for sensitization. The NOAEL may be used as the point of departure (POD) for application of the total uncertainty factor in derivation of health-based guidance values such as the RfD/RfC or ADI/TDI, but health-based guidance values may also be derived through the benchmark dose (BMD) approach (see EHC 239 [IPCS, 2009] for a detailed discussion). A health-based guidance value is meant as a reference point in safety assessment and for determining the need to perform a detailed risk assessment, as exposure at or below the RfD/RfC or ADI/TDI is considered to be safe. An integral step in the risk assessment process concerns the exposure assessment, in which information on previous, current or expected exposure is collected and quantitatively described. The final step of the risk assessment process is the risk characterization, in which the exposure assessment results are integrated with and assessed in relation to the hazard assessment results. In the case of threshold effects, an exposure below the RfD/RfC or ADI/TDI can be regarded to be without appreciable risk. However, when the health-based guidance value is exceeded, it cannot be automatically concluded that an adverse effect will occur. It is important to realize that the health-based guidance value is not the lower bound of toxicity in humans. The RfD/RfC or ADI/TDI is regarded as a safe dose, and there is generally a margin of safety between this value and the lower bound of toxicity. In many cases, short-term excursions above the health-based guidance value may be without appreciable risk. To judge for how long and by how much the health-based guidance value may be exceeded or to characterize the risk in the case of an exposure exceeding the health-based guidance value, an evaluation on a case-by-case basis is needed.

The TTC approach is a pragmatic aid in risk assessment for substances for which no or limited toxicological information is available. It is aimed at establishing a human exposure threshold value for chemicals, below which there is a very low probability of an appreciable risk for human health. Kroes et al. (2000) concluded that it is applicable for immunotoxicology. Further guidance on the application of the TTC principle in risk assessment is provided by Kroes et al. (2004) and Munro et al. (2008). Kroes et al. (2004) proposed a guidance for the application of the TTC principle in risk assessment of low molecular weight chemicals in food. This guidance includes a stepwise approach, allocating different TTCs to different classes of chemicals. Munro et al. (2008) proposed a further refinement of the application of the TTC approach in risk assessment.

This guidance document generally presents the deterministic approach to risk assessment. In some situations and cases, a probabilistic approach is feasible and may be more useful to quantify risk at the population level. For example, in the area of food allergy, a probabilistic approach has been developed and applied, among others, for quantifying risks of cross-contamination of food products with allergens (Spanjersberg et al., 2007, 2010; Kruizinga et al., 2008; Madsen, 2009).

3.2 Application of risk assessment principles to immunotoxicity

Immunotoxicity risk assessment should be performed according to the same principal approaches as applied in risk assessment for other (thresholded) toxicological end-points. There is no evidence suggesting fundamental differences between immunotoxicity and other toxicological areas that would demand a fundamentally different approach in risk assessment. However, the immune system manifests many special aspects (see chapter 2) that need specific consideration in risk assessment. In particular, the lack of harmonized guidelines or consensus regarding data requirements and study protocols for immunotoxicity assessment poses specific complexity for immunotoxicity risk assessment in comparison with other, more developed areas of toxicology.

Exposure to xenobiotics has the capability of producing any combination of the following recognized adverse outcomes (chapter 2): 1) focused or more extensive immunosuppression, 2) increased propensity for allergic disease, including atopy, food allergies and asthma, 3) hypersensitivity reactions directed at the chemical itself, 4) increased risk of autoimmune disease and 5) dysfunctional inflammatory responses of innate immune cells, producing tissue or organ damage or dysfunction.

Each type of immunotoxicity manifests different specific aspects and therefore requires different specific considerations for risk assessment. The different types of immunotoxicity and their specific aspects are addressed in the next chapters, giving risk assessment guidance for immunosuppression (chapter 4), immunostimulation (chapter 5), sensitization and allergic response (chapter 6) and autoimmunity and autoimmune disease (chapter 7). For immunosuppression and sensitization, and to a lesser extent for autoimmunity, risk assessment can be built upon well-established model systems, mechanistic data and experience. This is reflected in a higher level of detail in these chapters relative to that on immunostimulation.

As described in the following chapters, structured approaches can be followed in the risk assessment for each separate type of immunotoxicity. For risk assessors, not only is it important to be able to follow transparent and structured approaches for risk assessment for each type of immunotoxicity, but it is also important to have entry points for immunotoxicity risk assessment. Such entry points are based on the available data for a given chemical and are structured to help the risk assessor determine whether immunotoxicity needs to be considered, and what type of immunotoxicity needs to be considered, in a risk analysis. Therefore, an overview with possible entry points was developed for chemical risk assessors and is described in section 3.4.

Expert judgement of the available information and situation on a case-by-case basis is crucial. Experts in immunotoxicology may need to be consulted for this. The entry points for immunotoxicity risk assessment given in this chapter can also be considered to convey triggers (information retrieved from a data set that alerts for potential immunotoxicity) that might lead to an interaction between a risk assessor confronted with potential immunotoxicity and immunotoxicology experts.

The guidance given in this document should not be considered as an inflexible set of requirements, but rather as an aid to determine when and what type of immunotoxicity needs to be considered and what steps (stepwise approach in risk assessment) might be followed. The (stepwise) approach needs to be attuned to the objective of the risk assessment and the risk management questions. An inherent problem of any risk assessment is the question of how to

identify and deal with knowledge and data gaps. This should be an aspect of specific consideration in the evaluation of the health-related database.

3.3 General considerations for immunotoxicity risk assessment

3.3.1 Introduction

To accurately predict the risk of immunotoxicity from xenobiotic exposures in human populations, a scientifically sound immunotoxicity risk assessment framework is required that supports an accurate and quantitative interpretation of experimental and epidemiological studies and their application to human health risk assessment. Although age-related differences in immune function during development and old age are not well understood, concerns for the special vulnerability of these life stages have been established and must be taken into account. Chemical-related immunotoxicity may be expressed as hypersensitivity (chapter 6), immunosuppression (chapter 4), autoimmunity (chapter 7) or unintended stimulation of immune responses (chapter 5). Immunosuppression has been associated with increased susceptibility to infectious and neoplastic diseases, if the magnitude of suppression is sufficient to allow overgrowth of infectious agents or prevent destruction of spontaneously arising neoplastic cells (see chapter 4). Hypersensitivity (allergic) responses associated with chemical exposure may be the result of a direct allergic response to the chemical or modulation of the immune response that skews the immune response so that allergic responses are more common or of greater severity (see chapter 6). Autoimmune disease following chemical exposure may be caused by a loss of immune system ignorance (tolerance) of self-antigens, by chemical modification of tissues that renders them sufficiently foreign that they engender an immune response or by loss of other control mechanisms that results in self-reactivity (see chapter 7). Unintended stimulation of immune function has received very limited research attention and may or may not be a direct cause of disease (see chapter 5). However, chemicals that cause unintended stimulation have been associated with autoimmune disease and thus may signal dysregulation of homeostatic processes required to maintain the critical balance between adequate immune responses and pathological loss of immune system control.

3.3.2 Clinical and epidemiological data

3.3.2.1 Clinical data

Immunotoxicology data in humans may be derived from well-designed clinical or epidemiological studies, observational studies or case reports. Although controlled clinical studies represent the best opportunity to identify and characterize immunotoxicants, they are not routinely conducted for environmental or occupational chemicals for obvious ethical reasons. Where the ethical issues have been appropriately resolved, immune function data following controlled exposure in humans would require the least extrapolation and present the strongest data for estimating risk for the general population.

3.3.2.2 Epidemiological data

The primary study design employed for evaluation of potential immunotoxicity in humans following exposure to environmental or occupational agents has been the retrospective epidemiological study, usually in individuals with transient high-level occupational exposure or large cohorts with chronic low-level exposures. Although body burdens of the chemical

have been determined in some instances, it can be difficult to draw broadly applicable conclusions from these studies for a number of reasons. Many of the studies are based upon fairly small sample sizes, the subjects may have been exposed to additional chemicals other than those specifically addressed by the study, the population may be self-selected or characterization of exposure (duration and intensity) may have relied upon subject recall. Immunological testing is often limited to one or two tests and designed to identify very severe immunological effects, rather than mild to moderate changes. Studies evaluating changes in host resistance have been reviewed in detail by several authors (Thomas et al., 1995; Vial et al., 1996; Voccia et al., 1999; Luebke, 2002). Some of the more complete immunotoxicology studies in humans have examined persistent organochlorine compounds, formerly found in pesticides and industrial chemicals (e.g. PCBs), in children following prenatal or postnatal exposure (via maternal diet and breast milk) (reviewed in Luster et al., 2004). There is also considerable human evidence for an association between exposure to pesticides and autoimmunity (reviewed in Holsapple, 2002).

Surface marker analysis (immunophenotyping) and serum immunoglobulin levels are the most commonly employed tests to evaluate immunological changes in human studies. These tests are routinely conducted in large hospitals and have provided considerable information on the ontogeny and activation state of the human immune system, as well as assisting in the clinical diagnosis for immunological (e.g. primary immunodeficiency, acquired immunodeficiency syndrome [AIDS]) and haematopoietic disorders. However, care in both experimental design and technical performance is important in order to obtain the sensitivity that would consistently detect subtle changes in the immune system due to considerable interlaboratory and interindividual variability. Shearer et al. (2003), working with the Pediatric AIDS Clinical Trials Group to determine immunophenotype values in a large (n = 807) control population as a function of age, demonstrated variances within each age group that often exceeded 2-fold, despite efforts to control for interlaboratory and intralaboratory methodological differences and discarding the highest and lowest 10th percentile values. In addition to age, immunophenotype values can be greatly influenced by sex, ethnicity and environmental factors (Marti et al., 2002). In many human studies, statistically significant differences have been found between the control and case populations with respect to serum immunoglobulin levels and immunophenotypes. However, because of the large variability in historical control values, case values may be significantly different from control values while still falling within historically normal ranges, making interpretation of the results difficult. An example of this problem can be seen in CD8+ T cell enumeration derived from studies of children with human immunodeficiency virus–1 (HIV-1) infection (Shearer et al., 2000) and halogenated aromatic hydrocarbon exposure (Weisglas-Kuperus et al., 1995). Although differences in the number of CD8+ T cells were statistically significant in both experimental populations, the values were still within normal ranges. This is also an example of the interindividual and age variability that would likely be observed in an observational study. When evaluating routine immune system data collected during epidemiological studies or routine toxicity testing (e.g. immunoglobulin levels, white blood cell [WBC] counts, immunophenotyping), the assessor should generally be less concerned with whether values from the exposed population fall within typically broad historically normal ranges than with whether the changes are statistically different from values obtained in an appropriately matched control population or whether there is a shift in the number of individuals who fall outside of the normal range.

When interpreting human immune data, considerably more credence is given to those studies in which multiple immune tests were conducted and the resulting data provide a biologically

plausible interpretation. When a large number of immunophenotypic markers is examined, an abnormal value in one or two immunophenotypes is likely to result simply from a type 1 error. A more reliable indicator of immunotoxicity would be multiple changes consistent with a specific pattern. For example, it is unlikely that a significant decrease in immunoglobulin level would be observed without a concomitant decrease in certain lymphocyte markers, particularly those associated with B cells. A description of biomarkers in epidemiological studies for general immunotoxicity data is provided in "Report of the Bilthoven symposium: advancement of epidemiological studies in assessing the human health effects of immunotoxic agents in the environment and the workplace" (Van Loveren et al., 1999) and EHC 180: *Principles and methods for assessing direct immunotoxicity with exposure to chemicals* (IPCS, 1996). In addition, epidemiological considerations are presented for characterizing sensitization and allergic response in EHC 212: *Principles and methods for assessing allergic hypersensitization associated with exposure to chemicals* (IPCS, 1999b) and for autoimmunity in EHC 236: *Principles and methods for assessing autoimmunity associated with exposure to chemicals* (IPCS, 2006a). The risk assessor should refer to the epidemiology sections in EHCs 180, 212 and 236 for immunotoxicity, hypersensitization and autoimmunity end-points contained in the data set for the chemical in question to provide specific context, cautions and information that may assist in the interpretation of immunotoxicity data for risk assessment. Because the data for a given chemical may indicate one or more types of immunotoxicity, it is expected that the risk assessor may want to refer to the epidemiology sections of one or more of these three EHC documents and utilize one or more of the following chapters (i.e. chapter 4 for immunosuppression data, chapter 5 for immunostimulation data, chapter 6 for sensitization data and chapter 7 for autoimmunity data) on risk assessment for specific areas of immunotoxicity.

3.3.3 Laboratory animal data

3.3.3.1 Introduction

A basic understanding of the typical methodologies used to evaluate immunotoxicity in laboratory animal models is necessary to evaluate the database of studies for hazard characterization of a given chemical as the first step in risk assessment. Detailed discussions of end-points and methods utilized in characterizing general immunotoxicity are provided in EHC 180: *Principles and methods for assessing direct immunotoxicity with exposure to chemicals* (IPCS, 1996). A similar in-depth discussion of end-points and methods utilized in characterizing sensitization and allergic response is provided in EHC 212: *Principles and methods for assessing allergic hypersensitization associated with exposure to chemicals* (IPCS, 1999b). In addition, a detailed description and evaluation of end-points and methods utilized in characterizing autoimmunity are provided in EHC 236: *Principles and methods for assessing autoimmunity associated with exposure to chemicals* (IPCS, 2006a). The data set for most chemicals is unlikely to contain data on all of the described end-points. The risk assessor should refer to the assay descriptions in EHCs 180, 212 and 236 for immunotoxicity, hypersensitization and autoimmunity end-points contained in the data set for the chemical in question to provide specific context, cautions and information that may assist in the interpretation of immunotoxicity data for risk assessment. The risk assessor may utilize more than one of the following chapters (i.e. chapter 4 for immunosuppression data, chapter 5 for immunostimulation data, chapter 6 for sensitization data and chapter 7 for autoimmunity data) on specific areas of immunotoxicity, because the data for a given chemical may indicate more than one type of immunotoxicity. The following general considerations are provided for

evaluation of any and all data on immunotoxicity available for the chemical under assessment.

3.3.3.2 Considerations in evaluating immunotoxicity data

Many of the standard toxicity testing guidelines published by various agencies, including the USEPA, specify 28 days as the minimum multidose exposure period, although 14-day exposures are common in studies published before the middle to late 1990s and as part of some tiered approaches to immunotoxicity testing or screening, as in EHC 180 (IPCS, 1996). For immunotoxicity hazard characterization, the 28-day exposure period would typically be adequate to elicit a response. Furthermore, the USEPA testing guidelines (USEPA, 1996a,b, 1998) indicate that immunization of animals should be done towards the end of the exposure period and timed so that samples are taken the day after the final exposure. This scheme ensures that all phases of the host response to immunization take place in the presence of the test chemical and its metabolites, maximizing the chance of identifying potential hazards. A significant proportion of immunotoxicity studies published before the middle to late 1990s delayed immunization until after exposure ended, citing concerns that unpredictable or undetectable direct interactions between toxicant and antigen could potentially decrease the immunogenicity of antigens. That approach has largely been replaced by immunization during exposure, although similar results would likely be obtained by either scheme unless toxicity was minimal and toxicant clearance was extremely rapid.

The role of stress and the immune system in chemical-induced immunotoxicity has received considerable discussion. If immunotoxicity is observed at dose levels that do not induce overt toxicity, the test chemical can be considered immunotoxic, independent of whether it occurs via a direct effect on the immune system or an indirect effect, such as induction of a stress response. Given clear evidence of general toxicity, stress-induced immunotoxicity is usually determined using adrenalectomized animals. Changes in serum corticosteroid levels or characteristic leukograms (i.e. decreases in lymphocytes and elevated monocyte numbers) are supportive but not sufficient to definitively establish a stress-induced response.

(a) Sex considerations

Qualitative and quantitative sex-dependent differences in baseline levels of immune function are well known in humans and laboratory animals and in part have been linked to relative levels and response to sex steroids. Although the interaction between the endocrine and immune systems is complex, females typically mount a more robust antibody response than males, whereas cell-mediated responses are more robust in males. Resistance to infections requiring antibody or cell-mediated immune responses generally follows the same pattern of responses. Whereas there is some indication that females are the more reactive responder population to contact allergens (Rees et al., 1989), the weight of evidence suggests that females and males react similarly (Felter et al., 2002). Only recently, with the advent of adult immunotoxicity testing guidelines that parallel general toxicity testing, have male and female rodents been evaluated simultaneously. Historically, male mice have rarely been used in immunotoxicity testing, as they are generally caged separately to avoid the stress response associated with fighting; male rats are somewhat less prone to fighting than male mice and have found wide use in routine toxicity testing. Immune function in both rodents and humans is also known to be affected by the estrous cycle and pregnancy. Sex-dependent differences in the effects of chemicals on immune function have been described, particularly in developmental immunotoxicity studies. For example, female mice exposed to DES early in life are

dramatically immunosuppressed, whereas immune function in males quickly returns to normal (Kalland, 1980); various sex-related differences have also been described in the offspring of lead-exposed rats (Miller et al., 1998; Bunn et al., 2000, 2001a,b). However, there is only limited evidence that these alterations in immune function may affect disease in autoimmune disease–prone mice following developmental exposure (Stoll & Gavalchin, 2000). Data on the potential immunotoxicity in both sexes would reduce uncertainty in the evaluation of immunotoxicity for chemicals with known effects on the endocrine system and should be collected when the test chemical is suspected to be an endocrine disruptor, particularly those that may interfere with levels of sex steroids or end-points mediated by sex steroids. Given the known sex-dependent differences in immune function, consistency in response to immunotoxicants strengthens the weight of evidence for immunotoxicity, but is not required to demonstrate chemical immunotoxicity. A lack of consistency of chemical-induced immunotoxicity in specific assays between sexes does not necessarily represent conflicting data and may represent sex-dependent differences. Negative data from one sex do not disprove positive data from a well-conducted study in the other sex. Data from the more sensitive sex (i.e. with the lowest POD) based on an adverse immune effect demonstrating a dose–response relationship should be used in the risk assessment.

(b) Species and strain considerations

Although the number of chemicals with clear evidence of immunotoxicity in humans is limited, there is general consistency between human evidence from clinical and epidemiological studies and the experimental animal data (Descotes, 2003; see reviews by Vos & Van Loveren, 1998; Koller, 2001). Rodent data on immunosuppressive therapeutics have generally been good predictors of subsequent clinical observations after toxicokinetic adjustments. For example, a comparison of immunosuppressive effects of cyclosporin A among various species (mouse, rat, guinea-pig, dog, Rhesus monkey and human) demonstrated good quantitative and qualitative agreement (IPCS, 1996). Exceptions representing species-specific immunotoxicity exist, notably TCDD. Although TCDD is immunotoxic in multiple species (reviewed in Vos & Van Loveren, 1995, 1998), there are pronounced species differences in sensitivity (Luebke et al., 1994, 1995; Smialowicz et al., 1996). In vitro data may provide information to inform potential species and strain differences in sensitivity to immunotoxicity for a given chemical. For example, median inhibitory concentrations (IC_{50}s) for several known immunotoxicants (tributyltin chloride, cyclosporin A and benzo(*a*)pyrene) were species and assay dependent in several in vitro assays selected to detect potential immunotoxicity (cytotoxicity, cytokine release, myelotoxicity and antigen responsiveness) (Carfi et al., 2007). In cases where species or strain differences in immunotoxicity are demonstrated, data on toxicokinetics and the MOA(s) will help to select the best animal model for immunotoxicity in humans.

At this point, there are no reliable a priori bases for the prediction of species-specific sensitivity or which species would be most sensitive for a particular immunosuppressive effect from an immunotoxicant. A lack of consistency of chemical-induced immunotoxicity in specific assays across species or strains does not necessarily represent conflicting data and may represent species or strain differences. Negative data from one species or strain do not disprove positive data from a well-conducted study in a separate species or strain. In general, data from the most sensitive species or strain (i.e. with the lowest POD) based on an adverse immune effect demonstrating a dose–response relationship should be used in the risk assessment. Data from multiple animal species strengthen the human risk assessment implications of animal data, and therefore most testing guidelines recommend obtaining data from

multiple animal species. For example, immunotoxicity studies that are performed according to the harmonized USEPA Health Effects Test Guidelines for immunotoxicity (OPPTS 870.7800) state that unless absorption, distribution, metabolism and elimination (ADME) data are available and similar in rats and mice, both species should be used for testing (USEPA, 1998).

(c) Exposure duration

A majority of cells of the innate and adaptive immune systems have a relatively short lifetime, ranging from hours to a day for neutrophils and days for lymphocytes, except in the case of memory cells, which can survive for years. As such, the immune system is in a state of constant renewal by precursor cells produced by the haematopoietic system (adult bone marrow) or by clonal expansion of lymphocytes following antigenic stimulation. Soluble products of the immune system also have a limited lifetime as a result of protein catabolism. For example, the half-lives of circulating antibodies range from 3 to 28 days, depending on the immunoglobulin class. Severe suppression of the haematopoietic system or antibody synthesis should be detectable by simply counting circulating myeloid and lymphoid cells and measuring concentrations of serum immunoglobulin classes if chemical exposure spans one or more half-lives of the evaluated preformed immune system components. However, suppression of haematopoiesis or antibody synthesis is unlikely to occur following exposure to chemicals not specifically designed to target the immune system or to block DNA or protein synthesis, suggesting that such nonspecific end-points will lack sensitivity and have low predictive value in subchronic studies.

(d) Age at initial exposure

As the immune system develops and changes during life, this has consequences both for vulnerability to chemical exposure during various life stages as well as for the age at the time of assessment. The effects of chemicals on immunocompetence are most often evaluated in animals that are immunologically mature prior to chemical exposure. The persistence of effects in adult animals has not been systematically evaluated; however, based on data from humans exposed to potent immunosuppressive drugs, the expectation is that immune function returns to normal as immunotoxicants are cleared. In contrast, exposure during gestation or in the early postnatal period has been shown to have much longer lasting effects, persisting for weeks, months or even most of the lifespan of the host (Luebke et al., 2006a; Dietert & Dietert, 2007). Based on experimental animal studies, perturbations of the developing immune system may be manifested as a qualitative (i.e. affecting the developing immune system without affecting the adult immune system) or a quantitative (i.e. affecting the developing immune system at lower doses than in adults) difference. Immune maturation may simply be delayed by toxicant exposure and recover to normal adult levels over time, or, if exposure interferes with a critical step in the maturational process, it may result in lifelong defects in immune function (Dietert & Piepenbrink, 2006b; Luebke et al., 2006a; Dietert & Dietert, 2007).

While infectious diseases are also more common in the elderly than in young adults, it is not the immaturity of the immune system but rather age-related loss of immune responsiveness (i.e. immunosenescence) that is responsible for the increased susceptibility. In the simplest terms, the very young lack immunological experience, and the elderly, in spite of a wealth of experience, are no longer able to respond as well as the younger population. At this time, there are no data to demonstrate that the immune system of the elderly is uniquely susceptible

to immunotoxic agents. However, as a result of immunosenescence, a moderate loss of immune function may have more significant adverse effects in the elderly than in young adults.

(e) Route of exposure

There is no single standard exposure method for immunotoxicology studies, and the "best" method of exposure should match the most likely route of human exposure for a given chemical (IPCS, 1996; USEPA, 1998). For example, the relevant exposure of humans to JP-8 jet fuel occurs primarily through inhalation and dermal contact, and both routes of exposure (dermal: Ullrich, 1999; Ullrich & Lyons, 2000; inhalation: Harris et al., 1997) have been associated with reduced thymus weight and suppression of T cell proliferation in mice. It is generally accepted that the circulating concentration of immunotoxicant or immunotoxic metabolite(s) determines the level of immunotoxicity, and equal concentrations of immunotoxicant in the blood following different routes of exposure should result in similar levels of immunotoxicity. The potential for exposure route–associated differences in metabolism of the toxicant and resulting differential exposure of immune cells and immune tissues should be considered if data are available. Toxicokinetic determinations, performed in conjunction with immunotoxicity investigations, are extremely valuable. In the absence of toxicokinetic data, increased detoxification or clearance associated with dermal or oral exposure is presumed due to reduced absorption.

The possibility of local rather than systemic immune reactions is an additional consideration for immunotoxicity differences associated with route of exposure. For example, chemical immunotoxicity may suppress immune function at the site of exposure, such as inhalation-dependent suppression of the resident macrophage populations of the lung, without affecting immune function of macrophages elsewhere in the body. This may be due to either higher concentrations at the site of exposure or rapid clearance and detoxification as the toxicant enters the circulation. The distinction between local and systemic immunity is covered in greater detail in the following section.

3.3.4 Local versus systemic effects

The concept of distinct local toxicity related to the route of exposure is not unique to immunotoxicology and may result from direct exposure or increased local concentrations of toxicant at the exposure site. The contrast between local and systemic toxicity within general toxicology is illustrated by examples such as local route-dependent histopathological changes associated with the lowest inhalation concentration of furfural of 6 mg/kg body weight per day, well below any observed toxicity associated with oral exposure, in a study that examined route-dependent toxicity of furfural in Fischer 344 rats (Arts et al., 2004a). Route-associated local exposure effects may explain some aspects of local toxicity; in the case of inhalational toxicity of furfural in rats, the observed effects may be exacerbated by the increased local exposure resulting from the particular morphology of the rat nasal cavity (see Kimbell et al., 1997, for discussion of rat nasal exposure). However, unlike most toxicological end-points, for immunotoxicology, the route of exposure is not simply a local increased concentration or uptake issue. There are local, partially independent portions of the immune system associated with each of the major routes of exposure: inhalation, dermal and oral. Therefore, route of exposure is potentially more important for immunotoxicity end-points and the appropriate assay to use in testing for immunotoxicity, as local immunotoxicological effects may occur independent of systemic immunity.

3.3.5 (Ir)reversibility of effects

Human studies indicate that immune function returns to normal following exposure to potent immunosuppressive drugs, and the expectation is that immune function will return to normal as xenobiotics are cleared. Similarly, drug-induced autoimmunity is often ameliorated when the therapeutic that induces self-reactivity is discontinued (see chapter 7). However, persistence of effects in adult animals has not been systematically evaluated, and more long-lasting effects are expected to occur if exposure affects precursor or stem cells. This is not the case for exposure during gestation or in the early postnatal period, where immunotoxicity may have much longer lasting effects than in the adult, persisting for weeks, months or even most of the lifespan of the host (Dietert & Piepenbrink, 2006b; Luebke et al., 2006a; Dietert & Dietert, 2007).

The likelihood of persistence of immunotoxicity is a combined effect of dose, duration, timing and MOA (see additional discussion in section 4.7 of chapter 4, section 5.7 of chapter 5 and section 7.6 of chapter 7). For chemicals that bioaccumulate, increased duration will result in higher effective dose in certain tissues and a potentially longer relative dose after the chemical exposure is removed. As discussed previously in section 3.3.3.2(d) on age at initial exposure, timing of exposure is an important consideration, particularly for early developmental exposure, where the ability to produce persistent immunotoxicity may be categorically different from that associated with adult exposure. The MOA for immunotoxicity and the half-life of cells or mediators involved in the key events of immunotoxicity by particular chemicals directly affect the likelihood of persistence of immunotoxicity. Thus, the probability of persistence is increased for immunotoxicity that involves stem cells in the bone marrow and decreased when the MOA is restricted to short-lived cells, such as peripheral neutrophils.

3.3.6 Biological plausibility

The data for a given chemical may indicate one or more types of immunotoxicity, and therefore the risk assessor may utilize more than one of the following chapters (i.e. chapter 4 for immunosuppression data, chapter 5 for immunostimulation data, chapter 6 for sensitization data and chapter 7 for autoimmunity data) on risk assessment for specific areas of immunotoxicity. The following general considerations for a weight of evidence evaluation of the health-related immunotoxicity database should be performed for all data sets. Further consideration of specific areas of immunotoxicity and the use of risk assessment guidance provided in chapters 4–7 will depend on the data set for a given chemical. Each of these chapters presents a specific weight of evidence evaluation for the particular type of immunotoxicity discussed within the chapter. A general outline of possible entry points for chapters 4–7 based on the particular immunotoxicity data for a chemical of interest is described in section 3.4 to assist the risk assessor in the determination of which chapter or chapters will provide the appropriate guidance for a given data set.

3.3.6.1 Characterization of the health-related database

Each chemical is evaluated to judge whether the available data are sufficient to characterize the potential immunotoxicity hazard—that is, if the data collectively provide enough evidence to judge whether or not a human immunotoxicity hazard could exist. The approach presented below is based on three categories: 1) sufficient evidence for immunotoxicity, 2) sufficient evidence for lack of immunotoxicity and 3) insufficient evidence regarding

immunotoxicity. Data from all studies relevant to immunotoxicity are examined, whether or not immunotoxicity is detected. Data from humans have preference over animal data, provided that the data are of sufficient quality and cover adequate exposure scenarios and end-points. Currently, in vitro data alone are inadequate evidence for immunotoxicity; however, in vitro data can help to establish an MOA when accompanied by in vivo data. The evaluation should describe the database in terms of its strengths, weaknesses, uncertainties and data gaps.

An assessment of data quality contributes to the overall evaluation and characterization of the health-related database for immunotoxicity. The strengths and limitations of each individual study and of the broader array of available chemical-specific data should be considered in the weight of evidence assessment. Such factors as conformance with standard of study conduct (including principles of good laboratory practice), adequacy of study design (e.g. the use of a sufficient number of test subjects, the use of appropriate test methods) and the application of relevant and appropriate analytical techniques all contribute to a scientific judgement regarding data (and/or study) quality.

The weight of evidence considerations are applied to characterization of the data within the same or similar assays as well as across divergent measures of the immune system and across multiple species.[1] Host resistance assays in experimental animals, without immune test data, are sufficient for the determination of relevant immunotoxicity hazard for humans; however, negative data from host resistance assays do not necessarily preclude immunotoxicity. Conflicting data should be evaluated by the strengths and weaknesses (e.g. sample size, exposure duration) of the individual studies, as well as in the context of the remainder of the immunotoxicity database for a given chemical. The analysis evaluates the observed change in host resistance within the entire database, including the degree of suppression; number and type of end-points examined; relevance of dose, route and duration of exposure; and relevance of the challenge used. Although structure–activity relationships (SARs) are currently not well established for immunotoxicity, information on SARs, MOA, toxicokinetic data and other factors may affect the characterization of the database.

As mentioned above, the weight of evidence evaluation of the health-related database generally results in one of three conclusions:

1) *Sufficient evidence for immunotoxicity* indicates that, collectively, the data provide enough information to judge that a human immunotoxicity hazard exists. Data that support such a conclusion include, but are not limited to, the illustrative examples listed below:
   - ✓ Epidemiological studies (e.g. case–control or cohort studies) that demonstrate that exposure is associated with immunotoxicity. Case-studies may support the weight of evidence but are generally insufficient alone to comprise sufficient evidence.

[1] The predictivity of individual assays for a determination of general immunotoxicity (Luster et al., 1992) and host resistance (Luster et al., 1993) was systematically evaluated for over 50 chemicals in the NTP database. Some assays, such as leukocyte or WBC counts, had a concordance or predictive value of less than 50% for immunotoxicity, whereas others (e.g. measures of the antibody response with the PFC assay) had a concordance of up to 78%. The predictive power for general immunotoxicity or reduced host resistance was increased when two assays demonstrated immunosuppression, with certain combinations, such as the PFC and NK cell assays, resulting in over 90% concordance.

- ✓ Evidence of immunotoxicity is demonstrated by the results of a study or studies in humans (e.g. the human repeated insult patch test [HRIPT] for induction of contact sensitization or adequately controlled studies that demonstrate reduced resistance to common infections) or in one or more laboratory animal species that indicate a dose- and treatment-related effect on disease resistance assays and/or other parameters predictive for immunotoxicity (e.g. a local lymph node assay [LLNA] result indicating a contact sensitizer or a decrease in a functional measure of immunity, such as antigen-specific antibody production) that is not secondary to overt systemic toxicity.
- ✓ Evidence of immunotoxicity is demonstrated by data from multiple end-points with limited predictive evidence of immunotoxicity along with supporting evidence that suggests biological plausibility. The evidence of deficits in immune parameters may be supported by other data from in vivo laboratory animal studies (e.g. 14- or 90-day studies) that show gross and histopathological effects in the primary or secondary immune tissues of a nature and extent indicating a high likelihood of an adverse effect on immune function (e.g. lymphoid organ atrophy, reduced bone marrow function, significant changes in haematological end-points). A relationship of the chemical with other known immune system toxicants or SAR would also add to the supporting evidence.

2) *Sufficient evidence for lack of immunotoxicity* would be based on a database that demonstrated i) a lack of immunotoxicity for a range of end-points covering different aspects of the immune system in several species (preferably including humans) and ii) no other toxic effects that are expected to be predictive of immunotoxicity.

3) *Insufficient evidence regarding immunotoxicity* would include databases that i) are inadequate, ii) have conflicting outcomes that cannot be attributed to differences in study design (e.g. different species, strains or exposure durations), iii) present equivocal evidence interpreted as showing marginal deficits in immune parameters that may be chemically related (e.g. statistically significant changes in one or more parameters at middle or low doses in the absence of other supportive data) or iv) are limited by the insufficient number or types of studies that address immunotoxicity (e.g. many general toxicity studies only evaluate thymus weight and provide data that, in the absence of additional evidence, are insufficient to judge immunosuppression hazard). Data that could support such a conclusion include, but are not limited to, the illustrative examples listed below:
   - ✓ In vitro data alone are generally considered insufficient in the absence of in vivo functional data.
   - ✓ Statistically significant changes in the histology of the immune tissues or leukocyte counts without any clear effects on associated functional parameters (e.g. no changes in antibody production or no data on any functional immune measure) or deficits in one or more less sensitive end-points (e.g. lymphoproliferative responses) with no associated histological changes in the spleen, thymus, bone marrow or lymph nodes are generally equivocal evidence of immunosuppression.

As a default, any effect should be taken into consideration in the risk assessment, unless general knowledge or additional data indicate otherwise.

When the determination is made that the database for a given chemical provides sufficient evidence for immunotoxicity or lack of immunotoxicity, the risk assessor is directed to

perform a weight of evidence evaluation, as outlined in section 3.3.6.2, and complete the hazard identification step in the risk assessment. As described in section 3.1, it is expected that the assessment will continue through the hazard characterization (or dose–response assessment), exposure assessment and risk characterization steps.

Alternatively, when the determination is made that the database for a given chemical does not provide adequate evidence of immunotoxicity or lack of immunotoxicity, a separate analysis can be performed to evaluate the need for additional data to determine the potential immunotoxicity hazard. A detailed approach for this analysis is presented in section 3.3.6.3.

3.3.6.2 Weight of evidence evaluation of the health-related database

The NTP of the United States Department of Health and Human Services outlined five categories ranking the weight or strength of evidence of immune system toxicity from stronger evidence to uncertain findings (Germolec, 2009). The NTP's explanation of this system for summarizing the strength of evidence for immunotoxicity can be used for evaluating individual studies as well as bodies of evidence. Although the NTP's level of evidence explanation is focused on immunosuppression, the principles can be used to evaluate immunotoxicity in general.

The NTP describes the results of individual studies of chemical agents and other test articles and notes the strength of the evidence for conclusions regarding each study. Generally, each study is confined to a single laboratory animal species, although in some instances, multiple species may be investigated under the purview of a single study report. Negative results, in which the study animals do not exhibit evidence of immunotoxicity, do not necessarily imply that a test article is not an immune system toxicant, but only that the test article is not an immune system toxicant under those specific conditions. Positive results demonstrating that a test article causes immunotoxicity in laboratory animals under the conditions of the study are assumed to be relevant to humans, unless data are available that demonstrate otherwise. In addition, such positive effects should be assumed to be primary effects, unless there is clear evidence that they are secondary consequences of overt toxicity to non-immune organ systems.

It is critical to recognize that the "levels of evidence" statements described herein describe only immunological *hazard*. The actual determination of *risk* to humans requires exposure data that are not considered in these summary statements. This fact is particularly important to keep in mind when communicating study results to the general public.

Five categories of evidence of immune system toxicity are used to summarize the strength of the evidence observed in each experiment: two categories for positive results (*clear evidence* and *some evidence*); one category for uncertain findings (*equivocal evidence*); one category for no observable effects (*no evidence*); and one category for experiments that cannot be evaluated because of major design or performance flaws (*inadequate study*). Application of these criteria requires professional judgement by individuals with ample experience with and understanding of the animal models and study designs employed. For each study, conclusion statements are made using one of the five categories to describe the findings; if warranted, these conclusion statements should be made separately for males and females. These categories refer to the strength of the evidence of the experimental results and not to potency or mechanism.

The levels of evidence for evaluating immune system toxicity are as follows:

1) *Clear evidence of toxicity to the immune system*
   - Is demonstrated by data that indicate a dose-related[1] effect (considering the magnitude of the effect and the dose–response) on more than one functional parameter and/or a disease resistance assay that is not a secondary effect of overt systemic toxicity, or
   - Is demonstrated by data that indicate dose-related effects on one functional assay and additional end-points that indicate biological plausibility.

2) *Some evidence of toxicity to the immune system*
   - Is demonstrated by data that indicate a dose-related effect on one functional parameter with no other supporting data, or
   - Is demonstrated by data that indicate dose-related effects on multiple observational parameters without robust effects on a functional immune parameter or a disease resistance assay, or
   - Is demonstrated by data that indicate effects on functional parameters or a disease resistance assay that are not dose related with other data providing biological plausibility.

3) *Equivocal evidence of toxicity to the immune system*
   - Is demonstrated by data that indicate effects on functional parameters or a disease resistance assay that are not dose related without other data providing biological plausibility, or
   - Is demonstrated by data that indicate dose-related effects on a single observational parameter without effects on a functional immune parameter or a disease resistance assay, or
   - Is demonstrated by data that indicate effects on the immune system at dose(s) that produce evidence of overt systemic toxicity, or
   - Is demonstrated by data that are conflicting in repeat studies.

4) *No evidence of toxicity to the immune system*
   - Is demonstrated by data from studies with appropriate experimental design and conduct that are interpreted as showing no evidence of biologically relevant effects on the immune system that are related to the test article.

5) *Inadequate study of immune system toxicity*
   - Is demonstrated by a study that, because of major design or performance flaws, cannot be used to determine the occurrence of immune system toxicity.

When a conclusion statement for a particular study is selected, consideration must be given to key factors that would support the selection of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of immunotoxicity studies in laboratory animals, particularly with respect to the interrelationships between end-points, impact of the effect on immune function, relative

[1] The term “dose-related” describes any dose–response relationship, recognizing that the test article–related responses for some end-points may be non-monotonic due to saturation of exposure or effect, overlapping dose–response behaviours, changes in immunological manifestations at different dose levels or other phenomena.

sensitivity of end-points and specificity of the effect. Factors to consider in selecting the level of evidence of immune system toxicity are given below:

- Immunotoxicity is defined in the context that immune responses can be enhanced or suppressed by toxicants. As such, dose-related effects consistent with immunosuppression and immunostimulation will be considered in hazard identification.

- Functional effects, as defined as an alteration in the ability of the immune system to respond to a challenge or stimulus, should usually be weighed more heavily than observational parameters, such as alterations in cell counts.

- Increases in severity and/or prevalence (more individuals with the effect) as a function of dose generally strengthen the level of evidence, keeping in mind that the specific manifestation may be different with increasing dose. For example, histological changes at a lower dose level may reflect deficits in immune function at higher dose levels.

- Biological plausibility for immunotoxicity must be considered in the context of the nature of the response, the magnitude of the response and the pattern of the response, as well as the current understanding of immune system structure and function.

- Insights from supportive studies (e.g. toxicokinetics, ADME, computational models, structure–activity relationships) and immunological findings from other in vivo animal studies (NTP or otherwise) should be drawn upon when interpreting the biological plausibility of a change.

- The characterization of immunotoxicity must consider the impact of overt toxicity (e.g. effects on the immune system are not the direct effects of test article treatment, but are indirect effects mediated via stress and/or other dose-related responses).

- The characterization of immunotoxicity must consider the intended pharmacology of the test article. Immunotoxicity is reserved for unintended immunosuppression or immunostimulation.

- Results in one species or one sex are considered sufficient for evidence of immunotoxicity.

3.3.6.3 “Triggers” and the need for additional data to determine immunotoxicity hazard

Triggers for immunotoxicity testing may stem from available data on the chemical, such as epidemiological information or data from in vitro screening or mechanistic studies. Immune system–related data obtained in routine toxicity testing (e.g. immune system organ weights) are not definitive indicators of immunotoxicity but may be the only data available for a specific agent. Significant effects in the absence of overt toxicity suggest that functional testing would provide reliable data for risk assessment. A key issue in determining the need for special immunotoxicology studies is the identification of appropriate “triggers” or causes for concern. Several reviews have previously described potential triggers based on some evidence of immunotoxicity (Holsapple et al., 2005; Ladics et al., 2005). However, excluding extended histopathology (Germolec et al., 2004a,b), the ability of data that trigger specific immunotoxicity testing to accurately predict immunotoxicity has not been established. These

data may still provide sufficient evidence to support evaluation of immune function. Factors to consider include:

- *SARs and MOA alerts*: There are no public databases that can be used for computational determination of immunotoxic potential. Historical immunotoxicity data support the notion that chemicals with a similar structure or the ability to bind certain receptors may modulate immune function. Although structural classes are relatively broad, heavy metals (e.g. lead, cadmium), halogenated aromatic hydrocarbons (TCDD and dioxin-like PCBs), polycyclic aromatic hydrocarbons (e.g. benzo(*a*)pyrene and related bay region–containing compounds that are metabolized to diol epoxides) and various mycotoxins all cause adverse effects on immune function. MOA relationships are also somewhat general and include alteration of cellular proliferation or redox state and binding to intracellular or membrane receptors associated with immunomodulation. Examples include receptors for peroxisome proliferators, opiates, agonists and antagonists for steroid hormones (androgenic and estrogenic compounds) and muscarenic agents.

- *Routine toxicology studies*: A prime routine toxicology study is the 28-day oral toxicity study in rodents. This study involves screening a range of end-points across multiple organ systems, following exposure via the route that is expected to be most relevant to humans. Indicators of potential immunotoxicity include certain changes in haematological profiles, lymphoid organ weights and lymphoid organ histopathology. For example, whereas alterations in WBC numbers or differential WBC counts are not particularly sensitive indicators of immunotoxicity, neutropenia is a common finding in patients receiving immunotoxic drugs. Even small changes in WBC counts or phenotypes are associated with mortality risk in older individuals (Izaks et al., 2003) and development of cold sores in the general population (Parks et al., 2007). There are some cellular alterations that are nonspecific indicators of an effect on the immune response (e.g. increased numbers of macrophages in lung tissue or an increased incidence of inflammatory dermal lesions). Changes in lymphoid organ weights, including the thymus, spleen or bone marrow, may also indicate immunotoxicity. Thymus atrophy is a more sensitive indicator than weight changes in the lymph nodes or spleen, as the thymus is a primary lymphoid organ and the latter represent secondary lymphoid organs. Microscopic findings or histopathology of spleen, thymus and lymph nodes, when conducted under more defined conditions such as those proposed by Elmore (2006a,b,c,d,e), can also be a sensitive indicator of immunotoxicity.

- *Clinical and observational data*: Transmission of infectious disease is unlikely to occur in properly run specific pathogen–free animal facilities. However, a dose-related increase in spontaneous infectious disease in test animals is strong evidence of compromised host resistance and should trigger studies to determine the underlying cause of the increased infection incidence.

- *Range of values and statistical analysis*: When evaluating routine immune system data collected during epidemiological studies or routine toxicity testing (e.g. immunoglobulin, WBC counts, immunophenotyping), the assessor should put little weight on whether values within the exposed population fall within typically broad historically normal ranges and more on whether the changes are statistically different from values obtained in an appropriately matched control population. It should be cautioned,

however, that as immunotoxicity testing in humans normally includes performing multiple, often functionally overlapping tests, the most convincing evidence of altered immune function is alteration in several parameters that share a biologically plausible profile of change, rather than a statistically significant difference in a single parameter. Appropriate data analysis methods must be used when numerous markers are examined (e.g. a large suite of lymphocyte surface markers) to avoid type 1 statistical error.

3.3.7 Dose–response relationships and thresholds

The determination and evaluation of the dose–response relationship are important steps in the qualitative immunotoxicity hazard characterization. A dose–response relationship is a necessary criterion in demonstrating chemical immunotoxicity. In addition to identifying the shape of the dose–response curve and the effective dose range for immunotoxicity, factors such as exposure (route, timing and duration), toxicokinetics and other issues that might affect comparisons with human exposure scenarios are identified and discussed as part of the dose–response evaluation. The results from dose–response analyses can be used in various ways, depending on the goals of the risk assessment and the nature of the effect modelled: establishment of a health-based guidance value (RfD/RfC or ADI/TDI), estimation of the margin of exposure or quantitative estimation of the magnitude of the risk at the level of human exposure (see IPCS, 1999a, 2009, for detailed discussion). The interpretation of dose–response data should identify doses associated with the adverse effect (immunotoxicity), as well as doses associated with no adverse effects, to determine the most appropriate end-point(s) or critical effect(s). For sensitization and subsequent elicitation, there are likely to be different thresholds and dose–response relationships, and the elicitation dose is generally lower than the dose required to induce sensitization (see chapter 6 for further discussion). For the development of health-based guidance values, the critical effect or effects are used for the development of POD(s) from which an RfD/RfC or ADI/TDI can be calculated. The identification of a critical effect based upon immunotoxicity data is likely to involve considerable judgement and should therefore be facilitated by the inclusion of appropriate immunotoxicology experts in the risk assessment process.

3.3.7.1 Shape of the dose–response curve

As is the case for most non-cancer end-points, the dose–response functions for chemical-induced immunotoxicity are generally assumed to be non-linear[1] and to demonstrate a threshold dose below which effects on immunity would not be expected. Additionally, based upon our current understanding of immune processes in humans and available human study data (reviewed in Luster et al., 2005a), one would most likely assume that a linear relationship exists between the loss of immune function and increased disease. The immunotoxicology literature contains examples of non-linear and biphasic dose–response curves across the spectrum of immunological measures, such as lymphocyte proliferation, antibody production, phagocytosis, DTH and host resistance assays. The assumption of a threshold dose as the POD for risk assessment would be supported by the available data for these types of immunotoxicity-related end-points.

[1] The term “non-linear” is used here in a more narrow sense than its usual meaning in the field of mathematical modelling. In this guidance document, the term non-linear refers to threshold models (which show no response over a range of low doses that include zero) and some non-threshold models (e.g. a quadratic model, which shows some response at all doses above zero). In this guidance document, a non-linear model is one whose slope is zero at (and perhaps above) a dose of zero.

In addition to linear and biphasic (threshold) dose–response relationships, inverted U-shaped dose–response relationships have been demonstrated following exposure to immunotoxic compounds (reviewed by Calabrese, 2005). The existence and validity of non-linear effects in immunotoxicology have long been recognized (Dietert, 2005; Hastings, 2005; Holladay et al., 2005; Ladics & Loveless, 2005). Immune function and regulation involve the integration of the endocrine, immune and nervous systems; chemical exposure may produce a complex series of effects that result in stimulation of some measure or at some concentrations, while producing suppression of other measures or at other concentrations (Hastings, 2005). One example of this type of effect is the low-dose stimulation (3- to 6-fold) and high-dose suppression (0- to 3-fold) of the proliferative response of peripheral blood lymphocytes to phytohaemagglutinin (PHA) observed in rats exposed to methylmercury (0.35 μg/kg body weight per day and 35 μg/kg body weight per day, respectively) in drinking-water (Ortega et al., 1997). Holladay et al. (2005) suggested that non-linear responses within the immune system may represent different MOAs for different portions of the curve; that is, the stimulation portion of the curve may represent a different mechanism or a response from different subsets of immune cells compared with the suppression portion of the curve. In contrast, the increase in proliferation routinely observed at the low end of the dose–response curve in mitogen proliferation assays may simply reflect the activation of DNA repair mechanisms that would ensue following cellular damage. When evidence indicates that the observed response can be attributed to distinct effects of different dose-based or exposure duration–based MOAs, the NOAELs/LOAELs for each effect should be considered separately. Although interpretation of these types of responses may be complicated, it should not be assumed that because immunotoxicity is considered adverse, stimulation of the immune system should be considered positive or beneficial. Stimulation of the immune system can lead to hypersensitivity and asthma or autoimmune reactions with severe adverse consequences (interpretation of immunotoxicity associated with stimulation of the immune system is discussed in chapter 5 for immunostimulation, chapter 6 for sensitization and chapter 7 for autoimmunity).

3.3.7.2 Mode of action and dose metric

Information about the MOA for the identified immunotoxicity end-points (including characterization of the key events) and related toxicokinetic data may aid the risk assessor in evaluating proposed dose–response models. In particular, knowledge of the expected dose–response relationship (e.g. threshold, linear or inverted-U shaped) for the end-points of concern can support the selection of a specific model (in cases where available data support quantitative modelling). MOA information can also increase confidence in the methods used to extrapolate immunotoxicity risk from experimental animal data to humans.

The choice of an appropriate dose metric will more accurately reflect the relationship between exposure and response. The underlying assumption of the dose–response analysis is that the appropriate dose metric will reflect the same response at equivalent doses, independent of species and route of exposure, provided that there are no toxicodynamic (pharmacodynamic) differences (Andersen & Dennison, 2001; Clewell et al., 2002). The selection of the dose metric may include consideration of the active form of a chemical, the tissue or system affected, persistence of the chemical in the body and available MOA information indicating whether the specific effect of concern is more dependent on average, peak or frequency of exposure. Many metrics have been proposed, including the maximum tissue concentration and area under the concentration versus time curve (AUC). Use of metrics other than dose in milligrams per kilogram of body weight per day may suggest

consideration of alternative approaches or values to address interspecies extrapolation or intraspecies variability (see below). For example, the AUC for corticosterone has been used successfully as the dose metric for immunotoxicity of some end-points (e.g. antibody response to KLH) in several chemicals that induce a stress response; however, suppression of some measures (e.g. NK cell function) were greater than predicted on the basis of corticosterone, indicating other mechanisms of immunotoxicity and that AUC would not be appropriate in these cases (Pruett et al., 2003).

3.3.7.3 Dose–response analytical approaches

In the establishment of a health-based guidance value, margin of exposure or quantitative estimate of the magnitude of risk at the level of human exposure, dose–response assessment allows estimation of PODs from which an RfD/RfC or ADI/TDI can be derived (USEPA, 1994, 1995a, 2000b; IPCS, 1999a, 2009). The BMD or benchmark concentration (BMC) approach is often the preferred method for determination of POD(s) near the low end of the available data; for example, the BMD approach is preferred by the USEPA (1995a, 2000a,b). If the relevant data are not amenable to quantitative modelling, the NOAEL/LOAEL method may be used. The BMD methodology that is used for estimating both the BMD and the BMC is presented in detail elsewhere (USEPA, 1995a, 2000b; IPCS, 2009), and application of these procedures to immunotoxicity data is done with standard techniques. In general, PODs are developed from the most sensitive adverse immune end-point(s) from the most appropriate species (or the most sensitive mammalian species in the absence of information to determine the most appropriate species). The majority of immunotoxicity data are continuous; therefore, when applying BMD methodologies to data on immunotoxicity, "relative risks" are usually estimated. In contrast, histopathological data are likely to be dichotomous, and BMD methodologies estimate "extra risk" for this type of data. While directed immunotoxicity studies may not be available for many chemicals, histopathological examination of immune tissues is often performed as part of standard toxicological studies. Therefore, histopathology may be the only available immune-related data for some chemicals. Extended histopathological analyses of a semiquantitative nature have been shown to be good predictors of immunotoxicity. Studies that examined the sensitivity of extended histopathology suggest that lesions in the thymic cortex were the most consistent (Germolec et al., 2004b), and these lesions correlated with thymus to body weight ratios and, to a slightly lesser extent, antigen-specific antibody responses (Germolec et al., 2004a).

3.3.7.4 Dose–response risk assessment output: health-based guidance values

Current practices for health-based guidance value derivation and the application of dosimetric adjustments and uncertainty factors are detailed elsewhere (IPCS, 1999a, 2009; USEPA, 2002). The dose–response analysis should describe how the RfD/RfC or the ADI/TDI was calculated and should include a discussion of the assumptions, dosimetric adjustments and uncertainty factors used, as well as the confidence in the estimates. Discussion of the application of uncertainty factors specific to immunotoxicity is provided below in section 3.3.10. To derive health-based guidance values for immunotoxicity (i.e. use immunotoxicity data as the critical effect), the immunotoxicity-based POD for the adverse treatment-related response is divided by the total uncertainty factor (a multiple of either default uncertainty factors or chemical-specific adjustment factors [CSAFs]) to derive the RfD/RfC or the ADI/TDI. In each case, immunotoxicity data should be discussed in the context of other toxicity data.

Health-based guidance values are frequently derived for chronic exposure or intake over a lifetime that is considered to be without appreciable health risk. However, some organizations (e.g. WHO and some USEPA programme offices) have established practices for the application of less-than-lifetime risk calculations. The USEPA publication *A review of the reference dose and reference concentration* (USEPA, 2002) discusses the use of toxicity data in deriving reference values in risk assessments for exposures of various durations, including chronic, intermediate term and acute. WHO has published guidance for setting acute reference doses (ARfDs) that is specific for consideration of the acute effects of pesticides (Solecki et al., 2005). Immunotoxicity data that identify adverse outcomes following defined toxicant exposures are considered appropriate for these applications.

3.3.8 Groups at risk (developing immune system, elderly, immunocompromised)

Age-related physiological differences and immaturity of the immune system are both likely to play a role in increased susceptibility to chemical modulation. It has been demonstrated that for some chemicals, the immature immune system is more susceptible to chemicals than the fully mature system. Sequelae of developmental immunotoxicant exposure may be more persistent than effects observed following adult exposure, which generally occur at higher doses and are expected to resolve soon after exposure ends (Holladay & Smialowicz, 2000; Dietert & Dietert, 2007). Based on results obtained in various experimental animal studies, perturbations of the developing immune system may be manifested as a qualitative (i.e. affecting only the developing immune system) or a quantitative (i.e. lower doses affect the developing immune system) difference. Following developmental exposure, immune maturation may simply be delayed and recover to normal adult levels over time, or, if exposure interferes with a critical step in the maturational process, lifelong defects in immune function may follow (e.g. DES: Kalland & Forsberg, 1980; Kalland, 1984). The steps involved in human and rodent immune system maturation appear to be remarkably similar, but at different developmental stages, and no compelling evidence exists to suggest that effects observed in rodents are not representative of what might be expected to occur in humans. Thus, effects of rodent exposure shortly after birth are likely to reflect what may happen in humans exposed during late gestation, assuming that the chemical crosses the placenta. This concept was reviewed in detail by Holladay & Smialowicz (2000) and Holsapple (2003).

The elderly are also acknowledged as a potentially sensitive population, and age-related changes in immune function are described in EHC 144 (IPCS, 1993). The age-related decline in immune function and homeostasis at the molecular, cellular and organism levels is referred to as immunosenescence, a condition characterized by reduced immunocompetence (effector and regulatory function), increased rates of infection, autoimmune disease, inflammation and neoplasia (for reviews, see Hausman & Weksler, 1985; Miller, 1996; DeWitt & Luebke, 2009). Decreased immune system function associated with the advancement of age has been described for multiple aspects of the immune system (e.g. response to vaccination: Targonski et al., 2007; asthma: Vignola et al., 2003). Additionally, there may be a correlation between deterioration of normal immune function and higher incidences of some cancers (Cohen, 1994) and/or autoimmune diseases that are observed in the elderly (Goronzy & Weyand, 2003).

Enhanced susceptibility may also be a function of genetic disposition (i.e. genetic polymorphisms at the individual or population level), disease state (e.g. AIDS) or pharmaceutical intervention (e.g. organ transplant therapy). Sex differences in response to immunotoxic agents are not uncommon, either in laboratory animal studies or in epidemiological studies.

3.3.9 Acute versus chronic exposure

Developmental exposure to an immunotoxicant may result in quantitatively and qualitatively different immunotoxicity compared with exposure at other life stages (see discussion in chapter 2, in section 3.3.3.2(d) on age at initial exposure and in section 3.3.8 on groups at risk) and may result in long-lasting or permanent immunotoxicity after an acute or short-term exposure. Although there are no testing guidelines in widespread use that are designed to evaluate acute or developmental immunotoxicity at this time, the use of less-than-lifetime risk estimates may be appropriate for chemicals with a sufficient database demonstrating developmental immunotoxicity.

3.3.10 Uncertainty factors

As is the case for other areas of toxicological risk assessment, the use of uncertainty factors in immunotoxicity risk assessment and considerations in establishing overall factors based on various subfactors depend on the regulatory framework and/or the scope and purpose of the risk assessment. Detailed discussion of the application of uncertainty factors in human health risk assessment can be found elsewhere (IPCS, 1994, 1999a; USEPA, 2002; FAO/WHO, 2009), and the following discussion is focused on the use of uncertainty factors in the development of immunotoxicity risk assessments. Although generally applied values for the factors are discussed, it is emphasized that the numerical values for each factor (generally ranging from 0.1 to 10) need to be established on a case-by-case basis depending on the science, scope and regulatory framework. Particular caution is needed for preventing over-conservatism in combining various subfactors into one overall factor. Risk assessment for immunosuppression (chapter 4), unintended stimulation (chapter 5) and autoimmunity (chapter 7) should use all of the standard uncertainty factors (intraspecies, interspecies, database) as well as uncertainty factors addressing subchronic to chronic extrapolation and LOAEL to NOAEL extrapolation, as determined by the scope of the risk assessment and the data used for the POD. Considerations in the application of intraspecies (or interindividual), interspecies and database uncertainty factors for immunotoxicity data are described below. Uncertainty factors applied to hypersensitivity generally include intraspecies, interspecies, matrix, and use and time factors and may include database uncertainty factors (see chapter 6).

It should be noted that, in some cases, specific knowledge is available on the basis of which a certain correction could be made in the risk assessment—for instance, where there is information that dermal absorption for humans is higher or lower than for the animal species on which the toxicological data are based. In such a case, a data-derived adjustment factor may be applied in the risk assessment.

3.3.10.1 Intraspecies uncertainty factor

In the absence of information on the potential variability in susceptibility among the general population to the particular type of immunotoxicity considered for a POD, a default intra-species uncertainty factor of 10 is recommended, similar to that used for other non-cancer end-points. This uncertainty factor is used to address the variability in responses from one human to the next and protect sensitive subpopulations. The intraspecies uncertainty factor can be subdivided into toxicokinetic (e.g. $10^{0.5}$ or 3.2 in IPCS [1994, 1999a] or $10^{0.5}$ or 3 in USEPA [2002]) and toxicodynamic (e.g. $10^{0.5}$ or 3.2 in IPCS [1994, 1999a] or $10^{0.5}$ or 3 in USEPA [2002]) components that can be replaced by CSAFs when human toxicokinetic and toxicodynamic data exist for a given compound. As discussed in section 3.3.8, age-related

physiological differences and immaturity of the developing immune system are likely to affect susceptibility to immunotoxicity. As a result, the young (in utero and postnatal exposure or children) and the elderly may be at greater risk for immunotoxicity. Genetic polymorphisms are also potential sources of variability in susceptibility for immunotoxicity associated with chemical exposure, as has been demonstrated for the response to vaccination (e.g. Hennig et al., 2008; Ovsyannikova et al., 2008) and for the development of certain autoimmune diseases (e.g. Rose & Mackay, 2006). The risk assessor should consider a reduction in the intraspecies uncertainty factor if data for the POD are derived from the most sensitive subpopulation of humans.

3.3.10.2 Interspecies uncertainty factor

When PODs for immunotoxicity are derived from human data, no interspecies uncertainty factor is required, and it is set to 1. However, the availability of dose–response data in humans is generally limited, and therefore the extrapolation of laboratory animal data to humans is often necessary. Available data support this approach, because immunotoxicity data from experimental animals are generally good predictors for subsequent clinical data or epidemiological studies. The route and level of exposure in the data set should be compared with those of expected human exposures.

As for other non-cancer end-points, a default interspecies uncertainty factor of 10 is recommended to extrapolate from laboratory animal species to humans when animal data are used to derive PODs for immunotoxicity. Similar to the intraspecies uncertainty factor, the interspecies uncertainty factor can be subdivided into toxicokinetic (e.g. $10^{0.6}$ or 4 in IPCS [1994, 1999a] or $10^{0.5}$ or 3 in USEPA [2002]) and toxicodynamic (e.g. $10^{0.4}$ or 2.5 in IPCS [1994, 1999a] or $10^{0.5}$ or 3 in USEPA [2002]) components. Rather than applying these default interspecies uncertainty factors, data-derived uncertainty factors or CSAFs can be used when sufficient data are available to address species differences in the toxicokinetics for the chemical under evaluation. For example, the USEPA RfC process describes the interspecies adjustment from laboratory animal exposure to a human equivalent concentration via dosimetric adjustment factors (USEPA, 1994, 2002). This process applies to the toxicokinetic aspects of cross-species extrapolation and does not address toxicodynamic differences that may exist between species. Recent harmonization efforts at the USEPA advocate the adoption of body weight raised to the ¾ power ($BW^{3/4}$) scaling for RfD derivation in instances where there are limited data with which to perform an assessment (USEPA, 2011). Therefore, when species-specific data are available, there are procedures to apply CSAFs for both the RfC and the RfD (USEPA, 2002). In the case that information on interspecies variability is available—for instance, in the form of comparison of kinetic data or internal doses—a data-derived adjustment factor can be applied for the species-to-species extrapolation.

3.3.10.3 LOAEL to NOAEL uncertainty factor

As with other non-cancer end-points in toxicology, use of an additional uncertainty factor (usually a factor of 10, unless indicated otherwise, depending on the dose–response data and/or regulatory framework) is recommended when a LOAEL is used to derive the POD, rather than a NOAEL or BMD.

3.3.10.4 Subchronic to chronic uncertainty factor

The scope of the assessment outlined in the problem formulation phase will determine the need to apply an uncertainty factor for study duration. If the assessment is for risk over a lifetime of exposure (i.e. chronic) and the data used to derive the POD are from a subchronic exposure study, then an additional uncertainty factor (10 or 3, depending on study duration) is applied to extrapolate the risk from subchronic to chronic exposure. This uncertainty factor is often not applied to hypersensitivity data. First, hypersensitivity reference values for skin or respiratory sensitization are often derived for daily exposure, so the extrapolation would not apply. Second, sensitization and hypersensitivity may develop after relatively few exposures or may develop after years of exposure, as for hypersensitivity to halogenated platinum compounds (Merget et al., 2000). The use of a subchronic to chronic uncertainty factor for hypersensitivity is therefore a matter of debate and should be evaluated on a case-by-case basis for the compound in question.

3.3.10.5 Database uncertainty factor

Examination of the extent of the database, the quality of individual studies and data gaps will assist the weight of evidence determination of database sufficiency. Although there is no regulatory guidance for determination of database sufficiency, the following discussion provides examples where use of a database uncertainty factor for lack of immunotoxicity data may be appropriate.

The database for a chemical may not contain any toxicity studies specifically designed to determine immunotoxicity. Nevertheless, risk assessors determine which of the available data may provide information on immunotoxicity. In some cases, a limited data set may suggest the possibility of immunotoxicity without appropriate data to make a determination of immunotoxicity risk or perform a dose–response assessment. In such cases, the risk assessor should consider the use of the database uncertainty factor to indicate that the lack of information on this end-point may be significant. In addition, the risk assessor may be in the position of requesting additional data. The following discussion, while not comprehensive, is intended to present some examples of situations where additional data may be necessary to characterize the immunotoxicity hazard and therefore where a database uncertainty factor or request for additional data should be considered.

The database uncertainty factor is intended to account for the potential for deriving an underprotective reference value as a result of a database that leads to an incomplete characterization of a chemical's toxicity. When characterizing the overall toxicity of a xenobiotic, a database uncertainty factor is often applied in the absence of certain toxicity studies, such as a two-generation reproduction study; however, a value is generally not applied a priori for databases lacking immunotoxicity studies. Instead, evidence of immunotoxicity may contribute to the database uncertainty factor in several ways, depending on the weight of evidence analysis for immunotoxicity (see weight of evidence discussion in section 3.3.6.2 above). Therefore, the database uncertainty factor reflects the conclusion of the weight of evidence evaluation and the potential for additional data to affect the PODs for the particular reference value under development. Note, as illustrated in the following example, that the potential for additional immunotoxicity data to affect the POD is an important factor to consider in the application of the uncertainty factor:

- If sufficient evidence indicates that a human immunotoxicity hazard could exist and the limited data suggest that more extensive data might decrease the POD, this is taken into account in assigning the database uncertainty factor. On a case-by-case basis, the application of an uncertainty factor of 3 or 10 is suggested for this type of database deficiency.

There are several situations where insufficient evidence may be available to judge whether or not an immunotoxicity hazard exists. The implications for the database uncertainty factor differ depending on the reason why the evidence is insufficient to judge immunotoxicity and the purpose of the risk assessment. In cases of insufficient evidence, the risk assessor is faced with three options: 1) not performing a risk assessment, 2) performing a risk assessment on the most sensitive end-point without adjustment for the immunotoxicity-related uncertainty or 3) performing a risk assessment on another end-point with a database uncertainty factor to characterize the immunotoxicity-related uncertainty. An additional consideration is how to treat a complete lack of immune data to inform the evaluation of potential immunotoxicity for a given chemical. The following examples illustrate important considerations in the use of database uncertainty factors to address major gaps in the database that affect the ability to conclude whether or not a given chemical presents an immunotoxicity hazard:

- If the available data do not provide any evidence for immunotoxicity (i.e. no change in any immune parameter including immune organ weights in standard toxicological studies and a complete lack of immunotoxicity studies or no evidence of immunotoxicity from available studies), then immunotoxicity does not contribute to the database uncertainty, and the database uncertainty factor for immunotoxicity is set to 1. As discussed above, the risk assessor must determine if a complete lack of data on immunotoxicity requires the use of a database uncertainty factor (note: this is likely to differ depending on the purpose of the risk assessment and the regulatory mandates involved). The conservative approach is to use the maximum database uncertainty factor of 10 when there is a complete lack of immune data. Therefore, setting the database uncertainty factor to 1 under the conservative approach assumes that a minimum data set of studies with immune data is available for the chemical in question.

- If there is equivocal evidence, limited evidence or conflicting evidence that cannot be attributed to differences in study design, the potential that data to address the uncertainty might decrease a POD is taken into account in assigning the database uncertainty factor. On a case-by-case basis, the application of an uncertainty factor of 3 or 10 is suggested to address database deficiency. Additional considerations and examples wherein data from the available toxicology studies raise suspicions of immunotoxicity are detailed in section 3.3.6.3.

The size of the database uncertainty factor to be applied will depend on the available data for the weight of evidence evaluation of immunotoxicity as well as the completeness of the overall database of toxicity studies and on how much impact the missing data may have on determining a POD or PODs.

3.3.11 Exposure assessment

Exposure assessments are used to obtain an estimate of human exposure in order to help quantify the risk to a population. Specific guidelines on exposure assessment (e.g. USEPA, 1992; IPCS, 2006a, 2009) and guidance specific to assessing the exposure of children (e.g.

USEPA, 2005a; IPCS, 2006b) have been published separately and will not be discussed here. Rather, issues important for exposure assessment for immunotoxicity will be addressed. The resulting exposure estimate is a function of the behaviour of the exposed population and the amount of the agent available for potential exposure. Exposure assessments may consider the frequency, magnitude, duration, schedule, source and route of human exposure as well as bio-availability to humans and any special features of the population that will affect exposure. Information may be developed from monitoring data, from estimates based upon modelling of environmental exposures and from application of paradigms to exposure databases.

3.3.11.1 Exposure-related effects on severity and persistence

Interactions between the biology of various types of immunotoxicity and exposure paradigms are relevant for exposure assessment of immunotoxic agents. The exposure paradigm may be defined at the particular point in time when exposure occurred or may reflect cumulative exposures. Each approach makes an assumption about the underlying relationship between exposure and outcome. For example, using a cumulative exposure measure, a greater probability of an effect is assumed with greater total exposure or body burden. With a dichotomous exposure model (i.e. ever exposed or never exposed), the assumption is that the effect is irreversible. Models that define exposures only at a specific time may assume that only the present exposure is important. In the case of immunotoxicity, the magnitude of the adverse effect (e.g. infectious disease incidences) will be proportional to the severity of immune system damage as well as the length of time the effect remains (persistence). In biological terms, the adverse health outcome is proportional to both the severity and persistence of the immune effect. Thus, only the contribution of exposure to the severity of outcome may need to be considered if sufficient biological data are available indicating that the effect is persistent. This would be expected, for example, if the injury occurs in cells that cannot be replaced, such as long-term or short-term stem cells from the bone marrow, memory cells are left undisturbed and immunological tolerance is not induced. In contrast, an assumption can be made that any immune effects caused by an exposure are fully reversible under most conditions, if, for example, progenitor cells are left undisturbed and the effects are focused exclusively on clonally expanding immune cells (i.e. post–antigen challenge).

3.3.11.2 Exposure timing and susceptibility

Another consideration regarding exposure assessments for immunotoxicity is the life stage at which exposure occurs. Diseases associated with abnormal immune function, including some common infectious diseases, are considerably more prevalent in immature individuals and the aged. It is generally assumed that the immature immune system is more susceptible to chemicals than the fully mature system and that sequelae of developmental immunotoxicant exposure may be particularly persistent, in contrast to effects observed following adult exposure, which generally occur at higher doses and are expected to resolve soon after exposure ends (see review by Holladay & Smialowicz, 2000).

Certain types of infectious diseases are more common in the elderly than in neonates or young adults due to age-related loss of immune responsiveness (i.e. immunosenescence). In the simplest terms, the very young lack immunological experience, and the elderly, in spite of a wealth of experience, are no longer able to respond as well as the younger population. There are no data to support the notion that the immune system of the elderly is uniquely susceptible to immunotoxic agents. However, as a result of immunosenescence, a moderate loss of immune function and homeostatic controls may have more significant adverse effects

in the elderly than in young adults. An exposure assessment should characterize the likelihood of exposure of at least three general age groups—prenatal, young adult and elderly—and factor the susceptibility of the groups into the risk assessment to the extent possible.

3.3.11.3 Route of exposure and local immunity

The route of exposure as it pertains to potential systemic or local immunological effects needs to be considered in exposure assessment (see section 3.3.4 above for further discussion of local immunity). Whereas the route of exposure (i.e. respiratory, dermal or gastrointestinal) for most toxicological end-points need only be considered in terms of its influence on uptake to the potential target, for immunotoxicity, it is important to consider both systemic and potential local immunological effects. All three major routes of exposure to environmental toxicants are associated with the presence of local immune tissue and, to some extent, represent partially independent systems, such as the immune system associated with the skin (Elmets, 1994) or the lung (Selgrade, 2000). Several lines of evidence have suggested that although systemic immunity is not necessarily spared, the predominant immunological effect may occur at these local sites. For example, exposure to respiratory toxicants such as gallium arsenide or aerosolized JP-8 jet fuel may have a preferential effect on lung immunity and pulmonary defence mechanisms compared with non-pulmonary lymphoid tissues. Thus, exposure assessment for immunotoxicity should consider potential systemic uptake to central lymphoid organs (i.e. spleen, thymus and bone marrow) as well as exposure of local immune tissue. However, even when systemic immunotoxicity has been observed following respiratory or dermal exposure, in some cases, it may not be a direct result of the agent interacting with the central lymphoid tissue, but rather the release of immunomodulatory mediators from the local immune tissue. In this respect, the release of inflammatory/immunomodulatory mediators from the skin and lung following exposure may cause systemic effects (e.g. Rivas & Ullrich, 1994).

3.3.11.4 Exposure and toxicokinetic considerations

The toxicity of an exogenous chemical and the dose–response relationship for that chemical are both dependent upon the concentration of the toxicant at the site(s) of action (e.g. the target organ(s)). The disposition of a chemical in an organism is dependent upon the ADME processes, defined as toxicokinetic data (Renwick, 1994). Qualitative and quantitative information on each of these processes can be informative for study design and data interpretation, as well as in risk assessment.

Toxicokinetic information may provide the key to scientifically valid interpretation of the results of immunotoxicology studies. The determination of the toxicological relevance of events observed in individual laboratory animal studies may rely extensively on ADME data. For example, characterization of the metabolic profile of a toxicant may reveal species-specific differences in the bioavailability of active metabolites, which can influence the expression of adverse outcomes on the immune system. Toxicokinetic data that demonstrate in utero and/or lactational exposures can be valuable for interpretation of developmental immunotoxicity outcomes. Toxicokinetic data may also contribute to the analysis of and rationale for the MOA of the active moiety (Dybing et al., 2002). These data can be instrumental in building a weight of evidence approach for the use of immunotoxicity end-points in risk calculations. Establishing a valid physiologically based toxicokinetic (PBTK) or physiologically based pharmacokinetic (PBPK) model through the analysis and incorporation of toxicokinetic data can be an informative step in building the scientific foundation upon which

dose metrics are selected and risk calculations are based, both for adults and during development (Andersen & Dennison, 2002; Edler et al., 2002; Dybing, 2003; Faustman et al., 2005; USEPA, 2005b). As well, issues of species-to-species extrapolation and the use of CSAFs in the risk assessment are often reliant upon adequate toxicokinetic information (Suter et al., 2005). Toxicokinetic and toxicodynamic aspects to be addressed in uncertainty factor adjustment include identification of the active chemical species, the relevant internal exposure and the choice of the metric or end-point used in assessments (Gundert-Remy & Sonich-Mullin, 2002; Meek et al., 2002, 2003; Pelekis & Krishnan, 2004; Dorne & Renwick, 2005). Alternatively, toxicokinetic data may provide an important piece of the rationale for application of a database uncertainty factor when, for example, bioaccumulation of the test chemical in immune system tissues has been established, but no studies of immune system function are available.

3.3.12 Risk characterization

Risk characterization is the summary and integration portion of the risk assessment process in which the hazard characterization, quantitative dose–response assessment and exposure assessment are combined, along with a critical appraisal of the toxicity information. The critical evaluation contains a review of the overall quality of the assessment, including a discussion of uncertainties and a valuation of confidence in the conclusions. Ideally, a quantitative risk assessment is performed, but where the available data do not allow for such an assessment, a qualitative risk assessment may still be possible. For instance, the conclusion that a substance may be a sensitizer is an example of a qualitative risk assessment outcome. This basically is a form of hazard identification and is often used for classification and labelling purposes. Quantitative outcomes may be limited to a conclusion that there is a risk because exposure may be in excess of a health-based guidance value, but the risk characterization may also include a section describing risk in terms of the nature and extent of possible harm. The resulting summary is the final step in the risk assessment process, providing the risk manager with a useful synopsis of the risk assessment for a given chemical, including the following general components, to clearly illustrate the assumptions, uncertainties and methods used in the risk assessment process:

- the nature, reliability, consistency and variability of the data used;
- the reasoning behind the selection of key studies and critical effect(s), including relevance to human outcomes;
- occurrence of common versus rare immune deficits;
- use of historical control data to place the concurrent control into perspective;
- the consideration of sensitive populations and life stages;
- the qualitative and quantitative descriptors of the results of the risk assessment;
- the limitations of the available data, the assumptions used to bridge knowledge gaps in working with those data and the implications of using alternative assumptions;
- the strengths and weaknesses of the risk assessment and the level of scientific confidence in the assessment;
- the areas of uncertainty, additional data/research needs to improve confidence in the risk assessment and the potential impacts of new research;
- the science policy choices and the context of risk estimates relative to other similar risks or previous assessments.

Particular attention is to be given in the risk characterization phase to the interpretation of multiple or varying effects induced by a chemical. Different qualitative outcomes may, for

instance, appear with increasing doses or increasing exposure duration. In such cases, it is important to consider whether such variations may be attributed to changing outcomes of one and the same MOA or may be a result of different mechanisms with different dose–effect or exposure duration–effect relationships. All different effects need to be considered in the risk assessment and in the selection of appropriate safety factors.

At each of the first three stages of the risk assessment process—hazard characterization, quantitative dose–response assessment and exposure assessment—judgements are made on the relevance of the toxicity data for likely human exposure. Judgements made in one section may have implications for other portions of the assessment, and these decisions should be re-examined in the integration of the first three sections as part of the process of risk characterization. The immune system is highly complex, being composed of a number of interacting components. Thus, when reviewing immunotoxicology data, it is important that an immunological profile is evident. This is best established when multiple end-points are examined and the results suggest biological plausibility.

3.4 Entry points for immunotoxicity risk assessment

Prior to applying a risk assessment guidance for a specific type of immunotoxicity (see chapters 4–7), the questions as to how to judge whether immunotoxicity needs to be considered and what type(s) of (potential) immunotoxicity to address need to be addressed. Therefore, an overview of possible entry points for immunotoxicity risk assessment was prepared. The entry points are arranged in Table 3.1, starting on the next page, in order from more general immunotoxicological parameters to parameters that more specifically indicate separate types of immunotoxicity. It is to be noted that this overview is not exhaustive and that other indications of potential immunotoxicity may arise. It is advised that an immunotoxicology expert be consulted where indications for possible immunotoxicity are assumed, to assist the risk assessor in establishing the need for immunotoxicity risk assessment and the type of immunotoxicity to be addressed and for performing the required risk assessment.

In addition to these specific entry points, information on SARs and MOAs may form important entry points for immunotoxicity risk assessment as well.

Table 3.1: Entry points for immunotoxicity risk assessment.

Type of effect or observation	*Assess for potential risk*	*Chapter*	*Type of measure*
Human clinical, epidemiological or observational data and case reports			
Increase in incidence of neoplasms	Immunosuppression	Chapter 4	F
Increase in incidence of infections	Immunosuppression	Chapter 4	F
Changed antibody response to vaccination	Immunosuppression Immunostimulation	Chapter 4 Chapter 5	F
Increased incidence or exacerbation of autoimmune diseases	Immunostimulation Autoimmunity	Chapter 5 Chapter 7	F
Increased incidence or exacerbation of allergies	Immunostimulation Allergenicity	Chapter 5 Chapter 6	F
Changed DTH response	Immunosuppression Immunostimulation	Chapter 4 Chapter 5	F
Evidence of dermal, respiratory or oral sensitization or allergic effect elicitation	Allergenicity	Chapter 6	F
Changed lymphoproliferation	Immunosuppression Immunostimulation	Chapter 4 Chapter 5	F
Increased incidence of inflammation or increased levels of markers of inflammation	Immunostimulation Allergenicity Autoimmunity	Chapter 5 Chapter 6 Chapter 7	O
Increase in autoantibody levels, evidence of stimulation of the immune system	Immunostimulation Autoimmunity	Chapter 5 Chapter 7	O
Changed cytokine levels	Immunosuppression Immunostimulation Allergenicity Autoimmunity	Chapter 4 Chapter 5 Chapter 6 Chapter 7	O
Altered serum constituents, such as serum antibodies or complement factors	Immunosuppression Immunostimulation Allergenicity Autoimmunity	Chapter 4 Chapter 5 Chapter 6 Chapter 7	O
Changes in WBC counts or subpopulations	Immunosuppression Immunostimulation Allergenicity Autoimmunity	Chapter 4 Chapter 5 Chapter 6 Chapter 7	O
Potential accumulation in immune cells or tissues	Immunosuppression Immunostimulation Allergenicity Autoimmunity	Chapter 4 Chapter 5 Chapter 6 Chapter 7	O

Table 3.1 (continued)

Type of effect or observation	*Assess for potential risk*	*Chapter*	*Type of measure*
Laboratory animal data			
Changed host resistance to infectious agents or neoplasms	Immunosuppression Immunostimulation	Chapter 4 Chapter 5	F
Increased incidence or exacerbation of autoimmune diseases	Immunostimulation Autoimmunity	Chapter 5 Chapter 7	F
Exacerbation of allergies	Immunostimulation	Chapter 5	F
Alterations in immune function[a]	Immunosuppression Immunostimulation	Chapter 4 Chapter 5	F
Evidence of dermal, respiratory or oral sensitization or elicitation of allergic responses	Allergenicity	Chapter 6	F
Changes in ex vivo bone marrow cell proliferation or colony formation	Immunosuppression Immunostimulation	Chapter 4 Chapter 5	F
Stimulatory effects in popliteal lymph node assay	Immunostimulation Autoimmunity	Chapter 5 Chapter 7	F
Increased incidence of inflammation or increased levels of markers of inflammation	Immunostimulation Allergenicity Autoimmunity	Chapter 5 Chapter 6 Chapter 7	O
Increase in autoantibody levels	Immunostimulation Autoimmunity	Chapter 5 Chapter 7	O
Changed cytokine levels	Immunosuppression Immunostimulation Allergenicity Autoimmunity	Chapter 4 Chapter 5 Chapter 6 Chapter 7	O
Altered serum constituents, such as serum antibodies or complement factors	Immunosuppression Immunostimulation Allergenicity Autoimmunity	Chapter 4 Chapter 5 Chapter 6 Chapter 7	O
Changes in bone marrow cell numbers or subpopulations	Immunosuppression Immunostimulation	Chapter 4 Chapter 5	O
Changes in WBC counts or subpopulations	Immunosuppression Immunostimulation Allergenicity Autoimmunity	Chapter 4 Chapter 5 Chapter 6 Chapter 7	O
Changed cellularity of immune organs	Immunosuppression Immunostimulation Allergenicity Autoimmunity	Chapter 4 Chapter 5 Chapter 6 Chapter 7	O

Table 3.1 (continued)

Type of effect or observation	*Assess for potential risk*	*Chapter*	*Type of measure*
Immunohistopathological changes (e.g. in thymus, spleen, lymph nodes)	Immunosuppression	Chapter 4	O
	Immunostimulation	Chapter 5	
	Allergenicity	Chapter 6	
	Autoimmunity	Chapter 7	
Changed immune organ weight (thymus, spleen, lymph nodes)	Immunosuppression	Chapter 4	O
	Immunostimulation	Chapter 5	
	Allergenicity	Chapter 6	
	Autoimmunity	Chapter 7	
Preferential accumulation in immune cells or tissues	Immunosuppression	Chapter 4	O
	Immunostimulation	Chapter 5	
	Allergenicity	Chapter 6	
	Autoimmunity	Chapter 7	

F, functional; O, observational

[a] In primary or secondary or T cell–dependent or T cell–independent antibody response assays, in PFC assay, NK cell activity, DTH response, lymphoproliferation assays, phagocytic function of alveolar macrophages.

4. ASSESSMENT OF IMMUNOSUPPRESSION

4.1 Introduction

This section of the guidance document focuses on unintended immunosuppression, an area of immunotoxicity for which there is wide acceptance of the relevance of end-points in humans and laboratory animals for the determination of human risk (see reviews by Vos & Van Loveren, 1998; Koller, 2001; Descotes, 2003; Luebke et al., 2006a). Immunosuppression was first brought to the attention of the scientific community in the 1970s as a result of international workshops held by the United States National Academy of Sciences, the United States Food and Drug Administration (USFDA) and WHO (Luster et al., 1980a,b). Numerous experimental and epidemiological studies have since been published, suggesting that while immunosuppression is not a common occurrence, it is not rare and may arise as a result of chemical exposure in both humans and experimental animals. Immunosuppression represents a series of complex cascading cellular and organ-related events that can lead to an increased incidence and/or severity of infectious and neoplastic diseases (Luebke et al., 2004); thus, data from experimental immunotoxicological or epidemiological studies that are addressed in quantitative risk assessment may require careful interpretation. This is particularly true when the immunological effects are slight to moderate in nature, as may be expected from inadvertent exposures to immunosuppressive agents in human populations. This is in contrast to the severe immunosuppression that can occur in individuals with primary (i.e. genetic) immunodeficiency or AIDS. In cases of severe immunosuppression, the incidence of certain types of cancers and infections is greatly increased. In order to accurately predict the risk of immunosuppression from xenobiotic exposures in human populations, a scientifically sound immunosuppression framework should be established that will support an accurate and quantitative interpretation of experimental and epidemiological studies and application to human health risk assessment. This framework should also consider susceptible populations. In this respect, while age-related differences in immune function during development and old age are not well understood, concerns for the special vulnerability of these populations have been established.

4.2 Hazard identification

The questions to be answered during hazard identification are similar across all types of systemic toxicants: does the compound increase the risk of adverse effects, and are the effects likely to occur in humans? Data used to identify potential immunosuppressants may be generated as part of general toxicity studies or in dedicated immunotoxicity studies and take the form of observational or functional end-points (see Table 3.1). In general, observational end-points reflect changes in cells and products of the immune system, but, as a single point in time determination, do not reflect persistence of the change or necessarily indicate that immune function was sufficiently compromised to increase the risk of infection or neoplasia. In contrast, functional assays measure the response of the immune system to a challenge at the cellular or whole-animal level. The latter assay type provides the best evidence of immune system health by mimicking host responses that reduce the risk of infection (e.g. producing antibodies in response to immunization). Testing schemes may include tiers of observational and functional end-points (e.g. Luster et al., 1988) or one or two functional assays (e.g. OPPTS 870.7800: USEPA, 1998). Certain guidelines that include screening for potential immunotoxicants (e.g. OECD Test Guideline 407, WHO/IPCS's EHC 180, ICH S8 protocols, the EU's REACH) rely on changes in observational end-points to trigger

assessment of immune function. However, concordance analysis determined that many observational end-points have low predictive power for functional changes (Luster et al., 1992), leading to the requirement for functional testing in the USEPA's OPPTS 870.7800 guideline. Humans and laboratory animals often respond similarly to immune system challenges (e.g. immunization) and exposure to immunotoxicants, although there are documented differences. As such, human data should be used whenever available and should take precedence over extrapolation from laboratory animal data, provided that equivalent end-points are compared and the data are of sufficient quality and reliability.

4.3 Hazard characterization

Unintended suppression of immune function is clearly adverse. Even moderate suppression in humans may decrease responses to immunization and increase susceptibility to infection and certain types of cancer, particularly when exposure occurs during immune system development and maturation or under other circumstances (e.g. therapeutic suppression of immune function and inflammation, chronic stress, recreational drug use) that decrease immunocompetence. Specific examples and detailed discussion of immunosuppression are presented in sections 4.8.2 and 4.8.3.

4.4 Clinical and epidemiological data

4.4.1 Clinical data

Immunotoxicology data in humans may be derived from well-designed clinical or epidemiological studies, observational studies or case reports. Although controlled clinical studies represent the best opportunity to identify and characterize immunotoxicants, they are not routinely conducted for environmental or occupational chemicals for obvious ethical reasons. Pharmaceuticals that have been examined clinically for possible immunosuppression have been limited to cytoreductive drugs and transplantation agents (Descotes & Vial, 1994; Ryffel et al., 1994), with tests usually restricted to monitoring changes in WBC counts or recording opportunistic infections (e.g. herpes zoster, *Candida*, *Pneumocystis carinii*), both of which most likely reflect fairly severe immunosuppression (Luster et al., 2004). However, a common finding from these clinical studies indicates that opportunistic infections and/or neutropenia are frequently observed in patients on high-dose, acute exposure to drugs such as interferon-alpha (IFN-α), azathioprine, cyclophosphamide and methotrexate, whereas secondary neoplasms occur from long-term therapy (Neumann & Fauser, 1982; Lawson et al., 1984; Bradley et al., 1989; Antonelli et al., 1991).

Where the ethical issues have been appropriately resolved, immune function data following controlled exposure in humans would require the least extrapolation and present the strongest data for estimating risk to the general population. For example, Sleijffers et al. (2001) investigated the effects of ultraviolet B exposure on the antibody titres to the hepatitis B vaccine in human volunteers. The advantages and potential utility of measuring the response to vaccination as an indicator of immune effects associated with exposure to environmental agents have been suggested for adults as well as infants (Van Loveren et al., 2001; Gans et al., 2003).

4.4.2 Epidemiological data

In contrast to patients with a suspected primary immunodeficiency disease or HIV infection, it is considerably more difficult to detect mild to moderate states of immunodeficiency, as

would likely occur following exposure to immunotoxic chemicals, using common clinical immune tests. Testing for primary immunodeficiency diseases is normally undertaken by a stepwise (tiered) approach, which initially includes general parameters, such as complete blood counts, serum immunoglobulin levels, chest radiographs of the thymus and DTH tests, and is usually initiated because the patient presents with excessive infectious diseases (Noroski & Shearer, 1998). As has been shown in laboratory animal studies (Luster et al., 1992, 1993), such tests are not sensitive indicators of immunotoxicity and thus may not detect subtle immune changes. Further, in contrast to diagnoses of primary immunodeficiency disease in a child or adult, immunotoxicological studies are conducted routinely within an epidemiological framework, requiring relatively large populations and careful consideration of experimental design to account for absence of selection bias, exposure and outcome misclassifications, and confounding factors.

As discussed previously, from a population standpoint, small changes in the immune system have been shown to result in an increased risk of disease when studied under appropriate conditions. Changes in disease may not be apparent owing to limitations of study designs where, for example, the population is too small, the most appropriate infections were not being monitored or the population was not followed for a sufficient period to encounter infectious agents. When interpreting human immune data, considerably more credence is given to those studies in which multiple immune tests were conducted and the resulting data provide a biologically plausible interpretation. When examining a large number of immunophenotypic markers, an abnormal value in one or two immunophenotypes is likely to result simply from a type 1 error. A more reliable indicator of immunotoxicity would be multiple changes consistent with a specific pattern. For example, it is unlikely that a significant decrease in immunoglobulin level would be observed without a concomitant decrease in certain lymphocyte markers, particularly those associated with B cells. A description of biomarkers in epidemiological studies is provided in EHC 180: *Principles and methods for assessing direct immunotoxicity with exposure to chemicals* (IPCS, 1996). The risk assessor should refer to the assay descriptions in EHC 180 for immunotoxicity end-points contained in the data set for the chemical in question to provide specific context, cautions and information that may assist in the interpretation of immunosuppression data for risk assessment. In addition, it is recommended that the risk assessor consult an expert in immunotoxicology or clinical immunology to help interpret the biological plausibility of the study results.

Although data from functional immune assays (e.g. the antibody response to vaccine) are generally not available in humans, such data represent the strongest evidence of an immunosuppression. As described above, human data are generally restricted to immunophenotyping, cytokines and serum immunoglobulin levels, and these end-points are neither sensitive enough to detect mild to moderate immunosuppression nor predictive enough of an adverse response to be used as the sole indicator of an immunosuppressive effect of a given chemical. These end-points are, instead, useful to support the human relevance of laboratory animal data with evidence of immunosuppression, suggest that additional studies should be conducted to examine the possibility of immunosuppression or indicate that a database uncertainty factor may be appropriate to address the suggestion of immunosuppression.

4.5 Laboratory animal data

Rodent models have also been used to increase understanding of immune function end-points and disease resistance. Immunotoxicologists have historically used host resistance assays to validate the predictive value of other methods and extrapolate the potential for environmental

agents to alter host susceptibility in the human population, because altered resistance is a biologically plausible effect with clear relevance for potential adverse effects in humans. In these assays, groups of experimental animals are challenged with either an infectious agent or a transplantable tumour at a challenge level sufficient to produce disease in a small number of control animals. However, challenge agents in host resistance assays should be chosen to explore or confirm a known functional defect, not used to screen for effects. Non-lethal resistance models, in which numbers of tumour foci, viral titres or bacterial counts in target tissues are assessed, provide greater sensitivity than mortality, because the data provide a quantitative assessment of the host response to challenge and are a better reflection of protective immunity in the organism. Furthermore, the biological relevance of death as an end-point is questionable when most or all of the immunocompetent controls do not survive, because the virulence or number of the challenge agents simply overwhelms the initial response to infection, killing the host before a protective response can be mounted. In general, there is a good correlation between experimental animal data and human clinical and epidemiological data on chemicals for which there are studies of immunosuppression in both humans and laboratory animals (Descotes, 2003; see also reviews by Vos & Van Loveren, 1998; Koller, 2001). Rodent data on immunosuppressive therapeutics have generally been good predictors of subsequent clinical observations after toxicokinetic adjustments. For example, a comparison of immunosuppressive effects of cyclosporin A among various species (mouse, rat, guinea-pig, dog, Rhesus monkey and human) demonstrated good quantitative and qualitative agreement (IPCS, 1996).

One approach to examine the relationship between immunosuppression and disease has been to determine the concordance between immune tests routinely used in rodent studies of chemical-induced immunosuppression and host resistance models (Luster et al., 1993). Although it is rare for a single component of the immune system to be solely responsible for resistance to specific infectious agents or tumour types, certain immune measures show increased correlation with the outcomes of individual host resistance assays (Luster et al., 1988). Using mathematical models to assess the relationship between immune function tests and disease resistance models following exposure to cyclophosphamide, Luster et al. (1993) found that a majority of the immune function–host resistance relationships appeared to approximate a linear relationship. However, for several parameters, a linear-quadratic model best fit the relationship. Note that the models in Luster et al. (1993) describe the relationship between functional immune tests and disease resistance assays, not between chemical exposure (cyclophosphamide in this example) and immune function. While individual immune tests ranged from good (PFC assay, 73%; NK cell activity, 73%; DTH response, 82%) to poor (lymphoproliferative response to lipopolysaccharide [LPS], 54%) predictors of changes in resistance to any disease challenge, a combination of multiple immune tests, including those assays found to be good individual predictors of host resistance, could result in concordance rates as high as 100% (Luster et al., 1993).

A basic understanding of the typical methodologies used to evaluate immunotoxicity in laboratory animal models is necessary to evaluate the database of studies for hazard characterization of a given chemical as the first step in risk assessment. Detailed discussions of end-points and methods utilized in characterizing immunosuppression are provided in EHC 180 (IPCS, 1996). The data set for most chemicals is unlikely to contain data on all of the described end-points. The risk assessor should refer to the assay descriptions in EHC 180 for immunotoxicity end-points contained in the data set for the chemical in question to provide specific context, cautions and information that may assist in the interpretation of immunosuppression data for risk assessment. Chapter 3 presents a detailed discussion of general

considerations in the assessment of immunotoxicity data available for the chemical under assessment that should be consulted when evaluating the database for immunosuppression.

4.6 Local versus systemic effects

The concept of distinct local toxicity related to the route of exposure is not unique to immunotoxicology and may result from direct exposure or increased local concentrations of toxicant at the exposure site. The contrast between local and systemic toxicity within general toxicology is illustrated by examples such as local route-dependent histopathological changes associated with the lowest inhalation concentration of furfural of 6 mg/kg body weight per day, well below any observed toxicity associated with oral exposure, in a study that examined route-dependent toxicity of furfural in Fischer 344 rats (Arts et al., 2004a). Route-associated local exposure effects may explain some aspects of local toxicity; in the case of inhalational toxicity of furfural in rats, the observed effects may be exacerbated by the increased local exposure resulting from the particular morphology of the rat nasal cavity (for a discussion of rat nasal exposure, see Kimbell et al., 1997). However, unlike most toxicological end-points, for immunotoxicology, the route of exposure is not simply a local increased concentration or uptake issue. There are local, partially independent portions of the immune system associated with each of the major routes of exposure: inhalation, dermal and oral. Therefore, route of exposure is potentially important for immunosuppression, as local immunotoxicological effects may occur independent of systemic immunity.

Potential enhancement of respiratory infection as a result of exposure to an inhaled toxicant and subsequent immunosuppression are important considerations for risk assessors. Four decades of research demonstrate that the alveolar macrophage is an important target for inhaled toxicants and that suppression of this response increases the risk of bacterial pneumonia (reviewed by Selgrade & Gilmour, 2006). A widely accepted method for identification of this hazard is assessment of phagocytic function of alveolar macrophages contained in bronchoalveolar lavage fluid obtained from laboratory rodents following inhalation exposure to the toxicant. Several good methods are available using different types of particles (Lewis, 1995; Neldon et al., 1995; Brousseau et al., 1999; Tasat et al., 2003).

Mortality in response to inhalation or intratracheal challenge with a variety of extracellular bacteria has been shown to increase with exposures to environmentally relevant concentrations of criteria air pollutants (ozone, nitrogen dioxide and sulfur dioxide) in mice. Also, inhalation of certain particulate air pollutants, particulate-associated metals as well as certain soluble metal salts and volatile organics has been shown to enhance susceptibility to infection. In addition to enhanced mortality, many of the same studies have demonstrated impaired bacterial clearance from the lung and/or impaired alveolar macrophage function (reviewed by Selgrade & Gilmour, 2006). Pulmonary macrophages, however (through the production of cytokines in response to microbial exposures), provide an important link between the innate and adaptive cell-mediated response, and enhancement of these infections following exposure to air pollutants has also been associated with suppression of alveolar macrophage function (reviewed by Selgrade & Gilmour, 2006).

Early bacterial clearance is mediated by a dual phagocytic system involving both macrophages and neutrophils (Zhang et al., 2000). The alveolar macrophage is the first line of defence; for some bacterial species (e.g. *Staphylococcus aureus*), the alveolar macrophage is normally sufficient for clearance (Rehm et al., 1980). When the invading pathogens are too virulent or represent too large a load to be contained by the macrophage alone, alveolar

macrophages generate numerous mediators that recruit large numbers of neutrophils into the alveolar space to help defend the host against the bacterial invasion. However, this influx of neutrophils is a double-edged sword, because the resulting inflammation can cause pneumonia or bronchitis. When rats were exposed to ozone and challenged with *Streptococcus zooepidemicus*, bacterial clearance from the lung was initially impaired, and bacteria isolated from the exposed rats exhibited enhanced virulence factors, but no mortality was observed. This more favourable outcome in rats (compared with mice) appears to be related to a more timely influx of neutrophils, which peaked at 1 day post-infection in rats compared with day 2 or later (depending on the strain) in mice (Gilmour & Selgrade, 1993). Although the neutrophil response represents an overlapping resistance mechanism, ozone-exposed rats exhibit both prolonged infection and pulmonary inflammation well above those observed with bacteria or pollutant alone.

Studies with phosgene suggest that once the toxic insult is removed, alveolar macrophage function rebounds rather quickly. Hence, a single short-term exposure may create only a small window of vulnerability, during which an infectious agent might gain an advantage over the host. However, chronic exposure may result in a longer window of vulnerability, and there is no evidence that macrophages adapt to such insults (Selgrade, 1999).

4.7 (Ir)reversibility of effects

Human studies indicate that immune function returns to normal following exposure to potent immunosuppressive drugs, and the expectation is that immune function will return to normal in humans and laboratory animals as xenobiotics are cleared. However, persistence of effects in adult animals has not been systematically evaluated, and greater persistence of effects is expected if chemical exposure affects precursor or stem cells. This is not the case for exposure during gestation or in the early postnatal period, when immunotoxicity may have much longer lasting effects than in the adult, persisting for weeks, months or even most of the lifespan of the host (Dietert & Piepenbrink, 2006b; Luebke et al., 2006a; Dietert & Dietert, 2007).

The outcome of chemical exposure may be either reversible or irreversible in some cases, depending on the combination of dose and duration of the chemical exposure (see additional discussion in section 4.13 below). For example, it is known that many antiproliferative agents used in cancer chemotherapy can temporarily affect the immune response by diminishing the ability of immunocompetent cells to respond to antigen. The immune system would likely recover when the offending agent is removed. However, these same agents, when administered at higher concentrations or over prolonged periods of time, may affect the haematopoietic stem cells. This could result in either a delay in recovery, to allow sufficient time for the stem cell repopulation, or immune failure, in which the stem cells or stromal cell microenvironment is irreversibly damaged. Thus, the final outcome will depend upon the dose and duration of exposure, as well as the specific target within the microenvironment (e.g. stromal cells versus long-term stem cells versus short-term stem cells).

4.8 Biological plausibility

Biological plausibility is discussed in detail in section 3.3.6 of chapter 3. In particular, the reader is referred to section 3.3.6.1 for a discussion on the health-related database and the components of sufficient evidence for immunotoxicity and section 3.3.6.3 for a discussion of

"triggers" and factors to be considered in determining whether additional data may be required to complete the risk assessment.

4.8.1 Weight of evidence approach for assessment of immunosuppression

Hazard identification for immunosuppression should result in weight of evidence conclusions based on the available human and laboratory animal data for a given chemical. The risk assessor should consider the entire database of effects, both positive and negative. Data are evaluated within the same or similar assays, as well as across divergent measures of the immune system and across multiple species. For each assay, a dose–response relationship for chemical exposure in the absence of generalized overt toxicity is a necessary criterion in demonstrating immunosuppression.

The weight of evidence conclusions are strengthened by consistency (particularly across species, sexes or related end-points), biological plausibility and breadth (range of effects) of the evidence for immunotoxicity. A lack of consistency of specific assays or types of immunotoxicity across species, strains or sexes does not necessarily represent conflicting data and often represents species, strain or sex differences. Conflicting data should be evaluated by the strengths and weaknesses (e.g. sample size and exposure duration) of the individual studies, as well as in the context of the remainder of the immunotoxicity database for a given chemical. Additional information with which to interpret species, strain or sex differences may be gained by considering toxicokinetic data (when available) or the likelihood of sex differences resulting from hormonally active chemicals, such as endocrine disrupting chemicals. As with other non-cancer end-points, the weight of evidence evaluation should represent an expert judgement of the database to determine the potential for immunosuppression for a given compound in accordance with the following key considerations (Hill, 1965; IPCS, 1999a; Weed, 2005): experimental evidence, dose–response relationship, consistency of association, strength of the association, temporal association, biological plausibility, specificity, coherence and analogy.

The following outline presents a structured approach to organizing the available data for developing weight of evidence conclusions in the assessment of immunosuppression hazard identification through seven questions asking the risk assessor to evaluate the available data from the strongest and most predictive data (human data) through the least predictive (immune organ weight). While not exhaustive, the relative strength and predictability of different assays are presented below for major types of immunotoxicity data. This outline is presented in brief in Figure 4.1; however, the risk assessor should refer to the detailed text below for important considerations identifying key strengths and weaknesses for particular types of human and laboratory animal data.

As described below, some data (i.e. human disease incidence, human functional immune data, host resistance assays in experimental animals and immune function assays in experimental animals) present clear evidence of adverse immunosuppression, whereas other data (i.e. general immune assays, haematology, histopathology and immune organ weights) are more difficult to interpret and often present equivocal evidence without additional support for immunosuppression from other assays. It is particularly recommended that the risk assessor consult an expert in immunotoxicology or clinical immunology to help interpret the biological plausibility and adversity of these less predictive assays. The risk assessor should evaluate the weight of evidence for immunosuppression based on the database for a given chemical by considering all seven of the following questions, beginning with the extent or

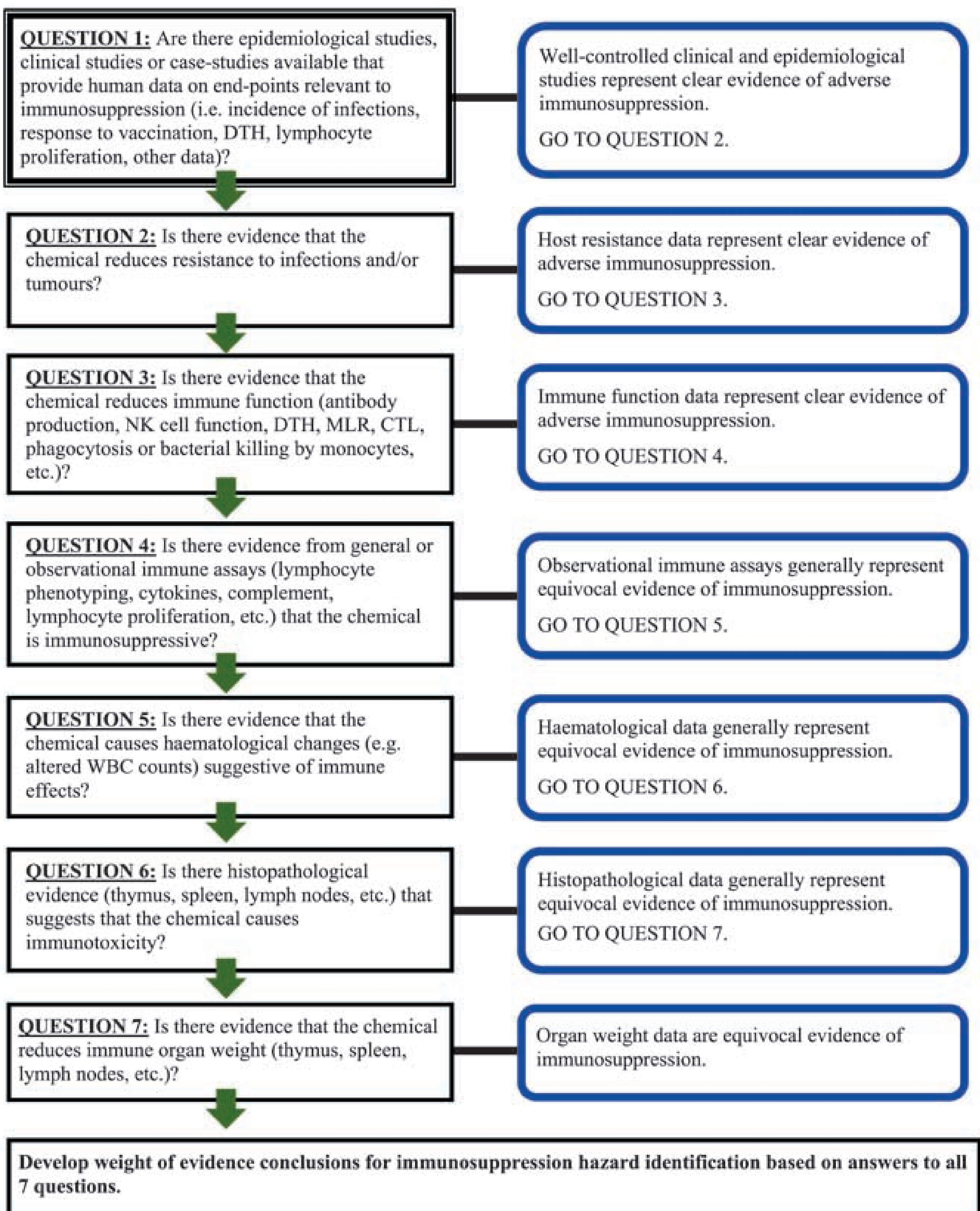

Figure 4.1: Weight of evidence approach for assessment of immunosuppression. The figure presents a structured approach for organizing all of the available data for developing weight of evidence conclusions for immunosuppression hazard identification. It presents a summary of categorical data binning, from the most to least predictive, as described in section 4.8.1, rather than a decision-tree. Note: If there are immunotoxicological data relevant to end-points other than immuno-suppression, evaluate those data in the appropriate chapter and include in weight of evidence evaluation for immunotoxicity. CTL, cytotoxic T lymphocyte; MLR, mixed leukocyte reaction.

availability of human data and ending with the availability of immune organ weight data from the animal literature:

1) *Human data*: Are there epidemiological studies, clinical studies or case-studies available that provide human data on end-points relevant to immunosuppression (i.e. incidence of infections, response to vaccination, DTH, lymphocyte proliferation, other data)?
   - ✓ Epidemiological studies demonstrating an association between chemical exposure and disease are considered clear evidence of adverse immunosuppression and appropriate to derive an effect level for immunosuppression, particularly if good exposure data are available. Controlled clinical studies with quantitative evaluation of immune function, such as primary antibody response to influenza vaccination, secondary antibody response to tetanus toxoid, DTH to naturally occurring antigens or disease resistance assays, represent clear evidence of adverse immunosuppression. These in vivo functional assays are appropriate to derive an effect level for immunosuppression; however, they are not frequently performed, because testing procedures require injection of the human subjects with antigen. Ex vivo functional assays (e.g. NK function, phagocytosis, bacterial killing by monocytes or polymorphonuclear leukocyte [PMNL] activity) also represent good human data that are considered clear evidence of adverse immunosuppression with the added benefit of providing functional immune data from peripheral blood samples without subjecting humans to an antigenic challenge. These ex vivo functional assays are appropriate end-points to derive an effect level for immunosuppression, but they should be evaluated with additional scrutiny; the risk assessor should closely evaluate the degree of suppression, consistency of data across studies and biological plausibility from supporting data, because the ex vivo tests do not directly test an intact immune system.
   - ✓ Human data are more likely to be enumeration of immune system components (for a more complete discussion of typical human data, see the above discussions in section 4.4). Immunophenotyping, serum immunoglobulin levels and in vitro assays, such as lymphocyte proliferation, are the most common assays used in human immunotoxicity studies, but individually are not predictive measures for immunotoxicity. These data are therefore not appropriate to derive an effect level for immunosuppression. These less predictive immune measures can be used to support laboratory animal data and should be considered with the weight of evidence evaluation of human and experimental animal data to determine biological plausibility and consider potential mechanisms or MOAs. Epidemiological studies with effects data and demonstrated chemical exposure lacking dose levels (as is frequently the case for retrospective studies) can be used to evaluate consistency of effects between the laboratory animal data and the available human data and to support the development of effect levels from the laboratory animal data. Available data support the notion that chemical immunosuppression is much more likely to result in mild to moderate immunosuppression than the severe suppression associated with primary immunodeficiency diseases and AIDS (see section 4.8.3 below for a more detailed discussion). However, epidemiological studies are generally not designed to detect mild to moderate changes in immune function or relatively small increases in incidences of infection, and therefore careful consideration of negative data is suggested.

2) *Host resistance (laboratory animal data)*: Is there evidence that the chemical reduces resistance to infections and/or tumours?
   - ✓ Suppression of a host resistance measure is considered clear evidence of adverse immunosuppression and appropriate for derivation of an effect level.
   - ✓ Suppression of the same host resistance assay in multiple species or multiple host resistance assays with concordance among end-points increases the strength of the data indicating suppression and provides data appropriate for derivation of effect level(s).
   - ✓ Suppression of a host resistance measure in combination with suppression of associated immune function(s) that mediated resistance to the challenge agent increases the strength of the data indicating suppression, supports determination of an MOA and provides data appropriate for derivation of effect level(s).
   - ✓ Suppression of a host resistance measure with additional evidence of immunotoxicity (e.g. immunophenotyping, cytokines, altered histology, immune organ weight) may support determination of an MOA.

3) *Immune function (laboratory animal data)*: Is there evidence that the chemical reduces immune function (antibody production, NK cell function, DTH, mixed leukocyte reaction [MLR], cytotoxic T lymphocyte [CTL], phagocytosis or bacterial killing by monocytes, etc.)?
   - ✓ Suppression of a single functional immune assay is considered clear evidence of adverse immunosuppression and appropriate for derivation of an effect level.
   - ✓ Suppression of the same functional assay in multiple species or multiple functional assays with concordance among end-points increases the strength of the data indicating immunosuppression and provides data appropriate for derivation of effect level(s).
   - ✓ Suppression of a functional measure in combination with additional evidence of immunotoxicity that supports an MOA or biologically plausible mechanism greatly increases the support for immunosuppression.
   - ✓ Suppression of a functional measure in combination with additional evidence of immunotoxicity (e.g. immunophenotyping, cytokines, altered histology, immune organ weight) increases the strength of the data indicating immunosuppression.
   - ✓ Some ex vivo functional assays (MLR, CTL) may be appropriate end-points to derive an effect level for immunosuppression, but they should be evaluated with additional scrutiny; the risk assessor should more closely evaluate the degree of suppression, consistency of data across studies and biological plausibility from supporting data, because the ex vivo tests do not directly test an intact immune system.
   - ✓ Nonspecific T or B cell proliferation assays have been listed as functional assays in some guidelines (e.g. IPCS, 1996), but they are not considered true functional tests and are covered below under general immune assays.

4) *General immune assays (laboratory animal data)*: Is there evidence from general or observational immune assays (lymphocyte phenotyping, cytokines, complement, lymphocyte proliferation, etc.) that the chemical is immunosuppressive?
   - ✓ Lymphocyte phenotyping, cytokines and other assays may add MOA information to support a biologically plausible description of immunosuppression.
   - ✓ Phenotyping, lymphocyte proliferation or altered soluble mediator concentrations are generally not considered to be reliable predictors of immunosuppression and

therefore should generally not be used to derive an effect level for immunosuppression.
- ✓ In vitro data alone are inadequate evidence of immunotoxicity.

5) *Haematology (laboratory animal data)*: Is there evidence that the chemical causes haematological changes (e.g. altered WBC counts) suggestive of immune effects?
   - ✓ Severe haematological changes alone are sufficient to demonstrate adverse immunosuppression and appropriate for derivation of an effect level; otherwise, haematological data should generally not be used to derive an effect level for immunosuppression.
   - ✓ Haematological changes may add MOA information to support a biologically plausible description of immunosuppression.
   - ✓ Haematological changes consistent with histopathological evidence may indicate immunotoxicity and constitute additional support for the weight of evidence of immunosuppression.

6) *Histopathology (laboratory animal data)*: Is there histopathological evidence (thymus, spleen, lymph nodes, etc.) that suggests that the chemical causes immunotoxicity?
   - ✓ Descriptive (extended) histopathological evidence from multiple immune organs alone may indicate immunotoxicity and support the weight of evidence of immunosuppression, but should not be used to derive an effect level for immunosuppression.
   - ✓ Limited histopathological evidence alone is equivocal.
   - ✓ Decreased immune organ weight may support histopathological evidence.

7) *Organ weight (laboratory animal data)*: Is there evidence that the chemical reduces immune organ weight (thymus, spleen, lymph nodes, etc.)?
   - ✓ Decreased immune organ weight may support other evidence of immunosuppression.
   - ✓ Immune organ weight change alone is equivocal and therefore should generally not be used to derive an effect level for immunosuppression.
   - ✓ Evidence for immunotoxicity based on reduced immune organ weight must be evaluated with caution, as reduced immune organ weight may be secondary to general toxicity, resulting potentially in a stress response.

The risk assessor should develop the weight of evidence for immunosuppression hazard identification based on answers to all seven questions. The weight of evidence conclusions for immunosuppression should also describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps. A small database with negative data is equivocal. Just as positive data on a range of assays strengthen the weight of evidence for immunotoxicity, negative data on a range of more predictive assays such as immune function data increase confidence to support a lack of immunotoxicity. The strength of the immune database will determine whether additional evidence is necessary to determine immunotoxicity. Incomplete or questionable data sets and high usage or high risk of exposure should trigger a request for additional data, if regulatory mandate allows.

When immunosuppression is indicated by the weight of evidence, these conclusions are then prepared to be brought forward to perform a dose–response assessment, beginning with the

selection of the most appropriate end-point(s) or critical effect(s) and the development of POD(s). Health-based guidance values or reference values are then calculated by dividing the POD(s) by the total uncertainty factor (see sections 3.3.7 and 4.9 for a detailed discussion of dose–response assessment and derivation of reference values). Data from human exposures (e.g. occupational exposure studies and case reports) are preferred for the critical effect, because fewer assumptions are required to determine the relative risk of immunotoxicity for the general population from human data compared with experimental animal data. Therefore, when human data are used for the critical effect and the POD, smaller uncertainty factors are generally utilized to derive the reference values. Nevertheless, all available data are considered for the critical effect. The quantitative risk assessment may be based on laboratory animal data even if there are human data for a given chemical in cases such as inadequate information on dose levels, no information on effects at low doses or absence of a no-observed-effect level (NOEL) in the human data set.

Dose-related changes in four principal types of data provide clear evidence of adverse immunosuppression appropriate for use as the critical effect for chemical-related immunosuppression: 1) increased human disease incidence, 2) reduced immune function in humans, 3) suppression of host resistance measure(s) in laboratory animals or 4) suppression of functional immune assay(s) in laboratory animals. In general, PODs are developed from the most sensitive adverse immune end-point(s) from the most appropriate species (or the most sensitive mammalian species, in the absence of information to determine the most appropriate species). Data from general immune assays, haematology, histopathology and immune organ weight changes may indicate potential immunotoxicity and are useful to support biological plausibility and potential MOAs for more predictive data (e.g. functional data). Observational end-points such as phenotyping, lymphocyte proliferation and altered soluble mediator (cytokines or complement) concentrations should generally not be used to derive an effect level for immunosuppression, because they are not considered to be reliable predictors of adverse immunosuppression. Similarly, haematological changes should not be used as a critical effect for immunosuppression unless they are severe. Major haematological changes are unlikely to occur in the absence of a related change in immune function (see discussion in section 4.8.2 below). Therefore, the risk assessor should consider available functional and host resistance data on related end-points when considering deriving an effect level from haematological data. Changes in immune organ weights and general histopathology may indicate potential immunotoxicity and can be used to support more predictive data (e.g. functional data); however, these data should not be used to derive an effect level for immunosuppression because of the low predictive value of these end-points when considered alone.

4.8.2 Biological relatedness of different outcomes

4.8.2.1 Diseases associated with specific types of immunosuppression in humans

Whereas both infectious and neoplastic diseases are associated with immunodeficiency, infectious disease incidence is often the focus of epidemiological studies, because changes in incidence are detectable over a shorter time span. The particular microorganism responsible for an infection may assist in identifying the qualitative and quantitative nature of the immunodeficiency. For example, extracellular pathogens, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, multiply outside phagocytic cells and cause disease when they resist phagocytosis. Facultative intracellular pathogens (e.g. *Mycobacterium tuberculosis*) are generally phagocytized but resist intracellular killing. Thus, infections from extracellular or facultative intracellular organisms will be more frequent in individuals in which impaired

phagocytic mechanisms exist, such as neutropenia, or when humoral (i.e. antibody) deficiencies are present. Obligate intracellular pathogens, which include all viruses, cannot multiply unless they are within a host cell and are more commonly observed in individuals with defects in cellular (T cell) immunity.

The importance of antibodies in controlling extracellular bacteria is sufficiently well known that a history of repeated infections with these organisms is a key diagnostic indicator of primary or acquired antibody deficiency in humans. The DTH response is used clinically to evaluate the potential to respond to intracellular infections and has been used to determine immunocompetence in populations where exposure to environmental contaminants has been suggested. A variety of naturally occurring antigens known to induce DTH (e.g. an extract of the fungus *Candida* or products of bacteria in the normal flora) to which all adults have been exposed are used as test antigens. Individuals who do not respond to challenge are considered to be at greater risk for certain infections.

Microbial agents associated with immunodeficiency disorders can also be classified into common, opportunistic or latent pathogens. Common pathogens, such as influenza viruses, occur in the general population at frequencies associated with their infectious nature (e.g. virulence, ease of transmission). The respiratory system is the most vulnerable target for most common pathogens, as it is directly exposed to the external environment and has a large surface area, 4 times the combined total surface areas of the gastrointestinal tract and skin (Patriarca, 1994). The low individual rates of common infections in the general population (only one or two episodes in an individual per year), combined with under-reporting, make it difficult to detect changes in infection rates. It is of significant impact, however, as influenza results in 3–5 million serious illnesses and 250 000–500 000 deaths annually worldwide (WHO, 2009).

While infections with common pathogens occur routinely in the healthy population, opportunistic infections typically occur in individuals with more severe immunosuppression, such as patients with AIDS, and cause disease in the general population at very low incidences. The microorganisms in this group are commonly encountered in food, water, dust or soil and include certain protozoans, such as *Toxoplasma gondii*, which causes cerebral infections and intractable diarrhoea, the fungi *Candida albicans* and *Pneumocystis carinii*, and bacteria in the *Mycobacterium avium* complex (Morris & Potter, 1997).

A third group of pathogenic microorganisms is responsible for latent infections. Cytomegalovirus (CMV), herpes simplex virus (HSV) and Epstein-Barr virus (EBV), all members of the herpes virus family, can remain in the tissue following primary infection for the duration of the host's life without causing disease. In healthy individuals, the immune system usually maintains viral latency, with cellular immunity playing a major role. When the immune response is compromised, viral replication can ensue, and reactivation occurs and, in rare instances, causes severe complications, including death. Preceding viral activation, a vigorous immune response to viral-specific antigens occurs in response to viral replication. Changes in virus-specific immune response or activation of latent viruses have been observed in individuals with secondary immunodeficiency disorders and, as will be discussed further, may reflect mild to moderate immunosuppression.

Immunodeficiency is also associated with an increased incidence of certain virus-induced tumours, such as non-Hodgkin lymphomas and skin tumours (Penn, 2000). In contrast to cancers of internal organs, such as the lung and liver, which are often induced by chemical

Table 4.1: Host resistance and infections associated with antibody deficiency.

Infections associated with antibody deficiency	*Organisms associated with antibody deficiency*
Recurrent pneumonia	**Bacteria**
Sinusitis	*Streptococcus pneumoniae*
Recurrent otitis	*Haemophilus influenzae*
Conjunctivitis	*Staphylococcus aureus*
Meningitis	*Streptococcus*
Septicaemia	*Pseudomonas*
Persistent infectious diarrhoea	*Campylobacter*
Viral hepatitis	**Viruses**
Persistent viral encephalitis	Enteroviruses
Paralytic poliomyelitis	Rotavirus
Chronic cystitis	**Protozoans**
Chronic urethritis	*Giardia lamblia*
	Cryptosporidium parvum
	Plasmodium yoelii[a]
	Trichinella spiralis[a]
	Pneumocystis carinii[a]

[a] Response involves both cell-mediated and antibody-mediated immunity.
Source: Adapted from Stiehm et al. (1986)

carcinogens, virus-induced cancers are considerably more immunogenic and therefore more likely to be influenced by immunological factors. Examples of cancers that are common in immunosuppressed individuals include leukaemia and lymphoproliferative disorders as well as cancers of the skin, seen in transplant patients, Kaposi sarcoma and EBV-associated B cell lymphomas.

4.8.2.2 Diseases associated with specific types of immunosuppression in laboratory animals

Reduction in NK cell activity has been shown to correlate with increased susceptibility to challenge with PYB6 sarcoma cells, B16F10 melanoma cells and murine CMV (Luster et al., 1988; Selgrade et al., 1992). Suppression of cell-mediated immunity, complement deficiency and depressed macrophage and neutrophil function have all been associated with decreased resistance to *Listeria monocytogenes* (Petit, 1980; Luster et al., 1988; Bradley, 1995). The relationship between suppression of humoral immunity (antibody response) and infections as well as specific challenge agents is illustrated in Table 4.1. The relationship between suppression of cell-mediated immunity (T cell–mediated response) and infections as well as specific challenge agents is illustrated in Table 4.2. Clearance of parasitic infections such as *Plasmodium yoelii* and *Trichinella spiralis* has both a cellular and a humoral component, and increased infectivity has been shown following depression of both arms of the immune system (Luebke, 1995; Van Loveren et al., 1995).

Deletion or functional block of specific immune components is another method that has been used to examine this relationship. This can be achieved via targeted gene disruption resulting in animals deficient in specific cell populations (e.g. CD4+ T cell knockout), administration of agents that affect cells or mediators involved in host defence (e.g. the use of gadolinium

Table 4.2: Host resistance and infections associated with reduced cell-mediated immunity.

Infections associated with effector T cell deficiency	*Organisms associated with deficiencies in T cell–mediated immunity*
Chronic enterovirus encephalitis	**Bacteria**
Vaccine-induced paralysis	*Mycobacterium tuberculosis*
Persistent parainfluenza infection	*Mycobacterium intracellulare*
Refractory mucosal candidiasis	*Listeria monocytogenes*
Progressive EBV infection	*Escherichia coli*
Staphylococcal pneumonia	*Serratia marcescens*
Recurrent cutaneous staphylococcal infections	*Salmonella*
Mycobacterial lymphadenitis	**Viruses**
Recurrent meningococcal infection	CMV
	HSV; herpes zoster
	EBV
	Rotavirus; adenovirus; enterovirus
	Respiratory syncytial virus
	Parainfluenza virus
	Protozoans
	Toxoplasma
	Cryptosporidum
	Plasmodium yoelii[a]
	Trichinella spiralis[a]
	Pneumocystis carinii[a]
	Fungi
	Candida albicans
	Cryptococcus
	Nocardia

[a] Response involves both cell-mediated and antibody-mediated immunity.

Source: Adapted from Stiehm et al. (1986)

chloride to block macrophage function) or the functional depletion of specific cell populations through the binding of monoclonal antibodies to cell surface receptors. It should be noted, however, that multiple genes are usually involved in disease resistance (Hickman-Davis, 2001). Previous studies have suggested that alterations in one or more lymphocyte subpopulations are predictive of changes in host resistance approximately 70% of the time, although no attempt was made to correlate changes in specific cell types with specific infection models (Luster et al., 1993). Studies by Wilson et al. (2001) were specifically designed to determine the magnitude of NK cell suppression that would translate into altered resistance in three host resistance models. The studies were conducted by blocking NK cell activity with an antibody to the cell surface molecule Asialo GM1 using a treatment regimen that did not alter any other of the standard immune function tests used in the assessment of immunotoxicity in rodents. These authors demonstrated that at low levels of tumour challenge, such as might occur spontaneously, NK cell activity would need to be suppressed approximately 50% or more before significant effects on resistance to an NK cell–sensitive tumour would be observed. These studies also demonstrated that the level of suppression needed to alter host

resistance was related to the number of tumour cells in the challenge. Conversely, studies that have used monoclonal antibodies to effectively deplete CD4+ and CD8+ T lymphocytes from the peripheral blood have found little evidence of altered resistance to challenge with PYB6 sarcoma cells, a model that is thought to be dependent on cell-mediated immunity (Weaver et al., 2001). The lack of effects may be related to the fact that cell populations in secondary lymphoid tissues such as the spleen and lymph nodes were unaltered.

Resistance to most common infections is mediated by multiple immune processes, and each effector mechanism may express differential sensitivity to a given toxicant. As individuals are typically exposed to a variety of chemicals, it may be difficult to predict the level of suppression for any one process required to increase the risk of disease.

4.8.3 Adversity and significance of mild to moderate immunosuppression

One question that often arises in interpreting laboratory animal studies is, "What degree of immune change constitutes a biologically significant effect for either a specific test or group of tests?" In one respect, this can be answered relatively simply, in that any statistically significant effect should be considered meaningful, provided the quality of the animal data is sufficient. This argument is based on the assumption that a linear relationship exists between loss of immune responsiveness and increased risk of developing disease. Although a linear relationship has never been definitively established and may never be, it is consistent with our understanding of immunological processes and is supported by both laboratory animal (e.g. Luster et al., 1993) and human studies (reviewed in Luster et al., 2005a) in which changes in immune tests correlated progressively with increased incidence of disease over a broad range.

In contrast to laboratory animals, for which the majority of data specific to the immune system will likely be quantitative evaluation of immune function, with disease resistance tests potentially included if immunosuppression is evidenced, human data are more likely to be an enumeration of immune system components, such as class-specific serum immunoglobulin concentrations and immunophenotyping, or evaluation of burden of disease following exposures, such as infectious disease incidence. It should be emphasized that small and transient changes in the incidence of common infectious diseases in exposed populations would be the most likely outcome of a mild to moderate immunosuppression resulting from exposure to chemicals in the environment, although this is difficult to capture in retrospective epidemiological studies (see below).

The clinical consequences of severe forms of immunosuppression, such as those that may occur in individuals with AIDS or primary (genetic) immunodeficiency diseases, are manifested as increased frequency or greater severity of certain types of cancers or infectious diseases. While these effects are profound, adverse effects that occur from mild to moderate immunosuppression, such as those associated with chronic stress, transplant therapies or even excessive exercise and that might occur in populations exposed to immunotoxic chemicals, are considerably more difficult to detect. The most comprehensive databases that address immunosuppression–adversity relationships, specifically primary immunodeficiency diseases and AIDS, are not included in these discussions, as these represent severe examples of immunosuppression, and neither the specific clinical diseases that result nor their eventual outcomes have much in common with those that occur in individuals with chronic mild to moderate immunosuppression. The issue of adverse health effects and immunotoxicology has been a topic of several recent reviews (e.g. Kimber & Dearman, 2002; Luster et al., 2005a).

To assist in assessing the health impact of mild to moderate immunosuppression, some of the most likely adverse effects that may occur from chronic mild to moderate immunosuppression associated with chronic stress, stem cell transplantation and organ transplantation are described below, as well as physiological factors and study design issues that can affect interpretation.

Chronic psychological factors (i.e. stressors), such as separation and divorce, caregiving for patients with Alzheimer disease or bereavement, produce low to moderate degrees of immunosuppression and increase infectious disease incidences (Cohen, 1995; Biondi & Zannino, 1997; Yang & Glaser, 2000; Kiecolt-Glaser et al., 2002). This immunosuppressive response to stress has also been confirmed in a controlled infectious challenge study in humans (Cohen et al., 1991). Although usually conducted in small cohorts, immune testing in other chronically stressed individuals has also provided insights into the relationship between mild to moderate immunosuppression and disease (Kiecolt-Glaser et al., 1986, 1987). In one chronic stress population showing an increased rate of infections, specific cell populations were reduced below mean control values by 20–40%; however, as with a number of immunotoxicology studies, some changes were within the range of normal values reported by the authors.

Associations have also been observed between chronic stress and reactivation of latent viruses, such as CMV, HSV-1 or EBV, as measured by either recurrence of symptoms or elevations in specific antibody titre (Kasl et al., 1979; Glaser et al., 1987, 1993; Esterling et al., 1993; Cohen, 1995; Biondi & Zannino, 1997; Yang & Glaser, 2000). Elevations in antiviral antibody titre (i.e. seroconversion), a reflection of viral reactivation and replication, precede disease onset, although only about 20% of those with elevated titres actually develop clinical disease. Studies have also been conducted to examine associations between psychological stress and the immune response to vaccination for hepatitis B, influenza virus or pneumococcus (Kiecolt-Glaser et al., 1996, 2002).

Haematopoietic stem cell transplantation, which came into general practice in the 1980s, is employed in the treatment of certain haematological malignancies, aplastic anaemia and inborn genetic errors of cells originating in haematopoietic stem cells. Following cell grafting, immunodeficiency can persist for well over a year due to pre-grafting radiation treatment (Ochs et al., 1995). Thus, prospective studies can help identify quantitative relationships between immune function and disease as the immune system recovers. The incidence of infections in these patients can be high, with 80% of the patients developing an infection during the first 2 years post-engraftment and 50% of the patients having three or more infections. Opportunistic infections predominate, with fungi being the most common type of organism causing disease, followed by bacteria and viruses (Ochs et al., 1995; Atkinson, 2000). Incidence data for upper respiratory tract infections are generally unavailable for these patients, as these infections are seldom monitored in allogeneic bone marrow recipients. Although infections that occur in the first month following transplant are most likely due to severe deficiencies in granulocytes, later infections appear to be due to deficiencies in CD4+ T cells and B cells (Storek et al., 1997, 2000; Small et al., 1999; Chakrabarti et al., 2001).

Studies in renal organ transplant patients have provided insights into the consequences of long-term (chronic) moderate immunosuppression. Although immunosuppressive therapies have greatly improved over the past 40 years, transplant patients are still predisposed to higher rates of malignancies (Jamil et al., 1999) and infections (Clark et al., 1993) compared with the normal population. Infection rates can range between 65% and 70% during the first 6

months post-transplantation, with CMV representing anywhere from 18% to 67% of the reported infections (Sia & Paya, 1998). As a result of the surgical procedure, urinary tract infections are commonly observed in all renal transplants, whereas severe bacterial infections (pneumonia and septicaemia) and systemic/invasive fungal infections were almost exclusively associated with the most immunosuppressed group. Wieneke et al. (1996) noted that reduced immunoglobulin G1 (IgG1) subclass levels and CD4+ T cell counts were the best predictors for infections.

Increased skin cancers have also been noted in patients on long-term immunosuppressive therapy. For example, the risk of developing skin tumours following renal transplantation is 10% after 10 years and 40% after 20 years, whereas the incidence of squamous and basal cell carcinomas is 10-fold and 250-fold higher, respectively, than in the general population (Hartevelt et al., 1990).

4.8.4 Mode of action/mechanisms

Information on the key events of the MOA can be used to evaluate human relevance and to help predict the types of adverse effect that might be expected to occur. For example, reduced antibody production would be expected to reduce resistance to infections caused by bacteria that live outside host cells, but not to those that live inside host cells. Defects in cellular and molecular immune function may result from a variety of mechanisms, including developmental arrest, blockage of metabolic pathways, abnormalities in cytokine synthesis or secretion, altered MHC expression, disrupted signalling pathways, impaired DNA synthesis and lymphocyte proliferation, and/or failure of normal apoptotic mechanisms. It is important to note that a chemical can have more than one MOA.

The MOA can also be used to evaluate the probability of a particular effect. For example, total suppression of haematopoiesis or antibody synthesis is unlikely to occur following exposure to chemicals not specifically designed to target the immune system or to block DNA or protein synthesis. However, if an immune defect occurs in cells that cannot be replaced, such as long-term or short-term stem cells from the bone marrow, persistent immune effects may occur. In contrast, any immune effects caused by an exposure may be fully reversible if, for example, progenitor cells are left undisturbed and the effects are focused exclusively on clonally expanding immune cells (i.e. post–antigen challenge). Thus, information about the MOA and potential key events may help predict the likelihood of persistence, even if the full MOA cannot be determined. For example, alterations in stem cells would likely have long-term effects, compared with alterations limited to secondary lymphoid organs, such as the spleen or lymph node.

4.9 Dose–response relationships and thresholds

A dose–response relationship is a necessary criterion in demonstrating chemical immunosuppression. The interpretation of dose–response data should identify doses associated with the adverse effect (immunosuppression), as well as doses associated with no adverse effects, to determine the most appropriate end-point(s) or critical effect(s). The critical effect or effects are then used for the development of POD(s) from which health-based guidance values or reference values (ADI/TDI or RfD/RfC) can be calculated by dividing the POD(s) by the total uncertainty factor, using either default uncertainty factors or CSAFs. The process for determining and evaluating the dose–response relationship for immunotoxicity data is presented in section 3.3.7 of chapter 3 and should be consulted for a more detailed discussion.

The dose–response functions for chemical-induced immunosuppression are generally assumed to be non-linear[1] and to demonstrate a threshold dose below which effects on immunosuppression would not be expected. Additionally, based upon our current understanding of immune processes in humans and available human study data (reviewed in Luster et al., 2005a), one would most likely assume that a linear relationship exists between the loss of immune function and increased disease incidence. The immunotoxicology literature contains examples of non-linear and biphasic dose–response curves across the spectrum of immunological measures, such as lymphocyte proliferation, antibody production, phagocytosis, DTH and host resistance assays. The assumption of a threshold dose as the POD for risk assessment is supported by the available data for these types of immunosuppression-related end-points.

In contrast, there are some examples, such as corticosterone-associated immunosuppression, for which linear dose–response curves have been demonstrated for the same end-points. For example, Pruett and colleagues (Pruett et al., 1999, 2000; Pruett & Fan, 2001) generated linearly proportional models for measures of immunosuppression (e.g. NK cell activity, lymphocyte subpopulations in the spleen and thymus, MHC class II expression and antibody response to the T cell–dependent antigen KLH) resulting from exposure to increased levels of either exogenous or endogenous (from restraint stress) corticosterone using the AUC for corticosterone as the dose metric. Pruett et al. (2003) also found that the corticosterone AUC was an appropriate metric for some measures, such as the antibody response to KLH, for chemicals known to induce a stress response (e.g. ethanol and atrazine) and that the magnitude of the effect was again linearly proportional to the corticosterone AUC (Pruett et al., 2003).

4.10 Groups at risk (developing immune system, elderly, immunocompromised)

Age-related physiological differences and immaturity of the immune system are both likely to play a role in increased susceptibility associated with the developing immune system. It has been demonstrated that for some chemicals, the immature immune system is more susceptible to chemicals than the fully mature system, and sequelae of developmental immunotoxicant exposure may be more persistent, in contrast to effects observed following adult exposure, which generally occur at higher doses and are expected to resolve soon after exposure ends (Holladay & Smialowicz, 2000; Dietert & Dietert, 2007). Based on results obtained in various experimental animal studies, perturbations of the developing immune system may be manifested as a qualitative (i.e. affecting only the developing immune system) or a quantitative (i.e. lower doses affect the developing immune system) difference (see chapter 2 for further discussion). Following developmental exposure, immune maturation may simply be delayed and may recover to normal adult levels over time, or, if exposure interferes with a critical step in the maturational process, lifelong defects in immune function may follow (e.g. DES: Kalland & Forsberg, 1980; Kalland, 1984). The steps involved in human and rodent immune system maturation appear to be remarkably similar, but occur at different developmental stages, and no compelling evidence exists to suggest that effects observed in rodents are not representative of what might be expected to occur in humans. Thus, effects of rodent

[1] The term "non-linear" is used here in a more narrow sense than its usual meaning in the field of mathematical modelling. In this guidance document, the term non-linear refers to threshold models (which show no response over a range of low doses that include zero) and some non-threshold models (e.g. a quadratic model, which shows some response at all doses above zero). In this guidance document, a non-linear model is one whose slope is zero at (and perhaps above) a dose of zero.

exposure shortly after birth are likely to reflect what may happen in humans exposed during late gestation, assuming that the chemical crosses the placenta. This concept was reviewed in detail by Holladay & Smialowicz (2000) and by Holsapple (2003).

Several studies have shown increased rates of certain infections in children following perinatal exposure to environmental agents (Luster et al., 2005b). For example, Weisglas-Kuperus et al. (2000) demonstrated that exposure to levels of polyhalogenated aromatic hydrocarbons normally found in highly industrialized countries is associated with increases in childhood infections and lower vaccination responses. Likewise, Karmaus et al. (2001) found that children with elevated levels of the dichlorodiphenyltrichloroethane (DDT) metabolite, dichlorodiphenyldichloroethylene (DDE), and PCBs or DDE and hexachlorobenzene (HCB) had more cases of inner ear infection than expected. Higher burdens of DDE alone increased the odds ratio (OR) for the development of asthma and elevated IgE levels. Limited human immune function data are available from offspring of women taking therapeutic doses of immunosuppressants during pregnancy, although maternal use of azathioprine (Price et al., 1976) or cyclosporin A (Tendron et al., 2002) during gestation has been reported to suppress immune function in human infants up to 1 year of age.

The elderly may also be at increased risk for immunosuppression, and, as described in EHC 144, the elderly represent an acknowledged sensitive population because of age-related changes in immune function (IPCS, 1993). Typically, immune system function decreases with the advancement of age (reviewed in Miller, 1996; Aw et al., 2007). The thymus continues a post-puberty process of atrophy, in which cellular components are replaced by adipose tissue, and the production of thymic hormones ceases by about 40 years of age (USEPA, 2005c). Altered immune system responses have been attributed to altered intracellular signalling in macrophages and neutrophils, reduced apoptosis in neutrophils and decreased stimulation of T and B cells by dendritic cells (Plackett et al., 2004). The presentation of IL-10, IL-12 and antigen by dendritic cells may also be altered (Uyemura et al., 2002). Other immune system functions that are often impaired in the elderly are the ability of T lymphocytes to increase in number in response to an antigen and the amount of antibody secreted by B lymphocytes. Owing to this normal process of immune system senescence, the elderly exhibit a poor vaccine response and are often more susceptible to infectious diseases, such as pneumonias, urinary tract infections or tuberculosis, and the responses to these diseases may be more severe in the elderly than in young, healthy adults (Kumar & Burns, 2008). Likewise, responses in the elderly to microbial contaminants in the environment may be differentially severe (USEPA, 2007). Additionally, the incidence of cancer increases with age in both humans and laboratory animals, suggesting that there is a correlation between immunosenescence and higher incidences of some cancers (Cohen, 1994; Anisimov, 2007).

Enhanced susceptibility may also be a function of genetic disposition (i.e. genetic polymorphisms at the individual or population level), disease state (e.g. AIDS) or pharmaceutical intervention (e.g. organ transplant therapy). Sex differences in response to immunotoxic agents are not uncommon, either in laboratory animal studies or in epidemiological studies.

4.11 Acute versus chronic exposure

Developmental exposure to an immunotoxicant may result in quantitatively and qualitatively different immunosuppression compared with exposure at other life stages (see discussion in chapter 2 and section 3.3.3.2(d) of chapter 3) and may result in long-lasting or permanent immunosuppression after an acute or short-term exposure. For example, a single injection of

TCDD at 1 mg/kg body weight suppressed the antibody response to the T cell–independent antigen trinitrophenyl (TNP)–LPS in mice (Smialowicz et al., 1996), and perinatal (gestational, lactational and juvenile) exposure to TCDD resulted in immunosuppression in rats, which lasted for 19 months (Smialowicz, 2002). Although there are no testing guidelines in widespread use that are designed to evaluate acute or developmental immunotoxicity at this time, the use of less-than-lifetime risk estimates may be appropriate for chemicals with a sufficient database demonstrating developmental immunotoxicity. Some authors have suggested replacing adult exposure assessment for immune evaluation with protocols to evaluate the developing immune system (Dietert & Piepenbrink, 2006b).

4.12 Uncertainty factors

Considerations in the application of uncertainty factors for immunotoxicity data are presented in detail in chapter 3 (section 3.3.10). All of the standard uncertainty factors (intraspecies, interspecies, database) as well as uncertainty factors addressing subchronic to chronic extrapolation and LOAEL to NOAEL extrapolation, as determined by the scope of the risk assessment and the data used for the POD, should be used to derive health-based guidance values for immunosuppression. Considerations in the application of intraspecies, interspecies and database uncertainty factors for immunosuppression data are described below.

4.12.1 Intraspecies uncertainty factor

In the absence of information on the potential variability in susceptibility among the general population to the particular type of immunosuppression considered for a POD, a default intraspecies uncertainty factor of 10 is recommended, similar to that recommended for other non-cancer end-points. This uncertainty factor is used to address the variability in responses among humans and protect sensitive subpopulations. As discussed in section 4.10, the young (in utero and postnatal exposure or children) and the elderly may be at greater risk for immunosuppression. Susceptibility may also be a function of genetic polymorphisms, as has been demonstrated for the response to vaccination (e.g. Hennig et al., 2008; Ovsyannikova et al., 2008). The risk assessor should consider a reduction in the intraspecies uncertainty factor if data for the POD are derived from the most sensitive subpopulation of humans.

4.12.2 Interspecies uncertainty factor

When PODs for immunosuppression are derived from human data, no interspecies uncertainty factor is required, and it is set to 1. However, the availability of dose–response data in humans is generally limited, and therefore the extrapolation of laboratory animal data to humans is often necessary. Similar to the situation for other non-cancer end-points, a default interspecies uncertainty factor of 10 is recommended to extrapolate from laboratory animal species to humans when laboratory animal data are used to derive PODs for immunosuppression. Available data support this approach, because immunosuppression data from experimental animals are generally good predictors for subsequent clinical data or epidemiological studies.

4.12.3 Database uncertainty factor

Examination of the extent of the database, the quality of individual studies and data gaps will assist the weight of evidence determination of database sufficiency. The database for a chemical may not contain any toxicity studies specifically designed to determine

immunotoxicity. Nevertheless, risk assessors determine which of the available data may provide information on immunosuppression. In some cases, a limited data set may suggest the possibility of immunosuppression without appropriate data to make a determination of immunosuppression risk or perform a dose–response assessment. In such cases, the risk assessor should consider the use of the database uncertainty factor to indicate that the lack of information on this end-point may be significant. The reader should consult section 3.3.10.5 of chapter 3 for a detailed discussion of the use of the database uncertainty factor to characterize the immunosuppression hazard. In addition, the risk assessor may be in the position of requesting additional data. A related discussion of "triggers" and the need for additional data to determine immunotoxicity hazard is presented in section 3.3.6.3 of chapter 3.

4.13 Exposure assessment

Exposure assessments are used to obtain an estimate of human exposure in order to help quantify the risk to a population. Specific guidelines on exposure assessment (e.g. USEPA, 1992) and guidance specific to assessing the exposure of children (e.g. USEPA, 2005a; IPCS, 2006b) have been published separately and will not be discussed here. Section 3.3.11 of chapter 3 should be consulted for general considerations important for exposure assessment for immunotoxicity, including contributions of dose and timing of exposure to the severity and persistence of effects, susceptibility based on timing of exposure, the importance of route of exposure for localized as well as systemic immune effects, and toxicokinetic factors of exposure that influence the immunotoxicity outcome(s).

4.13.1 Exposure-related effects on severity and persistence

For immunosuppression, the magnitude of the adverse effect (e.g. infectious disease incidence) will be proportional to the severity of immune system damage as well as the length of time the effect remains (persistence). In biological terms, this can be summarized as follows: the adverse health outcome is proportional to both the severity and persistence of the immune effect. Thus, the contribution of exposure to the severity of outcome may need to be considered only if sufficient biological data are available indicating that the effect is persistent. This would be expected, for example, if the injury occurs in cells that cannot be replaced, such as long-term or short-term stem cells from the bone marrow, memory cells are left undisturbed and tolerance is not induced. In contrast, an assumption can be made that any immune effects caused by an exposure are fully reversible under most conditions, if, for example, progenitor cells are left undisturbed and the effects are focused exclusively on clonally expanding immune cells (i.e. post–antigen challenge).

The timing of exposure to pathogens relative to the period of immunosuppression is also critical. Obviously, the outcome from exposure to influenza virus or a neoplastic cell could be very different if exposure to the pathogen occurs while the individual is immunosuppressed as opposed to exposure after recovery from immunosuppression. Persistence of immunosuppression following chemical exposure would result in a larger window of vulnerability to pathogens; however, short-term suppression may still have long-term effects. For example, if an individual who is immunosuppressed for only a few days as a result of chemical exposure also receives a vaccine (e.g. the influenza vaccine) while suppressed, the effect may be less than adequate response to vaccination and ultimately lead to being infected with influenza virus months after recovering from the chemical immunosuppression. Therefore, the issue of persistence and reversibility may be affected by the types of immune challenges that the organism faces during the period of suppression (either short or long term).

The issue of persistence versus reversibility may also be complicated by the dose or duration of the chemical exposure. For example, many antiproliferative agents used in cancer chemotherapy temporarily reduce the ability of immunocompetent cells to respond to antigen at low doses, whereas at high doses or for longer treatment periods, they may have a greater persistence of effects. This could result in either a delay in recovery, to allow sufficient time for the stem cell repopulation, or immune failure, in which the stem cells or stromal cell microenvironment is irreversibly damaged. Thus, the final outcome will depend upon the dose and duration of exposure, as well as the specific target within the microenvironment (e.g. stromal cells versus long-term stem cells versus short-term stem cells). For agents that bioaccumulate, increasing duration of exposure may also increase the extent of damage to the stem cell microenvironment. In some instances, a delayed effect on the immune system may result. This may occur if memory cells or circulating immunocompetent cells are affected, although this would be an unlikely occurrence.

Exposure in host resistance models used to assay immunosuppression is complicated by the dose and biology of the challenge agent as well as the dose of the chemical immunosuppressant. In laboratory studies, higher doses of pathogen and greater virulence are generally associated with increased severity of the outcome, including clinical score or mortality (Cohen, 2007). Although an effective immune response may be elicited, at higher doses, the associated disease can overwhelm the host. For example, a wide range of challenge doses of influenza virus resulted in similar influenza-specific antibody titres in mice; however, larger doses were associated with increased weight loss and mortality (Powell et al., 2006).

4.13.2 Exposure timing and susceptibility

Another consideration regarding exposure assessments for immunotoxicity is the life stage at which exposure occurs. It is generally believed that the immature immune system is more susceptible than the fully mature system to chemicals and that sequelae of developmental immunotoxicant exposure may be particularly persistent, in contrast to effects observed following adult exposure, which generally occur at higher doses and are expected to resolve soon after exposure ends (see review by Holladay & Smialowicz, 2000). Luebke et al. (2006a) recently reviewed adult and developmental animal data and, where available, human data on five diverse immunotoxic compounds for age-dependent differences. The chemicals reviewed included DES, diazepam, lead, TCDD and tributyltin oxide. For all five chemicals, the developing immune system was found to be at greater risk than that of the adult, because either lower doses caused immunotoxicity or adverse effects were more persistent, or both. When comparing doses that adversely affect the developing and adult immune systems, it is important to bear in mind that offspring may be exposed during pregnancy to only a fraction of the maternal dose, even though the maternal dose is typically referred to in the literature. The review by Luebke et al. (2006a) concluded that the exclusive use of adult animals in immunotoxicological studies is likely to underestimate the risk of exposure to chemicals during development and maturation of the immune system. These general conclusions are also presumed to pertain to humans, as the developmental processes between rodents and humans are relatively similar, except that immune maturation proceeds more slowly in rodents than in humans (Holladay & Smialowicz, 2000; Landreth, 2002; Holsapple, 2003).

Certain types of infectious diseases are more common in the elderly than in neonates or young adults owing to age-related loss of immune responsiveness (i.e. immunosenescence). In the simplest terms, the very young lack immunological experience, and the elderly, in spite of a wealth of experience, are no longer able to respond as well as the younger population.

There are no data to support the hypothesis that the immune system of the elderly is uniquely susceptible to immunotoxic agents. However, as a result of immunosenescence, a moderate loss of immune function may have more significant adverse effects in the elderly than in the young adult. An exposure assessment should characterize the likelihood of exposure of at least three general age groups—prenatal, young adult and elderly—and factor the susceptibility of the groups into the risk assessment to the extent possible.

4.14 Risk characterization in terms of reduced resistance to infections or tumours

Risk characterization is a synthesis of estimates of exposure levels and health risks. It is the summary and integration portion of the risk assessment process in which the hazard characterization, quantitative dose–response and exposure assessment are combined, along with a critical appraisal of the toxicity information. The critical evaluation contains a review of the overall quality of the assessment, including a discussion of uncertainties and a valuation of confidence in the conclusions. In addition to the thorough appraisal of the strengths and weaknesses of the assessment, risk characterization also includes a section describing risk in terms of the nature and extent of harm. Additionally, to the extent permitted by the available data, risk characterization indicates how risk varies with exposure and provides information to help risk managers evaluate a range of options. Ideally, a quantitative risk assessment is performed. Where the available data do not allow for this, a qualitative risk assessment may be possible.

A risk assessment for lead is used as a case-study to illustrate the application of the above guidance on immunosuppression risk assessment (see Case-study 1).

5. ASSESSMENT OF IMMUNOSTIMULATION

5.1 Introduction

Destruction of infectious agents and certain types of neoplasia requires activation and stimulation of the immune system, resulting in the release of potent cytokines, cellular proliferation and differentiation and multifunctional mediator release. Multiple feedback systems, including signals produced by cells of the immune, endocrine and central nervous systems, promote immune system homeostasis by balancing upregulatory and down-regulatory signals to minimize collateral tissue damage in the course of normal immune responses or clonal expansion of cells that inappropriately recognize and respond to host proteins. Failure to control the intensity and duration of normally protective immune responses is a well-documented cause of immune-mediated tissue damage. Nevertheless, deliberate artificial stimulation of the immune system is a routine and mostly beneficial clinical procedure. For example, chemical and/or biological adjuvants are routinely included in vaccines to increase and prolong the immune response and to improve the response to weak antigens; successful induction of protective immunity is often dependent on their activity.

Adverse effects associated with stimulation of the immune system by a xenobiotic include inappropriate stimulation or skewing of normally protective immune responses, direct allergenicity of the xenobiotic, induction or worsening of autoimmune disease and non-specific inflammation. Inappropriate stimulation of normally protective responses to infectious agents may increase inflammation, resulting in excess tissue damage or potentially exposing cryptic host antigens, one possible pathway to autoimmune disease. Skewing of the immune response to favour either inflammation or allergy can also occur; some studies have linked such changes with an increased risk of developing allergies and reduced resistance to certain infectious agents. Xenobiotics may act as complete allergens to induce allergic disease or may bind to host proteins, forming a complex that induces allergy or altering host proteins sufficiently that they are no longer recognized as "self", resulting in autoimmune disease. Allergy and autoimmune disease are covered in chapters 6 and 7 of this document, respectively. Inflammation is a normal component of tissue injury associated with toxicant exposure, although this type of injury is generally considered in the context of specific organ system toxicity and will not be addressed in this chapter. The possibility also exists that a chemical may act as a superantigen or cause a "cytokine storm", which can occur when cytokines, monoclonal antibodies to functional immune system epitopes or potent immune system regulators are given therapeutically. However, it is unlikely that this will be observed with chemicals (reviewed by Ponce, 2008).

This chapter will examine the evidence to support the hypothesis that unintended stimulation of either the innate or adaptive immune response should be considered as an adverse effect and taken into account in a weight of evidence approach to risk assessment.

5.2 Hazard identification

Immunotoxicity testing for hazard identification, exclusive of hypersensitivity and autoimmunity, has focused on suppression of immune function (see chapter 4). In some cases, assays used to detect immunosuppression have instead indicated responses at greater than control group values following exposure to certain pesticides, drugs and other chemicals of environmental concern. Although there is significant concordance between suppression of the

IgM response and increased susceptibility to infectious disease, it is unknown whether similar concordance exists between increased IgM responses and adverse outcomes. The same is true for other commonly assessed functional end-points (e.g. DTH or NK cell activity), although more data may be available for the primary antibody response simply because extant immunotoxicity testing guidelines require evaluating the primary humoral response, but not tests of cellular or innate function. Many immunotoxicologists consider unintended immune system stimulation to be a noteworthy finding, but one that may be difficult to characterize or unambiguously define as adverse, because assays currently used to screen for potential immunosuppression, allergy or autoimmunity may or may not be the most appropriate methods to detect immunostimulation.

5.3 Hazard characterization

Regulatory acknowledgement of unintended immune system stimulation as an adverse effect is limited. However, the USFDA Immunotoxicity Testing Guidance (USFDA, 1999) considers unintentional immunostimulation, immunogenicity and adjuvant activity as potentially adverse effects. According to the USFDA document, "Change in an immune function or level of immunological mediator may not necessarily appear as an 'adverse effect', but rather as immunostimulation. Caution must be exercised in such cases, because a non-specific enhancement of the immune response that might be interpreted as a beneficial effect may result in suppression of specific immunity against a particular infection." The USFDA also states that unintended stimulation may result in autoimmunity, hypersensitivity and chronic inflammation, but does not offer guidance on the best practices for interpretation of stimulation.

Allergy, hypersensitivity, inflammation and autoimmunity are clearly adverse. However, it can be argued that increased antibody production in response to immunization should not be interpreted as adverse per se, because current vaccination protocols typically rely on chemical adjuvants that stimulate antibody production. Exposure to xenobiotics that elevate antibody synthesis has been associated with increased production of autoantibodies or worsening of disease in autoimmune disease–prone animal models. These data suggest that adverse effects may occur in individuals with genotypes that are associated with allergy or autoimmunity, but tell us little about the level of risk that the general population will experience. As such, identification of elevated humoral responses in groups of experimental animals immunized during tier testing for immunotoxicity should not be ignored, as elevated responses are a clear indication that modulation of the immune system has occurred. Furthermore, as discussed in the examples provided in section 5.5 below, it is clear that upregulation of one functional end-point may be accompanied by suppression of other immune functions that mediate host resistance. Identification of unexpected stimulated immune function may therefore depend on which assays were used for hazard identification. The final decision on how to interpret the data is a policy decision. The regulatory mandate and the ability of the risk assessor to ask for additional testing will determine how, or if, additional testing will be pursued.

Because inflammation is a normal response to toxicity, the possibility exists that toxic exposures can synergistically or additively increase inflammatory responses to infectious or allergen challenge. In animal models, several types of chemical exposure, most notably to dioxin, have been shown to increase pulmonary damage caused by the immune response to influenza infection. Similarly, exposure to air pollutants has been shown to exacerbate respiratory responses to allergen challenge in rodent and human studies, and air pollutants act as adjuvants to promote allergic sensitization.

5.4 Clinical and epidemiological data

Human data may be the most appealing as a basis for risk assessment, although care must be taken when interpreting these data. Issues critical to interpreting clinical and epidemiological data are presented in sections 3.3.2.1 and 3.3.2.2 of chapter 3, respectively. Clinical data suggest that moderate stimulation of the immune system by adjuvants commonly used in vaccines or dietary supplements taken to "boost" the immune system is not associated with immune-mediated disease in the general population. However, individuals with pre-existing autoimmune disease may constitute a susceptible subpopulation in which activation of the immune system may have adverse effects. For example, the use of herbal supplements has been temporally associated with flare-up of pemphigus vulgaris in two patients and with the onset and later flare-up of dermatomyositis in another (Lee & Werth, 2004). Nevertheless, human studies suggest that routine vaccination against influenza and pneumonia is safe and effective in patients with various systemic autoimmune diseases, in spite of previously expressed concerns that immune system activation by components of the vaccine may activate or worsen systemic autoimmune disease (e.g. Elkayam et al., 2007; Holvast et al., 2007; Glück & Müller-Ladner, 2008).

In contrast, unintentional stimulation of human immune function by chemical exposure may have an adverse outcome. For example, occupational exposure to silica, reported to have an adjuvant-like effect on cells of the innate immune system, is associated with development of human autoimmune disease (Parks et al., 1999). The heavy metal mercury has been reported to polarize the response of stimulated human lymphocytes towards the Th2 phenotype by increasing production of IL-4 and IL-10, suggesting that mercury may upregulate ongoing immune responses (Hemdan et al., 2007). Unintended upregulation of human antibody production by mercury is suggested by a study in which removal of dental amalgam from individuals with autoimmune thyroiditis and mercury hypersensitivity was determined to reduce autoantibodies to thyroglobulin and thyroid peroxidase (Sterzl et al., 2006). Lead exposure also increases Th2 cytokine production and has been associated with increased human IgE synthesis, although conflicting results have been reported for other antibody classes (Dietert & Piepenbrink, 2006a).

Disruption of immune system balance by suppression of certain immune functions can lead to stimulation of other immune functions. For example, suppression of Th1 cells could increase Th2 responses (see chapter 4), and reduced regulatory T cell function may also result in stimulated responses, particularly against self-peptides. Hence, it is possible to enhance the risk of both infectious and allergic disease at the same time, an outcome that is supported by human data. For example, in utero exposure to cigarette smoke has been strongly associated with increased risk of developing asthma (Jaakkola & Gissler, 2004; Ng & Zelikoff, 2007), yet laboratory animal data demonstrated that such exposures increased susceptibility to tumour challenge in association with persistent suppression of cytotoxic T cell activity (Ng et al., 2006). Similarly, Soto-Peña et al. (2006) demonstrated that proliferation of peripheral blood mononuclear cells in response to PHA was significantly decreased in association with an increase in arsenic concentration in urine of children 6–10 years of age exposed chronically to arsenic. This same study demonstrated a tendency towards increased incidence of allergies and asthma among individuals with urinary arsenic concentrations higher than 50 µg/l. However, exposure to PCBs was associated with less shortness of breath and wheeze (Weisglas-Kuperus et al., 2000), whereas immunosuppression and increased infection were observed in similarly exposed populations (Dallaire et al., 2006; Heilmann et al., 2006). Thus, chemical immunosuppressants may or may not be associated with allergic risk. The

best evidence that environmental exposures may increase the risk of allergic disease is found in the air pollution literature. In addition to the tobacco smoke example above, several prospective studies support a modest increase in risk for asthma from other air pollutants, including oxidant gases and diesel exhaust particles (reviewed by Gilmour et al., 2006).

In aggregate, the human literature suggests that inadvertent stimulation of the immune response may have adverse effects. However, in almost all cases, there appears to be a genetic component associated with adverse effects, suggesting that adverse effects are most likely in sensitive individuals, rather than the general population (see section 5.9 below).

5.5 Laboratory animal data

Animal data will constitute the bulk of information available to the risk assessor, originating in response to a regulatory mandate or published in the peer-reviewed immunotoxicity literature. Many factors may influence the outcome of published studies, including sex, species and strain, route and duration of exposure, and age at initial exposure. Understanding how these factors influence immune system homeostasis and toxicity is critical; the reader is referred to section 3.3.3.2 of chapter 3 for a detailed discussion.

Immunotoxicity studies have consistently determined that exposure to various classes of xenobiotics (heavy metals, pesticides and endocrine disruptors) and certain drugs is associated with increased or stimulated immune function, particularly the T cell–dependent antibody response. In many cases, these same compounds were determined to hasten the onset or enhance the severity of autoimmune disease in genetically susceptible models.

Well-characterized methods for testing chemical exposure for adjuvant-type effects in rodent models are not currently available; however, animal studies have shown that exposure to a variety of air pollutants stimulates sensitization to common allergens, such as dust mites or ovalbumin (Gilmour et al., 2000; Steerenberg et al., 2005). Recent mechanistic studies have defined the prominent role of inflammation and oxidative stress in the proallergic immunological effects of particulate and gaseous pollutants (reviewed by Riedl, 2008).

5.5.1 Vaccination

Animal studies have evaluated the effects of vaccination on various autoimmune diseases. For example, repeated immunization with a commercial conjugated *Haemophilus influenzae* B vaccine was reported to cause a secondary IgA nephropathy in outbred mice (Kavukçu et al., 1997). In contrast, plasmid DNA vaccines caused a transient increase in circulating anti-DNA levels in BALB/c mice, but did not cause autoimmune disease (Mor et al., 1997). These results suggest that under some conditions, vaccination alone may be associated with at least transient signs of autoimmunity, if not autoimmune disease. Combining vaccination with exposure to an immunotoxicant, Ravel et al. (2004) found that repeated vaccination of mice concurrent with exposure to methylmercury increased serum IgG concentration, although the authors suggested that interpretation of the results was difficult because of the "extreme experimental conditions of this study". Direct comparison of the limited rodent data with human data is difficult, because most human studies evaluated the potentially exacerbating effects of vaccination in patients who were receiving immunosuppressive therapy to treat symptoms of their disease; in most studies, patients typically made a more modest response to immunization than disease-free and drug-free normal controls, although titres were generally judged to be protective.

5.5.2 Heavy metals

Exposure to heavy metals has been reported to increase serum immunoglobulin levels and antibody responses to T cell–dependent and T cell–independent antigens and worsening of autoimmune disease in genetically prone strains of rodents. For example, mercury(II) chloride injected subcutaneously (1 mg/kg body weight, 3 times per week) has been reported to increase the concentration of all serum immunoglobulin isotypes after 2 and 3 weeks of exposure; however, even with continued exposure, serum immunoglobulin concentrations returned to control levels by 6 weeks (Pelletier et al., 1988). Similar doses caused transient increases in antibodies to the T cell–independent antigen, TNP–bovine serum albumin, and to the T cell–dependent antigen, sheep erythrocytes, and induced autoimmune kidney disease in Brown Norway, but not in Lewis, rats (Hirsch et al., 1982). Cytokine production is skewed in favour of antibody production in Brown Norway rats; autoimmune kidney disease in this strain is antibody dependent. Lewis rats, in contrast, are biased towards Th1 (proinflammatory) cytokine production and are prone to developing inflammatory disease due to defective hypothalamic–pituitary–adrenal axis control of inflammation (Sternberg et al., 1989). Likewise, drinking-water containing lead at a concentration of 2072 mg/l for 10 weeks was reported to increase the antibody response to sheep red blood cells (SRBCs) in BALB/c mice (Mudzinski et al., 1986). Lead exposure was also associated with worsening of autoimmune disease in a genetically prone mouse model of lupus, but it did not induce disease in resistant strains of mice (Hudson et al., 2003). In contrast to enhanced humoral immune responses, other host-protective functions may be suppressed by heavy metals, including resistance to infection with the intracellular pathogen *Listeria monocytogenes* (Kishikawa et al., 1997). Furthermore, survival was decreased and body burdens of the extracellular pathogen *Salmonella enterica* serovar Typhimurium were increased in mice challenged with the organism after drinking water containing lead at a concentration of 2072 mg/l for 16 weeks (Al-Ramadi et al., 2006).

Dietert & Piepenbrink (2006a) reviewed the evidence that lead exposure may stimulate inflammatory responses. Increased production of the proinflammatory cytokines tumour necrosis factor–alpha (TNF-α), IL-1β and IL-6 by stimulated macrophages has been documented, as has production of reactive oxygen intermediates (in laboratory animals and children) by macrophages and neutrophils and increased eosinophil degranulation, all of which may lead to tissue damage.

Cadmium exposure has also been associated with increased IgM and IgG class antibody responses to sheep erythrocytes following a single exposure (Koller et al., 1976) or after short-term (3–4 weeks) or long-term (9–11 weeks) exposure to 50 or 200 mg/l in the drinking-water (Malavé & de Ruffino, 1984). In the latter study, long-term exposure to 300 mg/l suppressed the humoral response, suggesting that a dose threshold may exist for stimulation and that higher doses may lead to suppression. Furthermore, although recognition and proliferation in responses to allogeneic antigens were suppressed by subcutaneous injection of cadmium at doses of 0.11–1 mg/kg body weight for 5 days, the total numbers of splenic B cells producing IgM and IgG were increased (Hurtenbach et al., 1988). The increase in numbers of total spleen cells and both splenic T and B cells was also found in outbred ICR mice that drank water containing cadmium at 3, 30 or 300 mg/l for 10 weeks (Ohsawa et al., 1983). Moreover, Ohsawa et al. (1988) reported antinuclear antibodies (IgG class) in outbred ICR mice that drank water containing cadmium at 3, 30 or 300 mg/l for 10 weeks and increased numbers of spleen cells producing antibody that reacted with SRBCs in mice that had not been immunized. In contrast, the highest concentration of cadmium suppressed the

PFC response in immunized animals exposed to cadmium at 300 mg/l, but not at the lower doses. Unfortunately, nonspecific stimulation of antibodies that react with SRBCs has not been investigated following exposure to other chemicals that increase the antibody response to SRBCs. Such studies would help to determine whether this is a common finding and the extent to which this unexpected response may contribute to overall titres to SRBCs in exposed immunized animals. Cell-mediated immunity (DTH) was not affected. In contrast, antinuclear antibodies were detected only at the highest dose in inbred (BALB/c) mice. In a separate study, no effects on the antibody response to SRBCs or resistance to infection (survival rate) were detected in B6C3F1 mice exposed to cadmium at concentrations of 10, 50 or 200 mg/l in drinking-water for 90 days (Thomas et al., 1985). Furthermore, in auto-immune-prone mice (NZB/NZW, a model of spontaneous lupus nephritis), cadmium at 10 mg/l in drinking-water exacerbated immune complex deposition in the kidney and proteinuria after 4 weeks of exposure (Leffel et al., 2003). Compared across studies, these results reflect the importance of host genotype in susceptibility to modulation of the immune response by cadmium.

5.5.3 Pesticides

Rodgers et al. (1986) reported that a single high-dose (50% of the median lethal dose [LD_{50}]) exposure to the insecticide malathion increased the number of C57BL/6 mouse spleen cells that made IgM antibody when cultured with SRBCs 5 days, but not 1 day, after in vivo exposure. Changes in body and spleen weights, splenic cellularity and evidence of cholinergic activity were not detected at this dose. Spleen cell proliferation in response to the T cell mitogen concanavalin A or the B cell mitogen LPS was increased. Acute exposure did not affect body or thymus weights, splenic cellularity, generation of cytotoxic T cells or serum cholinesterase activity. Exposure to 10% of the LD_{50} for 14 days was without effect. An MOA study subsequently determined that mast cells have a central role in malathion-induced enhanced antibody synthesis (Rodgers et al., 1996). A later study (Rodgers, 1997) determined that oral exposure to malathion (33–300 mg/kg body weight per week, beginning at 6 weeks of age) accelerated the onset of autoimmune disease and increased autoantibody production in autoimmune disease–prone mice (MRL-lpr, a model of spontaneous systemic lupus erythematosus), but not in congenic resistant mice (MRL+/+). Studies in another laboratory corroborated malathion stimulation of the primary immune response to SRBCs; however, similar increases in numbers of antibody-producing cells were documented at oral doses ranging from 0.018 to 180 mg/kg body weight administered every other day over 28 days to SJL/J mice, a strain that can be stimulated to develop lupus-like disease (Johnson et al., 2002). Augmented antibody production occurred in the absence of apparent lymphocyte hyper-responsiveness to mitogenic signals (e.g. T and B cell mitogens) or activation of macrophages. This finding is of particular interest, because significant enhancement was at a dose that is lower than the upper end of the WHO-established ADI of 0–0.02 mg/kg body weight (FAO/WHO, 1998).

Route-dependent stimulation (oral and dermal exposure), suppression (intraperitoneal) and lack of effects (inhalation) were reported in C57BL/6 mice exposed to the carbamate insecticide aminocarb (Bernier et al., 1995). Although the toxicological significance of these results is questionable, it is noteworthy that class II MHC expression was upregulated in LPS-stimulated B cells isolated from the spleens of mice exposed by the oral and dermal routes only, suggesting that early B cell activation events were upregulated. The authors concluded that this compound was possibly autoimmunogenic, based on the apparent immune system stimulation. Intraperitoneal administration of another carbamate insecticide, propoxur,

was reported to increase antibody titres and numbers of spleen cells secreting SRBC-specific IgM at 2 mg/kg body weight per day for 28 days, a dose that was not associated with histopathological changes in lymphoid organs; suppression of the primary antibody response was observed at 10 mg/kg body weight per day, as were lesions in the spleen and thymus (Hassan et al., 2004).

The synthetic pyrethroids deltamethrin and α-cypermethrin were evaluated for immunotoxicity in male F344 rats (Madsen et al., 1996). Increases in the number of spleen cells producing antibody to SRBCs and in NK cell activity were reported at oral doses of 5 and 10 mg/kg body weight per day for 28 days. These effects were accompanied at the highest dose by reduced body weight and increased adrenal weight, suggesting that effects at the highest dose were accompanied by generalized toxicity.

In mice, the herbicide propanil (a single intraperitoneal injection of 50 or 150 mg/kg body weight) was reported to increase the number of spleen cells, but not bone marrow B cells, producing IgM, IgG2b and IgG3 isotype antibodies to the T cell–independent component of heat-killed *Streptococcus pneumoniae*, although concentrations of specific antibody isotypes in the serum were not affected (Salazar et al., 2005). The authors suggested that serum antibody concentrations were similar because antibody production in the bone marrow contributes significantly to serum titres in mice. This pattern of isotype enhancement suggests that propanil exposure did not skew Th cytokine production patterns; IgG2b synthesis is a Th2 response, and IgG3 requires Th1 cytokine. Responses to the T cell–dependent portion of the bacterium were similar in control and treated animals. The observed effects suggested a pattern of effects that are similar to those of estrogen (17β-estradiol). A second study by this group (Salazar et al., 2006) determined that while propanil did not bind to estrogen receptors, ovariectomy or treatment with an inhibitor of gonadotropin-releasing hormone blocked the increase in splenic antibody-producing cells, but pretreatment with estrogen or progesterone antagonists did not. Experiments in male mice corroborated the apparent estrogen independence. Why increased antibody production was confined to the spleen is unknown, but the results do provide an example of local versus systemic effects. If the underlying MOA depends on events or pathways unique to the spleen, it is unlikely that propanil-related upregulated antibody production in the spleen is a signal of potential adversity, at least with our current understanding of the role of antibodies in immunopathology and circulating levels of autoantibodies as an indicator of autoimmune processes.

The toxic effects of HCB were reviewed by Michielsen et al. (1999). HCB is present in the environment as a persistent and easily transported industrial waste product and was previously used to prevent fungus growth in seed grain. Exposure to HCB has been associated with immune system abnormalities, including increased immune function. A detailed discussion of immune system modulation in humans and rodents as a result of exposure to HCB is presented later in this document, as a case-study (see Case-study 2).

5.5.4 Endocrine disruptors

Sex is a well-documented host factor influencing immune function; as a general rule, estrogens increase and androgens decrease immune responses at physiological and pharmacological levels. Many laboratory animal studies have shown that gonadectomy and/or introduction of opposite sex hormones will mask or switch sex-typical immune response phenotypes. Although, at first glance, stimulated function may appear to be beneficial, the generally enhancing effect of estrogen on immune function is considered to be a major factor in the

preponderance of autoimmune diseases in females (see chapter 7). Thus, there is concern that exposure to estrogenic environmental chemicals may unintentionally increase immune function and ultimately result in inflammatory or autoimmune disease. However, the relationship between endocrine disruption and immune-mediated disease is complex, particularly if exposure occurs after puberty, and there are few studies that have focused on adverse effects related to inadvertent stimulation of the immune system.

Bisphenol A has been reported to increase the hormone prolactin in animal models, although not all studies have found this to be the case (Youn et al., 2002; Jung et al., 2007). In genetically susceptible mice, hyperprolactinaemia is associated with markers of immune system upregulation, including increased pro–B cell development, MHC class II expression by antigen-presenting cells and antibody synthesis (Orbach & Shoenfeld, 2007), as well as reduced negative selection of autoreactive B cells and worsening of autoimmune disease. In humans, hyperprolactinaemia is associated with the onset or worsening of autoimmune diseases, including systemic lupus erythematosus, multiple sclerosis and autoimmune thyroiditis (Orbach & Shoenfeld, 2007), suggesting a link between immune system upregulation and adverse health effects in a susceptible subpopulation.

Exposure to bisphenol A and DES, a synthetic estrogen, increases synthesis of autoantibodies in a mouse model of lupus by stimulating B1 cells, a subset of B lymphocytes that is self-renewing and associated with production of autoantibodies (Yurino et al., 2004), reinforcing the link between a genetic component of susceptibility and upregulation of the immune response.

5.5.5 Drugs

Although chronic cocaine intake suppresses the T cell–dependent antibody response in mice, acute exposure prior to immunization elevates the response; the latter effect is associated with elevated levels of corticosterone and can be reproduced by exogenous corticosterone under the same conditions (Stanulis et al., 1997b). The apparent MOA was identified as disruption of the Th1/Th2 cytokine balance; additional studies determined that acute administration of cocaine or corticosterone upregulated IL-4 and IL-10 production and modulated T cell–dependent, but not T cell–independent, antibody responses (Stanulis et al., 1997a). Shifts in cytokine production patterns are of concern, because host-protective responses against intracellular microbes are dependent on Th1 cytokines. One of the interesting observations made by Stanulis et al. (1997a) was that corticosterone did suppress Th1-dependent T cell function in this series of studies, but cocaine did not, indicating that generalized stimulation of the immune system was not responsible for increased antibody responses.

5.5.6 Air pollutants and other examples

Complementing the human epidemiological studies above, a number of studies in rodents have demonstrated that respiratory exposure to a number of air pollutants (nitrogen dioxide, ozone, residual oil fly ash and diesel exhaust) enhances both allergic sensitization to common allergens such as dust mites and respiratory responses to allergen challenge (Gilmour et al., 2000, 2006; Steerenberg et al., 2005). The role of pollutants in the induction and exacerbation of hypersensitivity is extensively discussed in chapter 6. In addition, oxidant gases (e.g. ozone) have been reported to exacerbate immune pathology associated with influenza infection (Selgrade et al., 1988). In similar studies with both aryl hydrocarbon receptor targets (dioxin) (Luebke et al., 2002; Teske et al., 2008) and ultraviolet radiation (Ryan et al.,

2002), decreased host resistance to influenza infection was associated with exacerbated immune pathology rather than suppressed immune defences.

5.6 Local versus systemic effects

A single study was identified that reported local versus systemic effects. The herbicide propanil (single intraperitoneal injection of 50 or 150 mg/kg body weight in mice) was reported to increase the number of spleen cells, but not bone marrow B cells, producing IgM, IgG2b and IgG3 isotype antibodies to the T cell–independent component of heat-killed *Streptococcus pneumoniae*, although concentrations of specific antibody isotypes in the serum were not affected (Salazar et al., 2005). The authors suggested that serum antibody concentrations were similar because antibody production in the bone marrow contributes significantly to serum titres in mice. Route-dependent stimulation (oral and dermal exposure), suppression (intraperitoneal) and lack of effects (inhalation) were reported in C57BL/6 mice exposed to the carbamate insecticide aminocarb (Bernier et al., 1995). Although the toxicological significance of these results is questionable, it is noteworthy that class II MHC expression was upregulated in LPS-stimulated B cells isolated from the spleens of mice exposed by the oral and dermal routes only. Although not investigated by these authors, route-specific effects may depend on differences in the toxicokinetics associated with each route of exposure.

5.7 (Ir)reversibility of effects

None of the adult studies evaluated for this section were specifically designed to evaluate the reversibility of the reported effects. The studies by Stanulis et al. (1997a,b) did determine that stimulation of antibody synthesis was the product of acute cocaine exposure, in contrast to suppression observed with chronic exposure. In adults, upregulation of the immune response is likely related to the half-life of active parent or metabolites. However, persistence of effects following exposure to immunosuppressive or immunostimulating compounds has rarely been investigated. Clinical data from human recipients of haematopoietic stem cell grafts indicate that the immune function returns over time, with a concomitant reduction of infections (Ochs et al., 1995; Atkinson, 2000); thus, the severity and persistence of effect may depend on the potency of the agent and the cell types affected. Irreversible effects of xeno-biotic exposure occur in mice genetically predisposed to spontaneous autoimmunity; disease may occur in younger animals or be more severe. These models typically follow an unrelenting course of disease that results in mortality, in contrast to many human autoimmune diseases characterized by flare and remission, at least in the early stages of disease. Auto-immunity is discussed in detail in chapter 7.

5.8 Biological plausibility

It is clear from the examples above that unintended immune system stimulation, relative to appropriate controls, may be an indicator of immunotoxicity and should not be ignored. Rather, the data should trigger the risk assessor to consider autoimmunity (see chapter 7), hypersensitivity (see chapter 6) or suppression (see chapter 4) of other immune system pathways as a consequence of exposure. Biological plausibility is discussed in detail in section 3.3.6 of chapter 3. In particular, the reader is referred to section 3.3.6.1 for a discussion of the health-related database and the components of sufficient evidence for

immunotoxicity and section 3.3.6.3 for a discussion of “triggers” and factors to be considered in determining whether additional data may be required to complete the risk assessment.

5.8.1 Weight of evidence approach for assessment of immunostimulation

As noted above, stimulation of the immune response is not adverse per se, given that most vaccine preparations include adjuvants to ensure that the immune response to microbes or their toxic products provides protective immunity. Clinical experience with biopharmaceuticals, particularly cytokines used to stimulate immune responses, or exposure to “superantigens” indicates that morbidity or mortality may be associated with accidental or deliberate exposure to potent immune system activators. In contrast, most reports of stimulated immune function following exposure to environmental agents or recreational drugs suggest that mild to moderate immune system activation is the most likely outcome. Whether or not immunostimulation represents a hazard to the host is likely the product of stimulating potency, persistence of elevated immune function and, perhaps the most important, host genotype. Nevertheless, stimulation of the immune system should be regarded as unintended immune system modulation and should not be disregarded. In most of the examples discussed in section 5.5, environmental agents that increase immune function (particularly antibody synthesis) have also been shown to adversely affect the course of disease in animals that are genetically prone to development of autoimmune disease. Likewise, adjuvant-like activity of some xenobiotics (e.g. diesel exhaust particles) has also been associated with stimulated antibody responses to allergens in susceptible rodent strains, as well as worsening of allergic hypersensitivity, including asthma (Chan et al., 2006). Although increased antibody synthesis does not necessarily constitute sufficient evidence of adversity, it should raise concern that susceptible populations may be adversely affected or that host resistance mechanisms not addressed in the suite of assays used for hazard identification may be suppressed.

Hazard identification for immunostimulation should result in weight of evidence conclusions based on the available human and laboratory animal data for a given chemical. The risk assessor should consider the entire database of effects, including data that support and do not support unintended stimulation. Data are evaluated within the same or similar assays, as well as across divergent measures of the immune system and across multiple species. For each assay, a dose–response relationship for chemical exposure in the absence of generalized overt toxicity is necessary to demonstrate immunostimulation.

The weight of evidence conclusions are strengthened by consistency (particularly across species, sexes or related end-points), biological plausibility and breadth (range of effects) of the evidence for immunotoxicity. A lack of consistency among specific assays or types of immunotoxicity across species, strains or sexes does not necessarily represent conflicting data and often represents species, strain or sex differences. Conflicting data should be evaluated by the strengths and weaknesses (e.g. sample size and exposure duration) of the individual studies, as well as in the context of the remainder of the immunotoxicity database for a given chemical. Additional information with which to interpret species, strain or sex differences may be gained by considering toxicokinetic data (when available) or the likelihood of sex differences resulting from hormonally active chemicals, such as endocrine disrupting chemicals. As with other non-cancer end-points, the weight of evidence evaluation should represent an expert judgement of the database to determine the potential for immunostimulation for a given compound in accordance with the following key considerations (Hill, 1965; IPCS, 1999a; Weed, 2005): experimental evidence, dose–response relationship,

consistency of association, strength of the association, temporal association, biological plausibility, specificity, coherence and analogy.

A series of questions similar to those used to organize immunosuppression data (see chapter 4) may be applied to immunostimulation data, particularly because data suggestive of unexpected upregulation of immune responses are most likely to be developed in tests designed to detect immunosuppression. The questions are arranged to evaluate the available data from the strongest and most predictive data (human data) through the least predictive (immune organ weight). The process is summarized below in Figure 5.1. Important considerations for identifying key data set strengths, weaknesses and utility of data for derivation of effect levels are presented below. The risk assessor should develop weight of evidence conclusions for immunostimulation hazard based on the database for a given chemical by considering all six of the following questions:

1) *Human data*: Are there epidemiological studies, clinical studies or case-studies that provide human data on end-points relevant to immunostimulation (i.e. unintended stimulation of cellular or humoral immune function, autoimmunity or allergy)?
   - ✓ Data from well-controlled clinical and epidemiological studies represent the strongest evidence to support immunostimulation. In some cases, data may be used to support stimulation and sensitization or stimulation and autoimmunity. Depending on the end-point, these data should be considered in the hazard identification of chapter 6 for sensitization and allergic response or chapter 7 for autoimmunity, as appropriate. Allergy, hypersensitivity and autoimmunity are clearly adverse; therefore, if all of the data can be considered in chapters 6 and 7, there is no need to evaluate the data for immunostimulation as well. In other cases, there will be data, such as increased antibody production in response to immunization, that are more appropriate to consider as measures of immunostimulation than for allergy or autoimmunity. Data can be used for derivation of effect level(s) for immunostimulation if there are concordant and biologically plausible human or animal data demonstrating stimulation of functional end-points. Human data will most likely come from individuals with unexpected worsening clinical symptoms of allergy or autoimmune disease that can be linked to xenobiotic exposure or from epidemiological investigations of disease clusters. As noted above, data do support the biological plausibility of xenobiotic-induced immunostimulation in human disease.
   - ✓ Induction or exacerbation of allergic or autoimmune disease or modulation of resistance to infectious agents, without evidence of concordant stimulation of functional end-points, should be considered in the hazard identification of sensitization and allergic response in chapter 6 or autoimmunity in chapter 7.

2) *Allergic, autoimmune or infectious disease (laboratory animal data)*: Is there evidence that exposure to the chemical is associated with exacerbation of hypersensitivity responses or induction or exacerbation of autoimmune disease or alters the outcome of host resistance assays?
   - ✓ Induction or exacerbation of allergic or autoimmune disease or modulation of resistance to infectious agents in multiple species, with concordant and biologically plausible stimulation of functional end-points, provides the strongest evidence to link stimulated responses to disease development and provides data appropriate for derivation of effect level(s).

- ✓ Induction of autoimmune or allergic disease or modulated resistance to infection in a single species and demonstration of suggestive, biologically plausible observational end-points in multiple species increase the support for immunostimulation as an adverse event and provide data appropriate for derivation of effect level(s).
- ✓ Increased immune-mediated disease development in combination with additional evidence of immunotoxicity (e.g. shifts in cytokine production or lymphocyte subpopulations, suggestive histopathology, immune organ weight) may suggest an MOA.
- ✓ Induction or exacerbation of allergic or autoimmune disease or modulation of resistance to infectious agents, without evidence of concordant stimulation of functional end-points, should be considered in the hazard identification of sensitization and allergic response in chapter 6 or autoimmunity in chapter 7.

3) *Immune function (laboratory animal data)*: Is there evidence that exposure to the chemical is associated with unintended stimulation of immune function (antibody production, DTH responses) or alters the balance of immunoregulatory cytokines?
   - ✓ Dose-related stimulation of immune system functional end-points is considered strong evidence of unintended immunostimulation and appropriate for derivation of effect level(s).
   - ✓ Stimulation of the same functional end-point in multiple species or of multiple functional assays with concordance among end-points increases support for unintended immunostimulation and provides data appropriate for effect level(s).
   - ✓ Dose-related stimulation of immune system functional end-points in combination with additional evidence of immunotoxicity (e.g. immunophenotyping, cytokine analysis, altered histology, immune organ weight) increases the support for unintended immunostimulation and may suggest an MOA.

4) *General immune assays (laboratory animal data)*: Is there evidence from general immune assays (phenotyping, cytokines, total immunoglobulins, etc.) that the chemical stimulates immune function?
   - ✓ Elevated concentrations of total IgM, IgG, IgA or IgE and elevated C-reactive protein concentration are suggestive evidence of unintended immunostimulation, but are inadequate for derivation of effect level(s).
   - ✓ Lymphocyte phenotyping, cytokine analysis, serum immunoglobulin concentrations and other assays may add MOA information to support a biologically plausible description of immunostimulation.
   - ✓ Lymphocyte phenotyping or proliferation and serum immunoglobulin or cytokine concentrations are generally not considered to be reliable predictors of immunostimulation and therefore should generally not be used to derive an effect level for immunostimulation.
   - ✓ In vitro data alone are inadequate evidence of immunotoxicity.

5) *Histopathology and haematology (laboratory animal data)*: Is there histopathological evidence or are there haematological changes that suggest that the chemical causes immunostimulation or modulates autoimmunity or allergy?
   - ✓ Certain major haematological changes alone (e.g. a significant increase in relative or absolute counts of lymphocytes, eosinophils or neutrophils; decreased albumin to globulin ratio; elevated concentrations of total IgM, IgG or IgE; elevated

C-reactive protein concentration) are suggestive evidence of unintended immuno-stimulation, but are inadequate for derivation of effect level(s).
- ✓ Haematological changes supported by histopathological evidence of increased immune system activity in non-immunized animals (e.g. increased germinal centre formation in spleen or lymph nodes) may support unintended immunostimulation.

6) *Organ weight (laboratory animal data)*: Is there evidence that the chemical increases immune organ weight (thymus, spleen, lymph nodes, etc.)?
   - ✓ Increased immune organ weight may support other evidence of immuno-stimulation.
   - ✓ Immune organ weight change alone is equivocal.
   - ✓ The strength of the database will determine whether additional evidence is necessary to identify unintended immunostimulation.

A schematic for organizing the data is presented in Figure 5.1.

The risk assessor should develop the weight of evidence for immunostimulation hazard identification based on answers to all six questions. The weight of evidence conclusions for immunostimulation should also describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps. A small database with negative data is equivocal. Just as positive data on a range of assays strengthen the weight of evidence for immunotoxicity, negative data on a range of more predictive assays, such as immune function data, increase confidence to support a lack of immunotoxicity. The strength of the immune database will determine whether additional evidence is necessary to determine immunotoxicity. Incomplete or questionable data sets and high usage or high risk of exposure should trigger a request for additional data, if regulatory mandate allows.

When immunostimulation is indicated by the weight of evidence, these conclusions are then prepared to be brought forward to perform a dose–response assessment, beginning with the selection of the most appropriate end-point(s) or critical effect(s) and the development of POD(s). Health-based guidance values or reference values are then calculated by dividing the POD(s) by the total uncertainty factor (see sections 3.3.7 and 4.9 for a detailed discussion of dose–response assessment and derivation of reference values). Data from human exposures (e.g. occupational exposure studies and case reports) are preferred for the critical effect, because fewer assumptions are required to determine the relative risk of immunotoxicity for the general population from human data compared with experimental animal data. Therefore, when human data are used for the critical effect and the POD, smaller uncertainty factors are generally utilized to derive the reference values. Nevertheless, all available data are considered for the critical effect. The quantitative risk assessment may be based on laboratory animal data even if there are human data for a given chemical in cases such as inadequate information on dose levels, no information on effects at low doses or absence of a NOEL in the human data set.

Dose-related changes in three principal types of data provide strong evidence of adverse immunostimulation appropriate for use as the critical effect for chemical-related immuno-stimulation: 1) human data on end-points relevant to immunostimulation (i.e. abnormal elevation of cellular or humoral immune function, autoimmunity or allergy) with concordant data supporting immunostimulation as the MOA, 2) induction or exacerbation of allergic or autoimmune disease, or modulation of resistance to infectious agents, in multiple laboratory animal species with concordant data supporting immunostimulation as the MOA and 3)

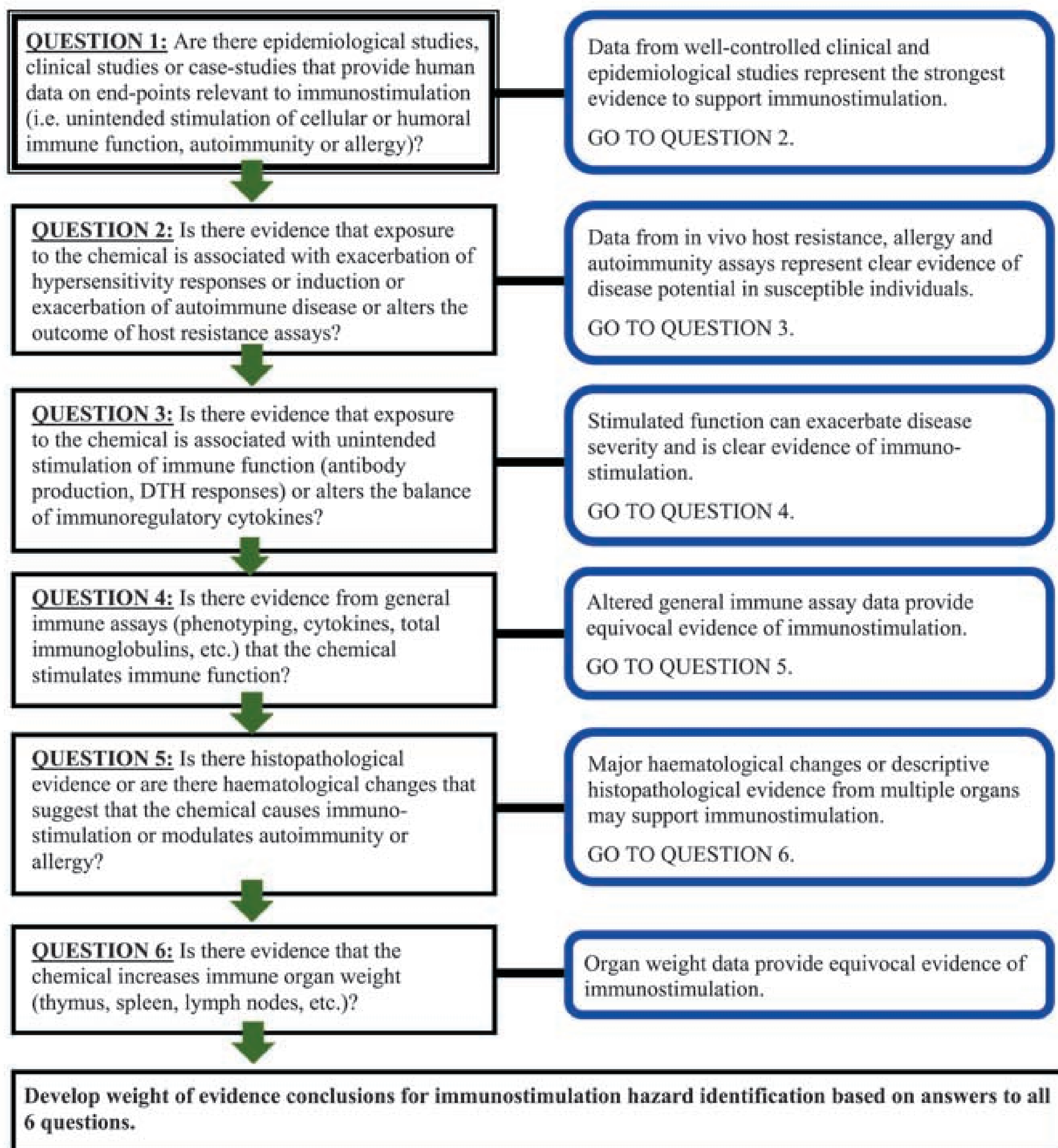

Figure 5.1: Schematic for organizing all available data for a weight of evidence approach for assessment of immunostimulation. The figure presents a summary of categorical data binning, from the most to least predictive, as described in section 5.8.1, rather than a decision-tree. Note: If there are immunotoxicological data relevant to end-points other than immunostimulation, evaluate those data in the appropriate chapter and include in weight of evidence evaluation for immunotoxicity.

stimulation of functional immune measures in laboratory animals. In general, PODs are developed from the most sensitive adverse immune end-point(s) from the most appropriate species (or the most sensitive mammalian species, in the absence of information to determine the most appropriate species). Data from general immune assays, haematology, histopathology and immune organ weight changes may indicate potential immunotoxicity and are useful

to support biological plausibility and a potential immunostimulation MOA for more predictive data (e.g. functional data). Observational end-points such as phenotyping, lymphocyte proliferation and altered soluble mediator (cytokines or complement) concentrations should generally not be used to derive an effect level for immunostimulation, because they are not considered to be reliable predictors of adverse immunostimulation. Major haematological changes are unlikely to occur in the absence of a related change in immune function. Therefore, the risk assessor should consider available functional and host resistance data on related end-points when considering deriving an effect level from haematological data. Changes in immune organ weights and general histopathology may indicate potential immunotoxicity and can be used to support more predictive data (e.g. functional data); however, these data should not be used to derive an effect level for immunostimulation because of the low predictive value of these end-points when considered alone.

5.8.2 Mode of action/mechanisms

Adverse effects of unintended immune system stimulation include allergy, hypersensitivity, autoimmunity and inflammation. Initiating and sustaining events that are responsible for allergy, hypersensitivity and autoimmunity are discussed in other chapters of this document. These effects are clearly adverse, and their underlying MOAs are fairly well documented. However, it is worth noting that these effects are typically observed in animal models that include a genetic propensity to develop allergic or autoimmune disease. In contrast, unintended upregulation of function has also been observed in the absence of readily apparent adversity (e.g. increased antibody responses to T cell–dependent antigens) in animal models that are not necessarily prone to allergy or autoimmune disease, but fewer studies have addressed MOAs under these conditions. Examples of studies that have addressed MOAs for stimulation of allergy and autoimmune disease are presented below.

Mercury(II) chloride increased antibody titres to T cell–dependent and T cell–independent antigens in allergy-prone Brown Norway rats by acting as a polyclonal activator of B and T lymphocytes, which may also be responsible for the observed autoimmune kidney disease that co-occurs in this rat strain (Hirsch et al., 1982). It is noteworthy that similar effects were not observed by Hirsh et al. (1982) in Lewis rats, a strain that is prone to developing inflammatory disease due to defective hypothalamic–pituitary–adrenal axis control of inflammation (Sternberg et al., 1989). Genotype appears to be pivotal in determining the outcome of exposure; a Th2 cytokine-driven response is responsible for both increased antibody synthesis and development of autoimmune disease in susceptible strains, whereas resistant strains (e.g. Lewis) experience immunosuppression following exposure (reviewed by Lawrence & McCabe, 2002). Lead in drinking-water for 10 weeks was reported to increase the antibody response to SRBCs in BALB/c mice, an allergy-prone strain that is biased towards Th2 responses, but not in C57BL/6 mice, which lack a Th2 bias (Mudzinski et al., 1986). These effects are supported by later studies (McCabe & Lawrence, 1991) that reported increased B cell differentiation and increased antigen presentation by Th2 clones exposed to lead.

Lead exposure is likewise associated with worsening of autoimmune disease in a genetically prone mouse model of lupus, but does not induce disease in resistant strains of mice (Hudson et al., 2003). Shifts in cytokine production patterns are linked to shifts in cellular (Th1) or humoral/allergic (Th2) function, such that significant overproduction of Th1 or Th2 cytokines is reflected in changing patterns of infection resistance and, in some cases, allergic disease. Significant loss of Th1 cytokine production has been associated with reduced resistance to intracellular pathogens; lead exposure (414.4 mg/l in drinking-water for 3 weeks) decreases

resistance to the intracellular bacterium *Listeria monocytogenes* due to overproduction of Th2 cytokines, a process that can be reversed by administration of the potent Th1 cytokine, IL-12 (Kishikawa et al., 1997). Although not directly related to the effects of lead on cytokine profiles, lead also reduces production of nitric oxide by macrophages, a key component of the intracellular bactericidal mechanism that clears listerial infection. Thus, although a portion of reduced resistance is attributable to altered cytokine expression, effects on cells other than lymphocytes also underlie reduced resistance. Furthermore, survival was decreased and body burdens of the extracellular pathogen *Salmonella enterica* serovar Typhimurium were increased in mice challenged with the organism after drinking water containing lead at a concentration of 2072 mg/l for 16 weeks. This level of exposure was associated with high levels of lead in blood (106.2 ± 8.9 µg/dl), increased (approximately 3 times) levels of IL-4, normal levels of protective IgG2a antibody to the bacterium and elevated levels of non-protective IgG1 antibody (Al-Ramadi et al., 2006). Th1 cytokines are required for production of protective IgG2a (Snapper et al., 1988). Thus, even though lead exposure was associated with an elevated antibody response to infection, the lead-induced shift in cytokine production resulted in reduced production of the protective antibody isotype.

Mechanistic studies suggest that the adjuvant effects of diesel exhaust particle extracts are due to oxidative stress in dendritic cells. This perturbation of dendritic cell function is accompanied by decreased IFN-γ and increased IL-10 induction in antigen-specific T cells. The data suggest that pro-oxidative diesel exhaust particle chemicals can interfere in Th1-promoting response pathways in a homogeneous dendritic cell population (Chan et al., 2006).

5.9 Groups at risk (developing immune system, elderly, allergic/autoimmune patients)

A general discussion of groups that may be at risk for immunotoxicity is presented in section 3.3.8 of chapter 3. In aggregate, immunotoxicological studies that have identified stimulation of immune function indicate that host genotype is an important factor in determining study outcome. Effects were often detected in strains of laboratory animals that have a bias towards production of Th2 cytokines; these same strains are often used to evaluate allergenicity and, less commonly, autoimmunity. Immunostimulation has also been detected in Th1-biased strains, and data from MOA studies indicate that upregulation of function by xenobiotics is not caused by a single event or MOA. Initial observations of increased function have been further evaluated for potential adversity by dosing strains of rodents that spontaneously develop autoimmune diseases or that are more sensitive to induction of autoimmune diseases under laboratory conditions. This may be an inherent bias in experimental design, as loss of immunological control or increased intensity or duration of immune responses is often observed in autoimmune diseases. As a group, however, these studies suggest that increased specific or nonspecific antibody synthesis is associated with more rapid onset, or greater pathology, of the spontaneously developing disease. The studies also suggest that some of these compounds may provide the environmental trigger portion of human autoimmune disease by inappropriate upregulation of immune function.

Endocrine hormones are responsible for a more robust immune response in females, and, as discussed in chapter 7, the greater preponderance of autoimmune disease is in females. A significant portion of xenobiotics that stimulate immune function are endocrine disrupting chemicals with estrogenic activity, suggesting that females may be more likely than males to experience unintended stimulation of immune function and, perhaps, at greater risk of immune system dysregulation resulting in autoimmune disease. Sex-dependent stimulation of

the antibody response to SRBCs is not an exclusive finding in females; increased numbers of antibody-producing cells were detected in male offspring of rats exposed to feed containing methoxychlor from gestational day 7 until postpartum day 51 (White et al., 2005). NK cell activity was increased in dams, but only at the highest dose (1000 mg/kg of feed), a dose that also caused maternal weight loss. Increased numbers of antibody-forming cells were detected in male offspring continually exposed to methoxychlor at concentrations of 100 and 1000 mg/kg feed until 11 weeks of age, although body weight was reduced at the highest dose. NK cell activity was also increased in males, but only at the 1000 mg/kg concentration in feed. In contrast, suppression, rather than stimulation, of immune function was reported for female offspring, along with reduced body weight, at 1000 mg/kg feed. Elevated immune function at 1000 mg/kg feed is of questionable significance, given the indications of generalized toxicity at this dose.

It is generally accepted that developing organisms are more susceptible, and often more sensitive, than adults to the effects of chemical exposure. Most developmental immuno-toxicity studies have focused on suppression (see chapter 4). The effects of gestational exposure to lead may provide one of the clearest examples of late effects of early exposure on increased immune responses. Increased IgE production occurs long after blood lead levels return to near normal, which may predispose offspring to allergy (reviewed by Dietert & Piepenbrink, 2006a). Likewise, maternal exposure of B6C3F1 mice to genistein enhanced IgE production in adult offspring challenged with the respiratory allergen trimellitic anhydride (Guo et al., 2005b).

5.10 Dose–response relationships and thresholds

A thorough understanding of dose-related effects is critical to a successful risk assessment. Prior to evaluating dose–response and threshold as applied to immunostimulation, the reader must be familiar with the issues discussed in section 3.3.7 of chapter 3. The relationship between inadvertent immune system activation and chemical dose is complex, and effects are not necessarily restricted to either end of the dose–response curve. As described in section 5.5 of this chapter, the administered dose may determine whether immune function is stimulated or suppressed by the same compound (e.g. propoxur: Hassan et al., 2004; lindane: Meera et al., 1992). In some studies, elevated function was reported for all doses, including those that caused signs of overt toxicity, suggesting that different MOAs may be responsible for immu-nostimulation and toxicity. Collectively, the observed patterns of stimulated function across various doses suggest that immunostimulation is not an artefact of very high or very low doses. From a practical standpoint, this observation suggests that stimulation can be detected at dose ranges that are appropriate for studies designed to detect immunosuppression.

The interpretation of dose–response data should identify doses associated with the adverse effect (immunostimulation), as well as doses associated with no adverse effects, to determine the most appropriate end-points or critical effects. The toxicity data and the shape of the dose–response relationship (e.g. an inverted U–shaped curve) may indicate that different qualitative outcomes occur as dose or exposure duration increases. In such a case, it is important to consider whether different outcomes are attributable to the same or different MOAs. If it can be determined that variable effects have the same underlying MOA, a risk assessment can be based on the lowest NOAEL or LOAEL. However, if evidence indicates that the observed response can be attributed to distinct effects of different dose-based or exposure duration–based MOAs, the NOAEL or LOAEL for each MOA should be consid-ered separately. In general, PODs are developed from the most sensitive adverse immune

end-points from the most appropriate species or the most sensitive mammalian species in the absence of information to determine the most appropriate species. Reference values (e.g. RfD/RfC or ADI/TDI) are then calculated by dividing the PODs by the total uncertainty factor, using either default uncertainty factors or CSAFs. These concepts are discussed in detail in chapter 3 (section 3.3.7).

5.11 Acute versus chronic exposure

Few studies have evaluated immunostimulation as a function of acute or chronic exposure. The studies by Stanulis et al. (1997a,b) determined that acute cocaine exposure stimulated antibody synthesis, in contrast to suppression following chronic exposure. Cadmium exposure has also been associated with increased IgM and IgG antibody responses to sheep erythrocytes after single (Koller et al., 1976), short-term (3–4 weeks) or long-term (9–11 weeks) exposure to cadmium concentrations of 50 or 200 mg/l in the drinking-water, suggesting a degree of insensitivity to exposure duration (Malavé & De Ruffino, 1984). However, in the latter study, exposure to cadmium at 300 mg/l for 10 weeks suppressed the humoral response, suggesting a dose threshold that favours suppression over stimulation. Finally, the primary antibody response was elevated 4 days into a 6-week inhalation exposure to crystalline silica, but not after 10 weeks of exposure, during a phase of pulmonary inflammation and granuloma formation (Langley et al., 2004). These results suggest that the temporal component of immunostimulation in this case was the direct result of changing cytokine production patterns that accompany disease progression.

5.12 Uncertainty factors

The predictive value of stimulated IgM antibody synthesis for disease development or progression has yet to be established. However, if IgM synthesis is elevated at doses that will change the POD, and in the absence of other immunotoxicity data (e.g. autoimmune or hypersensitivity data), this is taken into account in the database uncertainty factor. On a case-by-case basis, the application of an uncertainty factor of 3 or 10 is suggested for this type of database deficiency. The reader should consult section 3.3.10 of chapter 3 for a detailed discussion of uncertainty factors, particularly section 3.3.10.5 on database uncertainty factors.

5.13 Exposure assessment

Laboratory animal studies suggest that immunostimulation is not simply a high-dose or low-dose effect, but is dependent on the chemical and, in some cases, the animal model. Immunostimulation has been demonstrated following oral, dermal and inhalation exposure to xenobiotics. Route-appropriate exposure assessments, as described in other chapters of this document, are therefore also appropriate to assess stimulated immune function. A general discussion of exposure assessment is presented in section 3.3.11 of chapter 3, which addresses exposure-related severity and persistence, susceptibility based on timing of exposure, the consequences of exposure on localized and systemic immune tissues, and toxicokinetic factors that will influence the outcome of exposure.

5.14 Risk characterization

Deliberate enhancement of immune function, particularly in the context of vaccination, is a common therapeutic practice that is not generally associated with adverse effects.

Unexpected stimulation of the immune system following chemical exposure may likewise be without apparent adverse consequences or, as noted above, may be accompanied by suppression of critical functions that were not assessed during hazard identification. Data derived from models of inherited spontaneous autoimmune diseases or inadequate homeostatic control of immune responses suggest a causal link between host genotype (including sex), dose-dependent stimulation of immune function and exacerbation of allergy, hypersensitivity or inflammation. However, genotype is not an absolute requirement for chemically induced immunostimulation, because increased function has been reported in animal models not prone to autoimmunity or hypersensitivity. Further testing of these compounds often reveals increased disease in genetically susceptible strains; thus, unexpected stimulation of the immune system detected by screening assays may indicate an increased risk of autoimmune or hypersensitivity diseases in susceptible populations. See chapter 6 on allergy and hypersensitivity and chapter 7 on autoimmunity for detailed discussions of disease class–specific risk characterizations and section 3.3.12 of chapter 3 for a detailed discussion of the risk characterization phase of immunotoxicity risk assessment.

Chemical exposure may also result in activation of the innate immune system. Possible outcomes include symptoms similar to those of allergy but lacking the antigen-specific IgE component ("pseudoallergy") or direct tissue damage that induces inflammation by release of proinflammatory tissue factors. Both are beyond the scope of this document. A brief discussion of pseudoallergy is presented in section 6.3.3.3 (chapter 6) of this document.

As is true for all forms of immunotoxicity, ideally, a quantitative risk assessment for immunostimulation associated with chemical exposure is performed. In the case where the available data do not allow for this, a qualitative risk assessment may be possible.

6. ASSESSMENT OF SENSITIZATION AND ALLERGIC RESPONSE

6.1 Introduction

IPCS (1999b) defines allergy as:

> the adverse health effects resulting from hypersensitivity caused by exposure to an exogenous antigen [either in the form of an allergen when the molecule is large enough or as a hapten, i.e. a low molecular weight chemical that can combine with a larger (self) molecule to form a complete antigen] resulting in a marked increase in reactivity and responsiveness to that particular antigen on subsequent exposure. Allergy is not necessarily, or usually, the consequence of perturbed immune function, but the result of an immune system response to an [otherwise innocuous] antigen (in this case allergen) in such a way that a temporary or long-lasting disease results. The immunological processes that are involved in the development of allergic responses and allergic disease are in principle and practice no different to those that provide protective immunity and host resistance against potential pathogens.
>
> ….
>
> Although from the occupational[, consumer] and environmental health standpoint allergic contact dermatitis and respiratory hypersensitivity [e.g. allergic rhinitis and allergic asthma] represent the most important types of allergy induced by chemicals, it should not be forgotten that exposure to xenobiotics has been implicated in other forms of allergic disease. Certain drugs are associated with systemic allergic reactions that are sometimes reminiscent of autoimmune diseases. In addition, food components and food additives are implicated in adverse reactions, which in some cases take the form of an allergic response.

Hypersensitivity reactions to chemicals (or other allergens) pose some particularly challenging problems for quantitative risk assessment because they develop in two stages. First, there is a "learning phase" without symptoms (termed sensitization phase or induction phase), followed by the immune response effector phase (termed elicitation phase or challenge reaction). Consequently, the first contact (and often repeated contacts), even with relatively high concentrations of a sensitizing chemical, can go unnoticed, because no signs or symptoms of allergy occur. Nevertheless, this contact may induce sensitization—that is, cause the immune system to prepare for a reaction at the next contact. Once sensitization is established, contact with the same sensitizer, sometimes even at concentrations several orders of magnitude lower, may lead to symptoms of allergic disease. The dose–response relationships for sensitization and elicitation are different, but not entirely independent (Friedmann et al., 1983; Scott et al., 2002), and in practice it is sometimes difficult to determine the point at which sensitization ends and elicitation begins. For this reason, risk assessors have tended to deal with hypersensitivity responses as all-or-none responses. Recently, however, significant progress has been made, particularly with allergic contact dermatitis, in the development of dose–response relationships and thresholds.

Owing to the distribution of the cells of the immune system over diverse primary and secondary immune organs and tissues throughout the body and constant recirculation of immune cells via the lymphatic and blood vessels, the development of sensitization is always a systemic reaction, although allergic reactions may preferentially occur at localized sites: for example, in exposed skin areas (e.g. as delayed contact allergy or immediate-type contact urticaria), mouth, upper respiratory tract (e.g. as allergic rhinitis), lower respiratory tract (e.g.

as allergic asthma) or gastrointestinal tract (e.g. in food allergy) or as more systemic anaphylaxis. The site of reaction is not necessarily determined by the route of exposure; for example, dermal and food exposures may sometimes result in respiratory reactions.

In conclusion, it is clear that risk assessment for chemically induced hypersensitivity has two components: 1) the likelihood that a chemical will induce sensitization in a previously non-sensitized individual and 2) the likelihood that a chemical will provoke an allergic reaction in those who are already sensitized.

In this chapter, guidance will be developed for the conduct of risk assessments for both the induction and elicitation of skin allergy, respiratory allergy and oral (systemic) allergy. The most progress in this regard has been made with allergic contact dermatitis; tools for dealing with respiratory allergy are more limited, and systemic (oral) allergy has received the least attention to date. Decision-trees (see Figures 6.2A, 6.2B and 6.2C in section 6.4.1 below) have been developed as a guide through the process of assessing sensitization and allergy caused by exposure to chemical substances via the dermal, inhalation and systemic routes.

It should be emphasized that for the toxicological end-points addressed here, with the exception of allergic contact dermatitis, there are currently no internationally harmonized toxicity test guidelines that have been officially endorsed as a basis for quantitative risk assessment and that very limited databases exist for many compounds. Also, while quantitative risk assessment for skin sensitizers has become an industry standard to assess consumer exposure to sensitizing fragrance ingredients, it still lacks acceptance in other areas and has been employed in only a few cases by competent authorities and regulatory bodies.

The reader should note that some sensitizing chemicals may additionally cause other immunotoxic effects (or may constitute other toxic hazards besides immunotoxicity). For other immunotoxic effects, the reader is referred to Table 3.1, “Entry points for immunotoxicity risk assessment”, in chapter 3 of this guidance document.

6.2 Hazard identification

Sensitization hazard identification has been comprehensively discussed in IPCS (1999b):

> Testing [of the sensitizing potential] requires study of selected immunological effects and differs from conventional toxicity testing in the nature and content of its procedures, which are focused on responses of the immune system and not on general screening for changes in all body systems. In both types of testing, however, there will be some form of relation between dose (exposure) and effect, as the capacity of a substance to produce effects, its potency, will be represented by the dose (exposure) required to produce sensitization (or toxicity). A strong sensitizer will require only a small dose, whereas a less potent compound will require a higher dose, or multiple exposures. Unlike conventional toxicity, further exposure of a sensitized animal (or man) will elicit a harmful allergic reaction after a much smaller dose than that required for sensitization, although there will still be a graduation of the severity and nature of the hypersensitivity reaction, for example ranging from slight bronchoconstriction to fatal bronchospasm or anaphylaxis after respiratory challenge.

Identification of a sensitization hazard indicates, at the same time, that an elicitation reaction can be induced in sensitized laboratory animals or individuals, and often an elicitation reaction is the read-out in a sensitization test. Therefore, separate hazard identification tests for

elicitation are not required, but elicitation tests are usually performed to do hazard characterization (dose–response analysis).

An additional important difference between conventional toxicity and sensitization is that allergic sensitization (the induced state of hyper-reactivity to a substance) normally persists for a long time, even for life, whereas for many toxic responses, a state of lasting responsiveness is not induced. It is possible for different types of hypersensitivity and provocation to be effective in the same organ or tissue, but it is also possible for the route of sensitization and response to subsequent challenge to differ (e.g. sensitization via the skin and subsequent asthma on inhalation exposure). A special case of sensitization is photosensitization, where photodynamic compounds activated by sunlight cause the allergic response.

With regard to skin sensitization hazard identification, the development of in vitro, in chemico or in silico models for predicting the sensitizing potential and/or potency of chemicals has received widespread interest during recent years, mainly because of increasing public and political concerns regarding the use of animals for the testing of cosmetic ingredients.

Currently, the replacement strategy foresees that data from several non-animal test methods will need to be combined to produce adequate information on skin sensitizing potency (Jowsey et al., 2006; Natsch et al., 2009). Each alternative test method aims to address a key element in the induction of skin sensitization, such as dermal bioavailability (skin penetration), activation of keratinocytes and innate immune cells (phagocyte activation), chemical reactivity with skin protein, activation of epidermal Langerhans cells or dermal dendritic cells and the chemical-specific T cell activation. Besides well-established methods, such as in vitro skin penetration assays, novel alternative approaches for the identification of skin sensitizing chemicals include stimulation assays using human peripheral blood monocyte-derived dendritic cells (Aeby et al., 2004; Reuter et al., 2011), keratinocyte cell line–based reporter gene assay (Natsch et al., 2011), the direct peptide reactivity assay, the myeloid U937 skin sensitization test and the human cell line activation test (Maxwell et al., 2011). The latter four non-animal tests have been evaluated in interlaboratory ring trials and have recently been submitted to the European Centre for the Validation of Alternative Methods for formal prevalidation.

6.3 Hazard characterization (quantitative dose–response analysis)

Many predictive test methods serve simply to identify the inherent potential of a chemical to induce allergy but provide no indication of the potency with which it will do so. One problem is that some methods do not incorporate a dose–response analysis or identification of a threshold (or NOEL). According to IPCS (1999b):

> The other issue is that some tests measure activity as the frequency of responses [i.e. incidence, such as the number of animals showing an allergic response] rather than the severity of responses. The need is to have available information on potency defined as the quantity of chemical necessary to induce sensitization (or to elicit a reaction)….
>
> As in any form of toxic reaction, "dose" is important, in that initial sensitization requires at least a certain minimum exposure (concentration of allergen, its local availability at the site of administration, and the duration of contact). In someone already sensitized, the likelihood of producing a clinical disorder and its severity are also related to dose, although, by definition, the quantity of allergen required to produce an effect [may be] very much smaller than that associated with a conventional toxic action. This aspect of the extent or intensity of dose (= exposure) is [very]

> important [in risk assessment aiming at] preventing sensitization or protecting the sensitized individual….

6.3.1 Skin sensitization

The dose metric recommended for use in dermal sensitization risk assessment is the area dose—that is, the dose of a chemical applied per area of skin (µg/cm^2 per day). In experiments on patients or volunteers, the applied skin area dose can be determined by calculating the absolute amount of test substance from its concentration (in %) in vehicle and the applied volume and then dividing this amount by the application area of skin (in cm^2). In the same manner, the skin area dose in an LLNA can be calculated assuming that on each mouse ear, 25 µl of test substance in vehicle is applied onto 1 cm^2 of ear skin (Robinson et al., 2000).

It would be important to know the applied versus the intradermally delivered dose (but data are rarely available), as there are factors that can affect the effective amount of material delivered to the viable epidermis—evaporation, binding/sequestration in the skin and metabolism (inactivation and activation). Throughout the skin sensitization literature, both historical and current, allergen exposures are most commonly expressed in terms of percentage (i.e. weight of allergen per volume of vehicle applied to the skin). This leads to the assumption that in any given test system, an equal percentage exposure will lead to a similar incidence and/or severity of skin sensitization. Based upon the understanding of the immunological mechanism involved, it is logical to assume that for an immune response to be initiated, a certain number of Langerhans cells must be activated to initiate the cascade of events necessary for acquiring skin sensitization, thereby resulting in a threshold of induction of sensitization. This would suggest that for the induction of contact allergy, the application of an amount of allergen expressed as per cent weight per volume is not as important as understanding both the dose applied and the surface area over which the allergen is applied. Published data that support the use of this dose metric for the induction of skin sensitization are both robust and convincing in humans and experimental animals. There are a number of literature references to support this position (Kligman, 1966; Magnusson & Kligman, 1970; Friedmann & Moss, 1985; White et al., 1986; Rees et al., 1990; Upadhye & Maibach, 1992; Kimber et al., 2008).

6.3.1.1 Clinical and epidemiological data

(a) Induction

Human repeated patch testing methodology has evolved over more than 50 years. In essence, human repeated patch tests are experimental sensitization studies in humans. While they may generate valuable information for risk assessment in the species of interest, experiments on humans may cause fundamental ethical concerns and are not endorsed by all competent authorities and official bodies. According to McNamee et al. (2008), every human repeated patch method uses a number of

> … induction exposures, followed by a rest period and then a challenge exposure, but variations exist as to patch type, number of subjects, skin site, number of induction patches, patch application time, duration [of treatment phase] and rest period prior to challenge. In all, enhancement of the skin response after challenge over that seen during early induction exposures has been the criterion by which induction of contact allergy is measured…. The sample size of test subjects must be sufficiently large so that results are valid for the population at

> large, yet small enough … to be logistically feasible to conduct the study…. Test volunteers are typically healthy adults who are enrolled without restriction as to gender or ethnicity….

The test most typically conducted is the HRIPT. Generally, the induction phase in the HRIPT comprises a total of nine 24-hour occluded patch applications, and the application skin site is changed if moderate or strong skin reactions are observed at removal of a patch. In contrast, the human maximization test (HMT) induction phase typically consists of five alternate 48-hour patches applied to sodium lauryl sulfate–irritated skin if the test substance is not irritating itself. These conditions may be considered unsuitable for characterization of the skin sensitizing potency. Also, the HMT is no longer performed for ethical reasons. Therefore, HRIPT data are often given precedence over HMT data in the weight of evidence approach.

From HRIPTs done in the classical design using several different induction concentrations, a dose–response curve (induction incidence versus concentration or skin area dose), NOELs and lowest-observed-effect levels (LOELs) can be derived. When no NOEL is reported, doses producing sensitization rates below 50% may be extrapolated to a LOEL (it was felt that, without any dose–response curve, the uncertainty would be too large if extrapolation of higher incidences were to be done). To extrapolate to a suitable LOEL value, it has been suggested that a factor of 3 be applied to doses producing sensitization rates between 10% and 25% and a factor of 10 for sensitization rates between 25% and 50% (Griem et al., 2003).

Today, based on ethical considerations, skin sensitization hazard is usually in the first instance determined in laboratory animal assays (such as the LLNA, guinea-pig maximization test [GPMT] or guinea-pig Buehler test), whereas human sensitization tests are no longer used to determine hazard. The HRIPT is sometimes employed as a confirmatory assay to substantiate the lack of sensitization at an exposure level that was identified as a NOEL in an animal model or that was derived as a likely NOEL from quantitative structure–activity relationships (QSARs). When only one dose is tested, such a test will often deliver "a" dose without observable effect, but not necessarily "the" NOEL. In contrast, in many human hazard identification tests performed decades ago, only one high dose was tested, which causes problems when a high percentage of subjects were sensitized—that is, when no LOEL (and of course no NOEL) was identified. In Table 6.1 in section 6.3.3 below, a proposal is made for how such data may be used to derive a POD when more suitable data are lacking.

Besides experimental human studies, for certain substances, such as those that have already been used for some time in the workplace or in consumer products, epidemiological data may also be available. Such investigations can provide hazard identification and exposure assessment information. Data include studies of occupational or non-occupational cohorts, the general population or dermatology clinic patients and may consist of patch testing and/or questionnaire data. Although negative epidemiological data should not normally be used as proof of absence of a sensitizing hazard, the prevalence of acute contact dermatitis in reaction to a certain substance in an exposed subpopulation not only may indicate a sensitizing hazard, but also may provide dose–response information if the exposure that led to sensitization is adequately assessed and can be reported in terms of skin area dose (eventually using a quantitative exposure model). At best, a NOEL and LOEL or BMD can be derived from epidemiological data.

(b) Elicitation

The elicitation of an allergic skin response is usually tested in humans. However, only for a small number of sensitizing chemicals has the elicitation threshold been experimentally determined. This is due to the fact that for diagnostic purposes, often a single, relatively high concentration is employed in the patch test (e.g. 1% chemical in petrolatum) in order to reliably detect a sensitization, whereas the determination of the NOEL or LOEL for elicitation is usually not the aim of a diagnostic patch test study.

The dose–response relationship for elicitation of allergic contact dermatitis can be determined in different experimental setups. In clinical patch tests on allergic patients, the concentration of the sensitizer (in a suitable vehicle such as petrolatum) can easily be varied and an elicitation threshold determined. Alternatively, the repeated open application test (ROAT) or a product use test can be employed in which formulations with different concentrations of the sensitizer as well as a control formulation without the sensitizer are usually employed.

The patch test minimum elicitation threshold (MET)—for example, the MET inducing a threshold response in 10% of the subjects tested (MET_{10})—and the NOEL or BMD from a ROAT or use test have been proposed as PODs for risk assessment (Weaver et al., 1985; Sosted et al., 2006; Zachariae et al., 2006). The results from the patch test and the ROAT were shown to correlate quite well (Fischer et al., 2009).

The elicitation thresholds are usually determined in subjects who have had an established allergy for a long period of time. Tests in which elicitation thresholds were obtained using newly sensitized subjects (e.g. in the HMT or HRIPT) showed that elicitation thresholds in these subjects depend on the sensitization dose used; that is, the higher the sensitization dose, the lower the elicitation threshold (Friedmann et al., 1983). This dependency has also been found in mice (Scott et al., 2002). Thus, it seems that the elicitation threshold decreases with the time of established allergy and with the number of exposures. Although it has not been formally shown that a "minimum threshold" is finally approached over time, the thresholds determined in well-established allergic individuals seem more reliable than those determined after experimental sensitization.

With regard to elicitation of contact allergy, there is a considerable variation of the NOEL and MET both between individuals and when the test is repeated in the same individual (Jerschow et al., 2001). It should also be noted that when testing elicitation reactions in humans, it cannot be excluded that cross-reactions between chemicals forming "immunologically similar" haptens occur; therefore, a positive reaction against a chemical does not necessarily indicate that the individual has also been sensitized through contact with this chemical (see, for example, Tanaka et al., 2004; Ventura et al., 2006).

6.3.1.2 Laboratory animal data

(a) Induction

The LLNA (OECD Test Guideline 429) was originally used for qualitatively identifying sensitizing chemicals (hazard identification). A stimulation index (SI) of 3 or higher is used to differentiate sensitizers from non-sensitizers. As at least three test concentrations are used in the LLNA, it provides a dose–response curve for induction of sensitization. The sensitizing potency is expressed as the EC_3 value, which is the effective concentration of a chemical (percentage of chemical in vehicle) required to produce a 3-fold (i.e. threshold level) increase

in the proliferation of lymph node cells compared with vehicle-treated controls. The threshold of an SI of 3 or higher for the LLNA method using incorporation of radioactively labelled thymidine is replaced by an SI of 1.8 or higher for the non-radioactive LLNA: DA method (OECD Test Guideline 442A), which measures adenosine triphosphate content by bioluminescence as a surrogate of the number of living cells, and by an SI of 1.6 or higher for the non-radioactive LLNA: BrdU-ELISA method (OECD Test Guideline 442B), which measures 5-bromo-2′-deoxyuridine (BrdU) incorporation into replicating DNA by an enzyme-linked immunosorbent assay (ELISA).

In a number of studies, human NOELs and BMDs were compared with LLNA thresholds (EC_3 values), and it was found that the average ratio of both values is close to 1, indicating that area doses are directly comparable between mice and humans—that is, a sensitization threshold of 10 $\mu mol/cm^2$ in mice corresponds to a NOEL or BMD of 10 $\mu mol/cm^2$ in humans. Therefore, the LLNA EC_3 value has been suggested as a surrogate NOEL in quantitative risk assessment (Basketter et al., 2000, 2005b; Gerberick et al., 2001a,b; Griem et al., 2003; Schneider & Akkan, 2004; Api et al., 2008).

Tests in guinea-pigs (GPMT, Buehler test) have been used for decades to identify possible sensitization hazards. However, guinea-pig tests provide only poor information with regard to sensitizing potency. More recently, modified guinea-pig protocols have been proposed in order to generate useful potency data (Anderson et al., 1995; Van Och et al., 2001; Yamano et al., 2001), but these protocols have not yet been validated. The disadvantages with regard to the potency estimation that can be derived from guinea-pig experiments are, for example, circumventing the skin barrier by intracutaneous injection, elicitation of a local inflammatory reaction and activation of Langerhans cells by use of Freund’s adjuvant, the impossibility to express the dose as area dose (in $\mu g/cm^2$) and the dependency of the sensitization rate on the challenge concentration (also discussed in Basketter et al., 1997).

In cases where no quantitative hazard assessment can be performed (e.g. no NOEL can be derived), semiquantitative approaches using potency categories have been proposed (Gerberick et al., 2001a; Felter et al., 2002; ECETOC, 2003). Available data from experiments in guinea-pigs and murine LLNA as well as human experience can be used in a weight of evidence approach to put a substance into one of several potency categories. As a starting point for risk assessment, the lower boundary of the potency category is used into which a given sensitizing chemical is grouped. Category boundaries are expressed in units of specific area dose. Different systems with regard to the number of categories and their numerical boundary values have been put forward and are shown in Figure 6.1 (see, for example, EC, 2003; ECETOC, 2003; Akkan et al., 2004; Schneider & Akkan, 2004; Basketter et al., 2005a). The obvious disadvantage of a potency category system is that, by convention, “artificial” 10-fold steps are introduced into the continuum of sensitization potencies. However, category systems might be used in the future to make use of in vitro sensitization tests (see section 6.3.1.3).

A broad two-category system has recently been introduced for the classification and labelling of sensitizers under the Globally Harmonized System of Classification and Labelling of Chemicals (UN, 2008). Where data are sufficient, skin sensitizers are allocated either into subcategory 1A, for strong sensitizers, or into subcategory 1B, for other skin sensitizers. Sensitizers of subcategory 1A show a high frequency of occurrence in humans and/or a high potency in laboratory animals and can be presumed to have the potential to produce significant sensitization in humans. Severity of allergic reactions may also be considered. Human

evidence can include skin sensitization induction thresholds at or below 500 $\mu g/cm^2$ in HRIPT or HMT. Criteria for subcategory 1A in laboratory animal tests are an LLNA EC_3 value at or below 2%, at least 30% responding animals at or below 0.1% intradermal induction or at least 60% responding animals at 0.1–1.0% intradermal induction in the GPMT, and at least 15% responding guinea-pigs at or below 0.2% topical induction or at least 60% responding animals at 0.2–20% topical induction in the Buehler test.

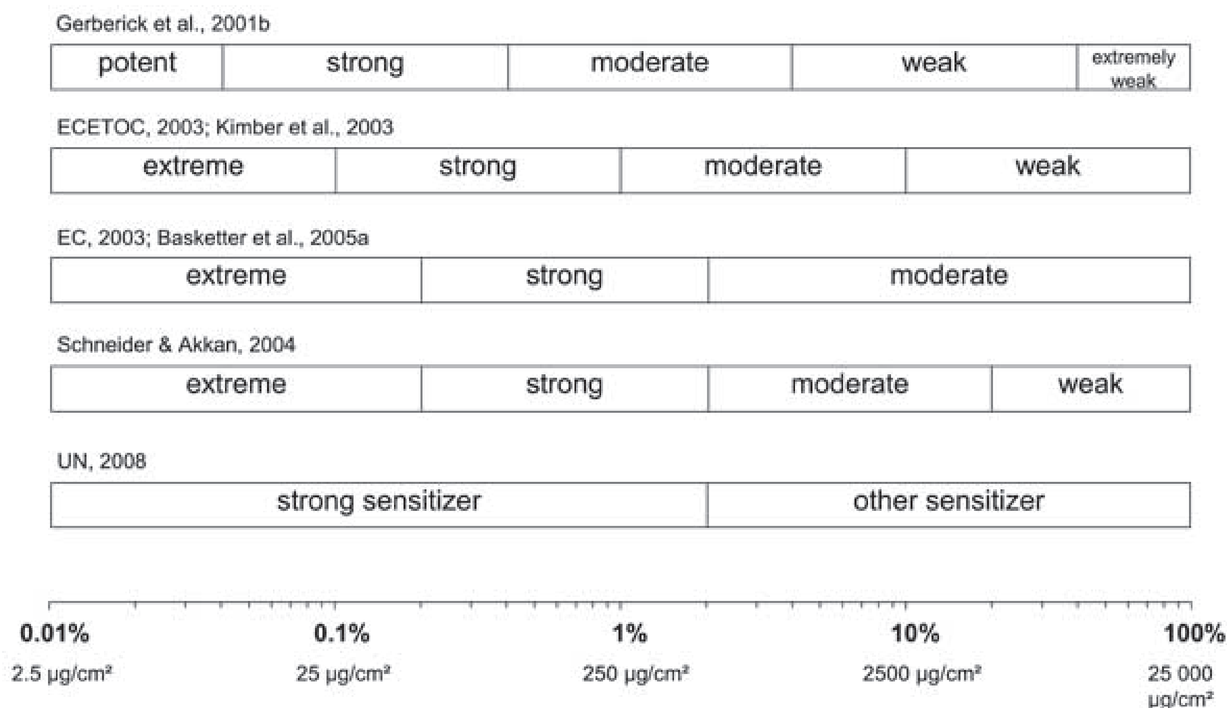

Figure 6.1: Overview of potency categories for skin sensitizers based on LLNA EC_3 values.

(b) Elicitation

As already discussed above, elicitation thresholds in newly sensitized animals depend on the frequency and dose used for sensitization. Therefore, elicitation NOELs are usually determined not in laboratory animal tests, but in human subjects with well-established skin allergy (see above).

6.3.1.3 In vitro data and general sensitization threshold approach

For both animal welfare reasons and compliance with requirements imposed by the chemical legislation in Europe (e.g. 7th Amendment of the Cosmetics Directive and REACH), there is an increasing emphasis on the development of in vitro methods for hazard identification and potency characterization.

In vitro approaches have to dissect the various elements of the immune response to skin sensitizing chemicals, such as skin penetration (bioavailability), quantitative measurement of chemical reactivity with glutathione, peptides or proteins with and without metabolic activation (e.g. Gerberick et al., 2007; Natsch et al., 2007; Maxwell et al., 2011), measurement of chemical activation of keratinocytes (e.g. Coquette et al., 2003; Natsch et al., 2011) and dendritic cells (e.g. Sakaguchi et al., 2006; Aeby et al., 2007; Maxwell et al., 2011) and

response of T cells against haptenated peptides. A single parameter measured in any of these individual elements of sensitization is unlikely to reflect the skin sensitization potential and/or potency of the chemical. Therefore, a method for the integration of the results of an in vitro test battery will be required. One possibility is to use the data to assign a potency category to the substance (Jowsey et al., 2006; Natsch et al., 2009).

In the tools of toxicological risk assessment, the TTC has evolved as a useful concept. It acknowledges that a human exposure threshold can be determined below which there is no appreciable risk to human health, even when the toxicological profile of a substance is not known (for review, see Barlow, 2005). Based on a similar approach, Safford (2008) analysed the distribution of 167 LLNA EC_3 values for sensitizing chemicals. The analysis indicated that, assuming that 20% of all chemicals are skin sensitizers, a 95% probability would exist that the LLNA EC_3 would not be lower than 289 µg/cm^2. Correcting the EC_3 dose area values with a factor derived from the correlation of skin area doses of LLNA EC_3s with NOELs from HRIPTs and applying a total sensitization assessment factor (SAF) of 100 for shampoos and 300 for deodorants, dermal sensitization thresholds of 1.64 µg/cm^2 and 0.55 µg/cm^2 for shampoos and deodorants, respectively, were derived. This concept may prove useful in the future for risk assessments of very low skin exposures to chemicals with insufficient data on sensitization hazard and/or sensitization potency.

6.3.2 Respiratory sensitization

A wide variety of synthetic chemicals are sensitizers, and they may be responsible for occupational rhinitis and asthma, if they can be inhaled as gases, vapours or aerosols, at any stage of their utilization, from their synthesis to their disposal. In addition, occupational respiratory allergy to macromolecular agents from animal, vegetal or microbiological origin is also an important problem in sectors such as the food industry, biotechnology and health care (Oberdörster et al., 1998). It should be noted that exposure via other routes, especially via the skin, has been implicated in the development of respiratory allergy and has also been described in experimental animals (Pauluhn, 2008).

Among respiratory allergies, allergic asthma has received the most attention from risk assessors because it is more serious than allergic rhinitis and laryngitis and occurs more frequently (or is at least identified more frequently) than hypersensitivity pneumonitis (extrinsic allergic alveolitis) and related conditions, such as chronic beryllium disease and hard metal lung disease, which are important and still incompletely resolved health problems in some workforces (Oberdörster et al., 1998). Allergic asthma can result from exposure to proteins (frequently enzymes) or from exposure to low molecular weight chemicals that, as with contact sensitizers, must be chemically linked to a protein carrier in order to activate the immune system. There is some debate as to whether the mechanisms underlying protein-associated asthma are the same as those underlying asthma associated with low molecular weight chemicals. More work has been done with protein allergies.

Currently, there are no universally accepted models applicable to humans that permit the determination of the dose–response relationship or relative potency of enzymes or low molecular weight chemicals for causing production of allergen-specific antibodies or symptoms of allergy via the inhalation route. Although the generation of total IgE or enzyme-specific IgE serum antibodies assessed by ELISA or cytophilic antibody (usually predominantly IgE) as measured by skin prick testing is often used as a component of the benchmark

value, it is not a disease state. Therefore, the generation of IgE antibodies is a conservative end-point on which to establish a benchmark.

In the detergent enzyme industry, more intense exposures have been associated with symptoms, whereas less intense exposures have been associated with production of allergen-specific antibodies (Sarlo & Kirchner, 2002). According to SDA (2005):

> The occupational data indicate that there are thresholds for the induction of antibodies and for the elicitation of symptoms. Experimental data in guinea-pigs support the observations made from the occupational experience [and vice versa]. The rate at which guinea pigs develop allergen-specific antibodies is dose related, with lower exposures leading to a low rate of animals having allergen-specific antibodies. However, combining low exposure with short peak exposures of enzyme allergen led to a greater number of animals with allergen-specific antibodies than expected. Intermittent peak exposures alone were also associated with allergen-specific antibodies. Elicitation was also dose-related, where symptoms in guinea pigs were only associated with the peak exposures.

Exposure routes in laboratory animal studies include inhalational, intradermal, intranasal and intratracheal routes. Although inhalation is the preferred route because of the similarity to human exposure, inhalation studies are labour intensive, time-consuming and expensive, and they present difficulties in delivering an accurate dose. Therefore, methods using other routes of exposure have been developed as surrogates for inhalation studies. Guinea-pigs are usually the species of choice for sensitization studies, although a mouse model is being developed for intratracheal and intranasal studies. Antibody responses of guinea-pigs showed that a dose inhaled over 6 hours/day has approximately the same effect as a single intratracheal dose (Ritz et al., 1993).

The time-weighted average (TWA) concentration (in mg/m^3) during a day or an 8-hour work-shift can currently be considered as the relevant measure of exposure. This can be justified because the uptake of antigen deposited in the respiratory tract by dendritic cells and their migration to the draining lymph nodes—as in skin sensitization—occur in a matter of hours. As mentioned below in this section, peak exposure concentrations or aggregate[1] exposure values have also been proposed as dose measures.

From a mechanistic point of view, the amount of antigen that gets to antigen-presenting cells in the airways and is presented to T cells and B cells affects the immune response (Bullock et al., 2000; Eisen, 2001). The delivered dose of enzyme is dependent on a number of factors, such as its concentration in the air, the rate of respiration, the particle or droplet size and the duration of exposure. Consideration of all the variables involved in determining the "delivered dose", perhaps assisted by applying computational deposition models, may help in describing exposure more accurately; currently, however, such information is available in only a few special cases.

As for skin sensitizers, a broad two-category system has recently been introduced for the classification and labelling of sensitizers under the Globally Harmonized System of Classification and Labelling of Chemicals (UN, 2008). Where data are sufficient, respiratory sensitizers are allocated either into subcategory 1A, for strong sensitizers, or into subcategory 1B, for other respiratory sensitizers. Sensitizers of subcategory 1A show a high frequency of

[1] In this document, the term aggregate exposure is used to refer to exposures to a single chemical from multiple sources and by all exposure routes over a given period of time (e.g. when hand soap is used 5 times per day).

occurrence in humans and/or a high probability of a high sensitization rate in humans based on laboratory animal or other tests. The severity of allergic reactions may also be considered.

6.3.2.1 Clinical and epidemiological data

(a) Induction

Dose–response data on the induction of respiratory sensitization may be obtained from clinical studies that are designed either as a prospective study (when a new compound or product is introduced in the consumer market) or as a retrospective study (in which cases of allergy are analysed post hoc as the inducing exposure, often involving experimental measurement of reconstituted exposure scenarios).

Heederik & Houba (2001) conducted an epidemiological study in bakery workers, analysing respiratory sensitization against wheat (measured as IgE antibodies). The prevalence of wheat allergy was positively correlated with exposure, no matter whether this was expressed as estimated average inhalable dust concentration (in mg/m^3) or as aggregate inhalable dust concentration (in mg-years/m^3). There was no indication of the existence of a threshold for wheat sensitization risk in any of the plots. For atopics, the sensitization risk levelled off at higher exposure levels and decreased at even higher levels (average exposures of approximately 4 mg/m^3 for inhalable dust or 10 $\mu g/m^3$ for wheat allergens). When a more rigid definition of sensitization was applied (anti-wheat IgE titre of 0.7 kU/l instead of 0.35 kU/l), the exposure–response relationship shifted somewhat to the right, and elevated risks were observed only in the highest exposure category. The same thing happened when sensitization in combination with the presence of work-related symptoms was used as the end-point in the analyses (rhinitis, asthma). The exposure–response relationship obtained for sensitization accompanied by symptoms is the result of superimposing the exposure–response relationship for exposure and symptoms on the relationships between exposure and sensitization. In this study, the steepest relationship was obtained for sensitization in combination with rhinitis symptoms, compared with sensitization only or sensitization in combination with asthmatic symptoms. The analysis of aggregate inhalable dust exposure suggested a concentration around 11.97 mg-years/m^3 as a no-observed-effect concentration (NOEC). The long-term average exposure over an 11.7-year period was estimated as 11.97/11.7 = 1.02 mg/m^3.

Dusty laundry products caused allergy to enzymes in some consumers in Sweden during the late 1960s and early 1970s (Belin et al., 1970; Zetterstrom & Wide, 1974). An analysis of 1645 individual serum samples showed that 15 individuals had enzyme-specific IgE antibodies (0.91%). These 15 also had a positive skin prick test (SPT) in reaction to the enzyme. Exposure data have been generated retrospectively to simulate the exposure to these materials that occurred from filling a sink with water and adding laundry detergent for hand laundering. The results suggested an average peak level of 212 ng/m^3 for this use scenario. This example demonstrates the effects resulting from high exposure over a short duration that occurred on a regular basis. Some of these 15 individuals reported symptoms of allergy when they used the dusty enzyme-containing laundry powder. A provocation test with some of these consumers showed that 8 out of 12 patients who had IgE antibodies to enzymes had symptoms after challenge with an enzyme-containing product (laundry powder mixed with enzyme). Several retrospective studies revealed that for indoor pouring of liquid laundry products, exposures ranged between 0.01 and 1 ng/m^3, which were considered safe, as no additional new cases of allergy were observed (SDA, 2005).

Mapp et al. (2005) suggested in their review that enough data for assessment of exposure–response relationships are available for several other high molecular weight occupational allergens—namely, for cedar, fungal α-amylase and laboratory animal proteins.

Baur (2003) reviewed clinical epidemiological and exposure evaluation findings as well as theoretical relationships between concentrations of respiratory allergens in workplaces and prevalence of sensitized workers, lung function impairment, and symptoms and/or frequency of occupational asthma. Corresponding slopes were found in the nanograms per cubic metre range for latex, purified enzymes and rat urinary proteins; in the micrograms per cubic metre range for wheat flour allergens, cyanates and platinum salts; and in the milligrams per cubic metre range for acid anhydrides, wood dust and the rather heterogeneous bakery flour dust (i.e. 1000-fold differences exist, partly depending on the purity of the allergen).

Prospective epidemiological studies in workers have been reported for toluene diisocyanate and several organic acid anhydrides used in epoxy resins (reviewed in Arts et al., 2006). In the prospective study performed in toluene diisocyanate–exposed workers, it was shown that accidental exposure to high concentrations of toluene diisocyanate resulted in IgE antibody formation. In contrast, exposure to low concentrations of toluene diisocyanate (at or below 0.14 mg/m^3) for up to 3 years did not result in any cases of toluene diisocyanate hypersensitivity or in the production of toluene diisocyanate–specific antibodies.

Other low molecular weight respiratory sensitizers for which dose–response relationships have been reported include colophony and platinum salts (reviewed in Mapp et al., 2005) (see also Case-study 3 on halogenated platinum salts).

From epidemiological investigations, NOECs or BMCs can be derived that may serve as PODs for risk assessment. However, appropriate data seem to be available for only a very limited number of allergens.

(b) Elicitation

Dose–response relationships and thresholds for the elicitation of respiratory allergy can be derived from epidemiological studies or experimental/diagnostic provocation tests.

Yokota et al. (1999) reported on two condenser plants using epoxy resins containing methyltetrahydrophthalic anhydride. Air concentrations of methyltetrahydrophthalic anhydride were higher in plant A than in plant B (mean concentrations approximately 25–64 $\mu g/m^3$ and 4.9–5.5 $\mu g/m^3$, respectively). Of a total of 95 workers, 24 workers (65%) in plant A and 38 workers (66%) in plant B had methyltetrahydrophthalic anhydride–specific IgE antibodies. In sensitized workers in plant A, eye, nose and pharynx symptoms were observed at a higher incidence when compared with sensitized workers in plant B. Furthermore, only 15% of persons often displayed work-related symptoms among the 20 symptomatic workers in plant B, compared with 73% of the 26 symptomatic workers in plant A. In plant B, the minimum level that was associated with work-related symptoms was 15–22 $\mu g/m^3$, indicating a threshold level for elicitation of about 15 $\mu g/m^3$.

Similar occupational studies have been reported for other organic acid anhydrides and for isocyanates (reviewed by Arts et al., 2006). For high molecular weight antigens, positive relationships between allergen exposure and work-related symptoms were also published. The prevalence of sensitized workers experiencing symptoms, the frequency of symptoms

and their severity (e.g. measured as forced expiratory volume in 1 second) were correlated with the mean exposure concentrations of antigen in air for antigens such as wheat flour, seafood protein and wood dust (see Arts et al., 2006).

Baur et al. (1998) reported that allergen exposures were not associated with an increased occurrence of asthma symptoms if levels stayed below threshold limits. Corresponding data were available for wheat flour (1–2.4 $\mu g/m^3$), fungal α-amylase (0.25 ng/m^3), natural rubber latex (0.6 ng/m^3), western red cedar (0.4 $\mu g/m^3$) and rat allergens (0.7 $\mu g/m^3$).

Studies investigating the variability of the NOELs or threshold concentrations in bronchial provocation tests are virtually lacking. While thresholds are mainly described for protein allergens, it should be noted that when testing elicitation reactions in humans, it cannot be excluded that cross-reactions between chemicals forming "immunologically similar" haptens occur; therefore, a positive reaction against a chemical does not necessarily indicate that the individual has also been sensitized through contact with this chemical.

6.3.2.2 Laboratory animal data

(a) Induction

For protein allergens, such as detergent enzymes, guinea-pigs and mice have been used to demonstrate thresholds for induction (Kawabata et al., 1996; Sarlo et al., 1997; Robinson et al., 1998). Guinea-pigs were exposed by intratracheal instillation with different levels of enzyme protein, and sera from the animals were evaluated for allergic antibody to the enzyme. The amount of antibody produced in response to an enzyme was compared with the amount of antibody produced in response to the same protein dose of the reference allergen subtilisin A (Alcalase).

Dose–response relationships and thresholds were also demonstrated in mice exposed 4 times by involuntary aspiration to a total protein extract from *Penicillium chrysogenum* (a common indoor mould) (Chung et al., 2005). Using four doses (10, 20, 50 and 70 µg), dose-dependent increases in eosinophil in bronchoalveolar lavage fluid, total IgE levels in serum and bronchoalveolar lavage fluid, antigen-specific IgE levels in serum and IL-5 levels in broncho-alveolar lavage fluid and increased severity of histopathological lesions were demonstrated, with a NOEL of 20 µg protein. Increased allergen-triggered immediate respiratory responses as well as nonspecific airway hyper-responsiveness to methacholine as assessed by baro-metric whole-body plethysmography were demonstrated only at the 70 µg exposure level. Because these exposures included all the mould proteins extracted, it is not possible to draw conclusions about administered dose of specific allergens.

Matheson et al. (2005) investigated a murine model of toluene diisocyanate asthma following either low-level subchronic or high-dose acute inhalation exposure to toluene diisocyanate. C57BL/6 J mice were exposed to toluene diisocyanate by inhalation either subchronically for 6 weeks (0.14 $\mu g/m^3$, 4 hours/day, 5 days/week) or by a 2-hour acute exposure at 3.6 $\mu g/m^3$. Both groups were challenged 14 days later via inhalation with toluene diisocyanate at 0.14 $\mu g/m^3$ for 1 hour. Mice that underwent the subchronic exposure regimen demonstrated a marked allergic response evidenced by increases in airway inflammation, eosinophilia, goblet cell metaplasia, epithelial cell alterations, airway hyper-responsiveness, Th1/Th2 cytokine expression in the lung, elevated levels of serum IgE and toluene diisocyanate–specific IgG antibodies, as well as the ability to transfer these pathologies to naive mice with lymphocytes or sera from toluene diisocyanate–exposed mice. In contrast, mice that received acute toluene

diisocyanate exposure demonstrated increased airway hyper-responsiveness, specific IgG antibodies and pathology in the lung consistent with asthma, but without the presence of elevated serum IgE, lung eosinophilia or increased cytokine expression.

In a similar study in guinea-pigs, Karol et al. (1980) demonstrated that animals exposed for 6 hours/day, 5 days/week, for 70 days to toluene diisocyanate at 1.8 µg/m^3 showed antibody production, whereas no toluene diisocyanate antibodies were observed after exposure to 0.14 µg/m^3. The aggregate exposures were (61.7 mg/m^3)·h for the 0.14 mg/m^3 group, compared with a (26.7 mg/m^3)·h aggregate exposure that had induced antibody formation at 1.8 µg/m^3 (3 hours/day for 5 days, NOEL 0.85 µg/m^3) in the Karol (1983) study. In the latter study, a linear relationship was observed between log concentration (range 0.85–6.8 µg/m^3) of toluene diisocyanate and the antibody response as well as the percentage of animals producing antibody to toluene diisocyanate. It was concluded that the exposure concentration (in combination with the duration of exposure) was important for establishment of antibody response, rather than total exposure.

Brown-Norway rats were exposed to diphenylmethane diisocyanate (MDI) by inhalation on 5 consecutive days according to two concentration × exposure time ($C \times t$) regimens: 1000, 5000 or 10 000 (mg/m^3)·min at exposure durations of either 10 or 360 minutes (Pauluhn & Poole, 2011). Challenge exposures to MDI of 30 minutes each were done at 40 mg/m^3 on days 20, 25, 50 and 65. After the last challenge, changes in breathing patterns and broncho-alveolar lavage fluids were examined. The most sensitive end-points were the number of neutrophils in bronchoalveolar lavage and physiological measurements of respiratory changes. The high concentration delivered for 10 minutes elicited a more vigorous response than the similar $C \times t$ product at 360 minutes, suggesting that short, high-level exposures have a higher sensitizing potency than equal $C \times t$ products at longer exposure periods.

NOECs or BMCs can be derived from inhalation studies in experimental animals and may serve as PODs for risk assessment. However, owing to the lack of a harmonized test guideline for respiratory sensitization, uncertainty exists as to how such tests should be designed in terms of the lengths of the daily exposure, the total exposure period (total number of exposure days) and the challenge concentration and read-out parameters. For the example of toluene diisocyanate, the same NOEC of 0.14 µg/m^3 was identified from workplace epidemiological studies and from a subchronic study in guinea-pigs, indicating that on a case-by-case basis, a POD may be derived from repeated exposure studies in laboratory animals. With regard to aerosols and dusts, it is currently unknown whether the external exposure concentration used in laboratory animal studies is relevant for risk assessment or whether this value should be corrected through considering amounts of substance deposited on the surface of respiratory tract subcompartments by applying computational deposition models.

One note of caution is that whereas there is no doubt that IgE antibody plays an important role in respiratory allergy to proteins, there is some debate about the suitability of IgE antibody for the development of occupational asthma in response to low molecular weight compounds. Although there is evidence that all known chemical respiratory allergens induce specific IgE in some symptomatic subjects, other subjects do not exhibit this response, and late-onset responses occur in the absence of an immediate response, particularly in allergy and asthma associated with diisocyanates (Cartier et al., 1989; Bernstein, 1996; Park et al., 1999; Bernstein et al., 2002). The animal models described above clearly model human subjects who develop an IgE response. The debate is whether information developed from these models would be protective for humans who do not develop IgE responses.

(b) Elicitation

Most data on elicitation after inhalation exposure obtained in animal models of respiratory sensitization were obtained after a single or a few induction exposures, but not after long-established respiratory sensitization. In addition, induction was often done by injection (Botham et al., 1989; Pauluhn & Mohr, 1994) or dermal application (Arts et al., 1998, 2004b; Zhang et al., 2004; Pauluhn, 2008) of the test substance. Challenge was done with either free chemical or chemical–protein adducts. Parameters measured included functional respiratory and histopathological parameters as well as antibody titres. It is uncertain whether the dose–response relationships (which often were not very clear) and NOEL/LOEL values reported are relevant for the situation in humans. Without further research into the topic of comparison of elicitation thresholds in humans and laboratory animals after long-established respiratory allergy, it is not recommended that a POD for risk assessment be derived from laboratory animal studies.

An example for the research on elicitation of respiratory tract reactions in laboratory animals is given in the following: Arts et al. (1998) investigated specific functional and histopathological airway reactions to trimellitic anhydride following inhalation challenge as an extension to the IgE test. Brown Norway rats were topically exposed to a fixed dose on days 0 and 7, total serum IgE was measured on day 20 or 21, and animals were subsequently challenged with various concentrations of trimellitic anhydride (16, 31 and 52 mg/m^3 on day 21 or day 22). A significant decrease in respiratory rate during challenge, followed by an increase in breathing rate with a reduced tidal volume 24 hours after challenge, but without a dose–response relationship, was observed at all concentrations tested. A challenge concentration–dependent histopathological response to trimellitic anhydride challenge was observed in the larynx and lungs. In a similar study using a wider range of challenge concentrations (0.2–61 mg/m^3), a concentration-dependent increase in functional and histopathological changes and unspecific airway hyper-responsiveness were observed. The NOEC was 0.2 mg/m^3 (Arts et al., 2004b). Interestingly, an elicitation NOEC of 0.2 mg/m^3 was also found in Brown Norway rats treated dermally with trimellitic anhydride powder under occlusion on days 0, 7, 14 and 21 and challenged by inhalation at concentrations of 0.2–40 mg/m^3 on day 35 (Zhang et al., 2004).

Brown-Norway rats were exposed to MDI by inhalation on 5 consecutive days at $C \times t$ products of 1000, 5000 or 10 000 (mg/m^3)·min for either 10 or 360 minutes (Pauluhn & Poole, 2011). Challenge exposures to MDI of 30 minutes each were done at 40 mg/m^3 on days 20, 25 and 50. On day 65, a challenge using a dose escalation regimen (5, 15 and 40 mg/m^3) was used to determine the elicitation threshold (measured as elevated numbers of neutrophilic granulocytes in bronchoalveolar lavage fluid). An elicitation NOEC of 5 mg MDI-aerosol per cubic metre at 30 minutes' duration was identified. In topically sensitized rats, this NOEC was estimated to be 3 mg/m^3 (estimated by linear extrapolation), suggesting that elicitation NOECs in rats sensitized to MDI by inhalation or skin exposure show no essential differences.

6.3.3 Oral and parenteral sensitization

As noted above, all allergic responses are systemic, in that sensitized immune cells can circulate throughout the body and can respond when challenge occurs at sites other than the original site of sensitization. However, for the allergic diseases described above, the response to challenge is usually localized at the site of challenge. Oral exposure (e.g. ingestion of food or medicaments) or parenteral administration of substances (e.g. drug injection) can also lead

to sensitization. In these cases, allergic reactions may occur locally, such as at the site of injection or in the mouth or the stomach, but often involve more systemic responses. Food allergy is an example of a more systemic response. IgE-mediated food allergies can cause symptoms in the skin (acute urticaria/angio-oedema and atopic dermatitis), the upper and lower respiratory tract, as well as the gastrointestinal tract. Typically, a sensitized individual will develop symptoms within minutes after ingesting the food. In addition, IgE-mediated reactions to food allergens have been reported to be one of the leading causes of anaphylaxis (a multiorgan reaction involving disseminated release of inflammatory mediators and circulatory collapse) seen in emergency departments. Hymenoptera (e.g. honey bee) stings and administered drugs are the other common causes of anaphylactic reactions seen in medical facilities (Treudler et al., 2008). Immune-related problems are the largest single area of adverse events that are not detected by preclinical testing of drugs (Olson et al., 2000). Many of these events are dermal reactions associated with systemic administration of drugs, although anaphylaxis is a more worrisome occurrence (Weaver et al., 2003). Both oral and parenteral allergic reactions have been difficult to model in animals, in part because these reactions occur with low frequency in the human population and because genetic susceptibility is a significant factor.

Estimates of the prevalence of food allergies vary depending on the study. Approximately 6–8% of children suffer from food allergy during their first 3 years of life (Sampson, 2005). Many of these children then go on to develop tolerance, and the prevalence of food allergy in adults is approximately 3% (Moneret-Vautrin & Morisset, 2005). Only a few foods are known to cause the vast majority of food allergies. In children, reactions are most commonly caused by eggs, peanuts, milk, soy and wheat, whereas the most common reactions for adults are to shellfish, fish, tree nuts and peanuts (Bernstein et al., 2003). True food allergy is distinct from food intolerance, in that the latter does not involve immune mechanisms, although the symptoms generated may be similar. Food allergies can be caused by IgE-mediated or non-IgE-mediated mechanisms, although IgE-mediated events are the most common and have generated the most attention. The SPT is typically used to diagnose IgE-mediated food allergy; however, double-blind placebo-controlled food challenge tests remain the gold standard for diagnosis (Sampson, 2005). The application of biotechnology to food production, particularly genetic modification to confer pest resistance or increase nutritional value, has created the need to consider the potential that novel proteins introduced into the food supply could induce oral sensitization. The introduction of new foods into a population by conventional methods (e.g. importation of kiwi) also occurs (Lucas et al., 2004).

The study of oral sensitization is complicated because the normal response (in both rodents and humans) to ingestion of antigen is tolerance, a state of antigen-specific unresponsiveness (Saklayen et al., 1984; Strobel & Mowat, 1998; Christensen et al., 2003). This phenomenon is preferentially directed against IgE and DTH responses and in laboratory animal studies had been adoptively transferred by transplanting T cells (probably CD25+CD4+ regulatory T cells). Tolerance induction is genetically determined, and high sensitivity is co-inherited with low IgE responder phenotype, such that 1000- to 10 000-fold greater allergen exposure is required to tolerize high IgE responders. Tolerance induction also appears to be a function of age, in that allergen exposure of neonates primes the individual for subsequent T cell reactivity rather than tolerance, presumably due to delayed postnatal maturation of one or more key elements of mucosal immune function that are rate limiting in inducing tolerance. Food allergy appears to be the result of a breach of oral tolerance.

The authors of this guidance document think that in light of the currently available scientific data, it is premature to propose a quantitative risk assessment approach for allergic reactions following oral or parenteral exposure. Currently, no validated test methods for hazard identification are available, and test systems delivering NOELs that could be used as starting points for a quantitative risk assessment are virtually lacking (with the exception of allergy elicitation in sensitized individuals). Regarding the elicitation of food allergy reactions in the general population, a probabilistic risk characterization approach has been proposed and successfully applied in some cases (Spanjersberg et al., 2007; Kruizinga et al., 2008; Madsen et al., 2009). However, the suitability of this method for oral and parenteral sensitization to chemicals has yet to be demonstrated, and therefore it is not recommended as an established standard method. A short overview of available data is given in the next sections.

6.3.3.1 Clinical and epidemiological data

(a) Induction

There is no clinical information available on the dose–response relationship associated with sensitization to chemicals, drugs or food constituents after oral or parenteral exposure. Published data on anaphylactic responses to drugs are generally detected as a result of post-market surveillance, where the dose of drug administered is based on clinical efficacy. In the case of food allergy, induction of oral tolerance versus induction of sensitization complicates potential dose–response relationships. There is growing evidence that introduction of small amounts of peanuts early in life may prevent sensitization (Khakoo & Lack, 2004), and variations in factors that contribute to potential sensitization (Burks et al., 2008), including relevant route of exposure, form of allergen (Bowman & Selgrade, 2008b), age of sensitization, intestinal flora (Calder et al., 2006) and others, in addition to the large genetic component, are still poorly understood and would all likely affect the sensitization dose–response relationships. There are no known cases in which introduction of novel proteins via genetic modification of crops has resulted in a new food allergen for humans.

(b) Elicitation

Double-blind placebo-controlled food challenge, generally used as a diagnostic tool, has only recently been adapted to provide data on thresholds. These studies have indicated a wide variability in the allergic subpopulation, and it is difficult to determine how well the patients tested represent the overall population and thus interpret findings in a population context. Regulatory authorities, such as the USFDA and European Food Safety Authority, have been reluctant to use currently available data to set thresholds. However, it is clear that there are thresholds for individuals, and standardized protocols have been developed that could be used to generate sufficient data to draw conclusions about specific populations, at least for some of the most allergenic foods. It should be noted that the challenge matrix should be one of the real food matrices (Crevel et al., 2007, 2008).

In one example of a double-blind placebo-controlled food challenge, data from 125 positive oral challenges to egg, 103 to peanut, 59 to milk and 12 to sesame seeds were analysed. LOEL values of 2 mg of egg, 5 mg of peanut, 0.1 ml of milk and 30 mg of sesame seed were found (Morisset et al., 2003).

While thresholds are mainly described for protein allergens in food, it should be noted that when testing elicitation reactions in humans, it cannot be excluded that cross-reactions

between chemicals forming "immunologically similar" haptens occur; therefore, a positive reaction against a chemical does not necessarily indicate that the individual has also been sensitized through contact with this chemical. Examples of cross-reactivity have been reported for drugs, such as β-lactam antibiotics (Antúnez et al., 2006).

In order to perform a probabilistic risk characterization (e.g. for food allergy), the statistical distribution of minimum eliciting doses in the sensitized population is necessary, rather than a POD, such as a NOEL or BMD (Spanjersberg et al., 2007; Kruizinga et al., 2008; Madsen et al., 2009).

For drugs, there is little incentive to determine a threshold for elicitation, because this would likely be below the level needed for clinical efficacy.

6.3.3.2 Laboratory animal data

Currently, a number of animal models have been used to study various aspects of oral sensitization and oral tolerance, but none have been validated or adopted for use in hazard identification or the generation of dose–response data. For genetically modified foods, hazard identification employs a weight of evidence approach that takes into account a variety of factors and approaches for an overall assessment of allergenic potential. These various recommendations are based on what is known about allergens, including the history of exposure and safety of the gene source; amino acid sequence identity to human allergens; stability to pepsin digestion in vitro; protein abundance in the crop and processing effects; and, when appropriate, specific IgE binding studies or skin prick testing (FAO/WHO, 2003).

(a) Induction

A recent report demonstrated that it was possible to distinguish allergenic from non-allergenic food extracts using oral exposure of C3H/HeJ mice with cholera toxin and antigen-specific serum IgE levels as the read-out. Dose–response relationships were demonstrated within a limited range (Bowman & Selgrade, 2008a). A second complementary model also distinguished between allergenic and non-allergenic food extracts based on the induction of tolerance following oral exposure (Bowman & Selgrade, 2008b). Neither of these models produced anaphylactic reactions. Another test proposed for hazard identification is the IgE test in BALB/c mice employing intraperitoneal injection of the test protein without adjuvant followed by determination of specific IgG and IgE formation (Dearman & Kimber, 2008).

(b) Elicitation

Another recent report demonstrated systemic anaphylaxis in BALB/c mice sensitized transdermally and challenged by the oral route. No attempt was made to develop a dose–response relationship (Birmingham et al., 2007). Both this model and those cited above require additional work before they can be applied to risk assessment.

6.3.3.3 Pseudoallergic reactions

Although pseudoallergy is beyond the scope of this document, it is important to recognize that in some cases, exposure to chemicals can result in symptoms that mimic symptoms of allergy, but the underlying mechanisms are not specific immune-mediated responses. For example, aspirin-induced asthma may result from inhibition of cyclooxygenase by aspirin and

the shunting of arachidonic acid to the lipoxygenase pathway. Under certain circumstances, this results in increased production of cysteinyl leukotrienes, which cause bronchoconstriction and/or increased responsiveness of the airways (Stevenson & Szczeklik, 2006; National Heart, Lung and Blood Institute, 2007), resembling the symptoms of allergic asthma. However, aspirin-induced asthma does not involve antibody production or specific immune response. Certain drugs, including radiocontrast media, liposomal drugs and micellar solvents, cause complement activation–related pseudoallergy. These agents activate complement through both the classical and the alternative pathways, giving rise to C3a and C5a anaphylatoxins that trigger release of mediators from mast cells and basophils (Szebeni, 2005). Occasionally, food intolerances cause symptoms similar to those of food allergies. Substances that can trigger pseudoallergic food intolerances include additives such as sulfites, tartrazine and glutamate. Pseudoallergic reactions can be triggered in various ways, such as interactions with the central or peripheral nervous system, nonspecific release of mediators, enzyme inhibition due to hereditary or pharmacologically induced enzyme deficiencies and pharmacological properties of some natural food constituents, such as biogenic amines.

6.3.4 Derivation of point of departure

Table 6.1 summarizes the possible ways to derive a POD for risk assessment for skin, respiratory and systemic sensitization induction and elicitation. It is generally recommended that risk assessors not look at only a single test result, although that study might be fully valid and provide a threshold or NOEL that may be used as a POD, but integrate all available information, both from laboratory animal studies and from human studies, case reports or otherwise documented experience in a weight of evidence approach.

For the evaluation of HRIPTs, criteria for a robust HRIPT have been proposed (see, for example, McNamee et al., 2008). An example of a guideline for applying the weight of evidence approach to human and laboratory animal skin sensitization induction data, which gives human data precedence over laboratory animal data, has been proposed by Api et al. (2008) for the assessment of fragrance ingredients. Here, however, in the larger context, it is purposely left open as to whether human data should be preferred over laboratory animal data, because the scientific and ethical acceptance of studies in volunteers differs between geographical regions and also depends on the regulatory framework.

Sometimes several studies with the same (or very similar) experimental method are available; for example, for a given chemical, several LLNAs using the same or different vehicles may exist. In this case, a NOEL as a POD can be derived only after discussion of the weight and relevance of each result, considering, for example, whether the study with the lowest LOEL should be given preference, whether studies can be combined to derive a new LOEL (e.g. as a BMD), how studies with non-standard vehicles should be weighted or whether studies with vehicles and/or exposure conditions most closely resembling human exposure conditions should be regarded as best evidence.

For the LLNA, it has been proposed that a vehicle-based mean EC_3 value be used (Api et al., 2008). If more than one EC_3 is available for a particular vehicle, a mean value for that vehicle is calculated first, and then the mean over all vehicles is derived (see worked example in Case-study 4 on citral). Use of a vehicle-weighted mean, rather than the lowest EC_3 value, is justified, because LLNA EC_3 values, when tested repeatedly, tend to vary within a factor of 2–3 from the mean value, and the variability of the EC_3 value caused by different vehicles

Table 6.1: Derivation of a POD for risk assessment for skin, respiratory and systemic sensitization induction and elicitation.

Type of data	*Value used as a POD*	*LOEL to NOEL extrapolation*
Skin sensitization: Induction		
Human data		
HRIPT (or HMT)	NOEL (or BMD_5) (µg/cm^2 skin per day)	If NOEL is lacking and results with sensitization rates below 50% are available, the LOEL may be extrapolated by applying a factor of 3 to doses producing sensitization rates of 10–25% and a factor of 10 for sensitization rates of 25–50% (as proposed in Griem et al., 2003).
Laboratory animal data		
LLNA in mice	EC_3 (µg/cm^2 skin per day)	None required (see text)
GPMT or Buehler test in guinea-pigs	Generally not suitable for derivation of POD	Not applicable
Weight of evidence approach for grouping substance into a skin sensitizing potency category	Use the lower boundary of potency category (expressed as µg/cm^2 skin per day)	Not applicable
Skin sensitization: Elicitation		
Human data		
Patch test	BMD, e.g. MET_{10} (µg/cm^2 skin per day)	Not applicable
ROAT or product use test	NOEL or BMD (µg/cm^2 skin per day)	Not applicable
Laboratory animal data	Currently not considered suitable for derivation of POD	
Respiratory sensitization: Induction		
Human data	NOEC or BMC from epidemiological study (TWA concentration in mg/m^3 or aggregate dose in (mg/m^3)·h)	Based on weight of evidence evaluation, when a NOEC is lacking and the lowest exposure level inducing sensitization can be regarded as the LOEC, the NOEC might be extrapolated by applying a factor of 3 or 10 to the LOEC.
Laboratory animal data	Only on case-by-case basis and using weight of evidence approach, NOEC or BMC from experimental study with repeated inhalation exposure (TWA concentration in mg/m^3 or aggregate dose in (mg/m^3)·h)	Based on weight of evidence evaluation, when a NOEC is lacking and the lowest exposure level inducing sensitization can be regarded as the LOEC, the NOEC might be extrapolated by applying a factor of 3 or 10 to the LOEC.
Respiratory sensitization: Elicitation		
Human data	NOEC or BMC from epidemiological or experimental study (TWA concentration in mg/m^3 or aggregate dose in (mg/m^3)·h)	Based on weight of evidence evaluation, when a NOEC is lacking and the lowest exposure level inducing sensitization can be regarded as the LOEC, the NOEC might be extrapolated by applying a factor of 3 or 10 to the LOEC.

Table 6.1 (continued)

Type of data	*Value used as a POD*	*LOEL to NOEL extrapolation*
Laboratory animal data	Currently not considered suitable for derivation of POD	
Oral and parenteral sensitization: Induction		
Currently, the database is not considered adequate for developing a quantitative risk assessment approach.		
Oral and parenteral sensitization: Elicitation		
Currently, the database is not considered adequate for developing a quantitative risk assessment approach. Thresholds for elicitation of systemic sensitization have been reported for allergenic food proteins, but not so far for chemicals. A probabilistic risk assessment approach, as proposed for food allergens, might be considered.		

BMD_5, benchmark dose for a 5% response

leads to uncertainty in the risk assessment that is taken into account in setting the matrix assessment factor.

6.4 Biological plausibility

6.4.1 Weight of evidence approach for assessment of sensitization

Hazard identification for sensitization should result in weight of evidence conclusions based on the available human and laboratory animal data for a given chemical. The entire database of effects, both positive and negative, should be considered in this process. For skin sensitization, evidence from epidemiological investigations or several case-studies from more than one clinical centre reporting allergic contact dermatitis are usually considered sufficient evidence for identifying a skin sensitization hazard. Also, positive findings from laboratory animal tests that were performed according to OECD test guidelines under GLP provide sufficient evidence for this end-point. For respiratory sensitization and oral/parenteral sensitization, epidemiological studies and several case-studies from more than one clinical centre are usually considered sufficient evidence. Laboratory animal studies on oral and parenteral sensitization are currently thought to provide equivocal evidence of systemic sensitization. For respiratory sensitization, laboratory animal studies that demonstrate allergic responses in animals that have been sensitized and challenged by the inhalation route are considered to provide some evidence of respiratory sensitization. Studies reporting sensitization after sensitization via instillation or topical or intradermal application are considered as equivocal evidence of respiratory sensitization.

The weight of evidence conclusions are strengthened by consistency (particularly across species, sexes or related end-points), SAR evaluations and biological plausibility. Conflicting data should be evaluated by the strengths and weaknesses of the individual studies, as well as in the context of other effects on the immune system.

Decision-trees to address skin sensitization, respiratory sensitization and systemic hypersensitivity are illustrated in Figures 6.2A, 6.2B and 6.2C, respectively. Depending on the data situation and on the scope of the risk assessment, it may be advisable to address all routes of exposure, that is, to use all three decision-trees, or it may be sufficient to use only one decision-tree, if the relevant sensitization route has already been clearly identified:

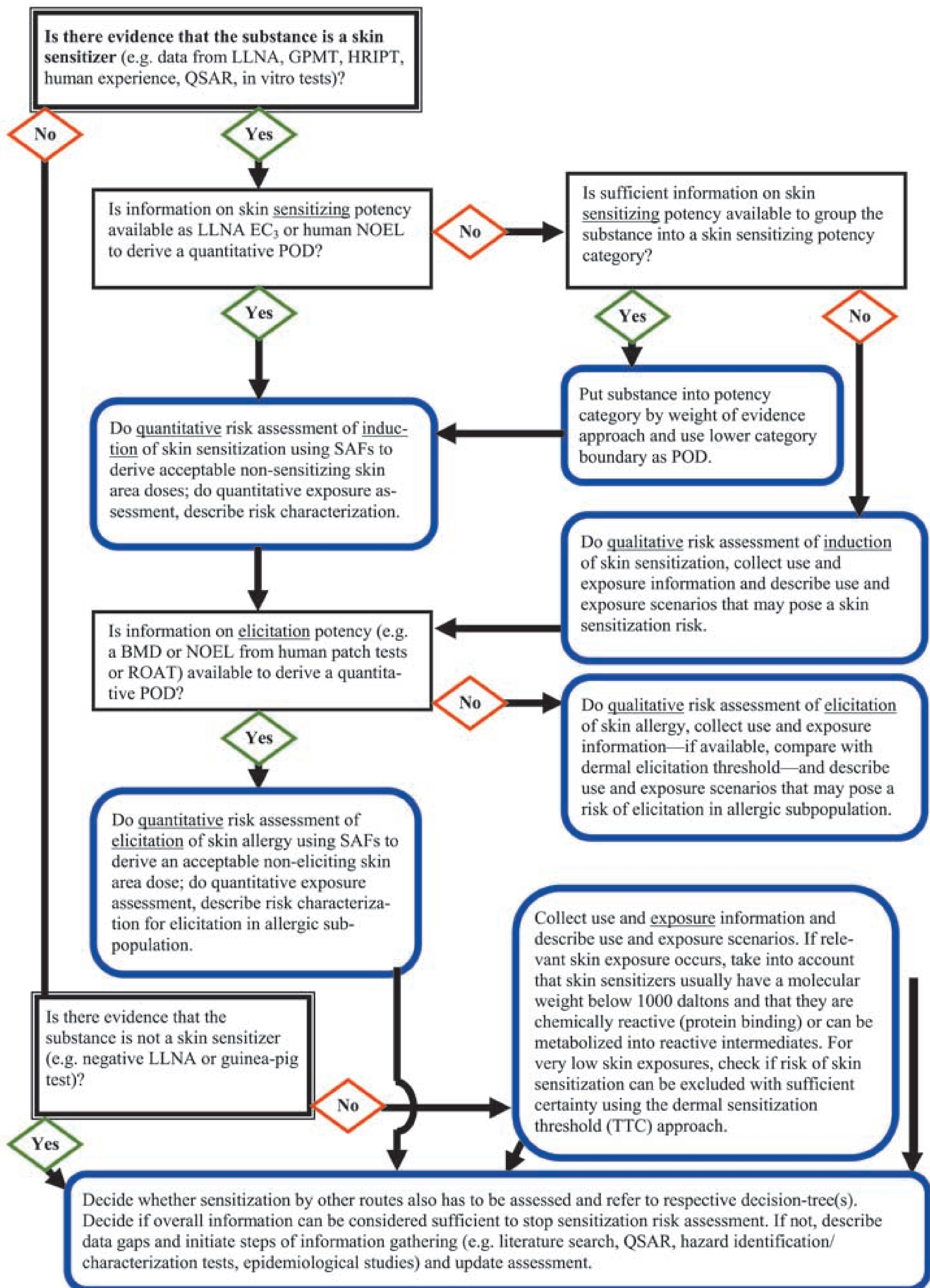

Figure 6.2A: Decision-tree for the assessment of sensitization and allergic response: skin sensitization.

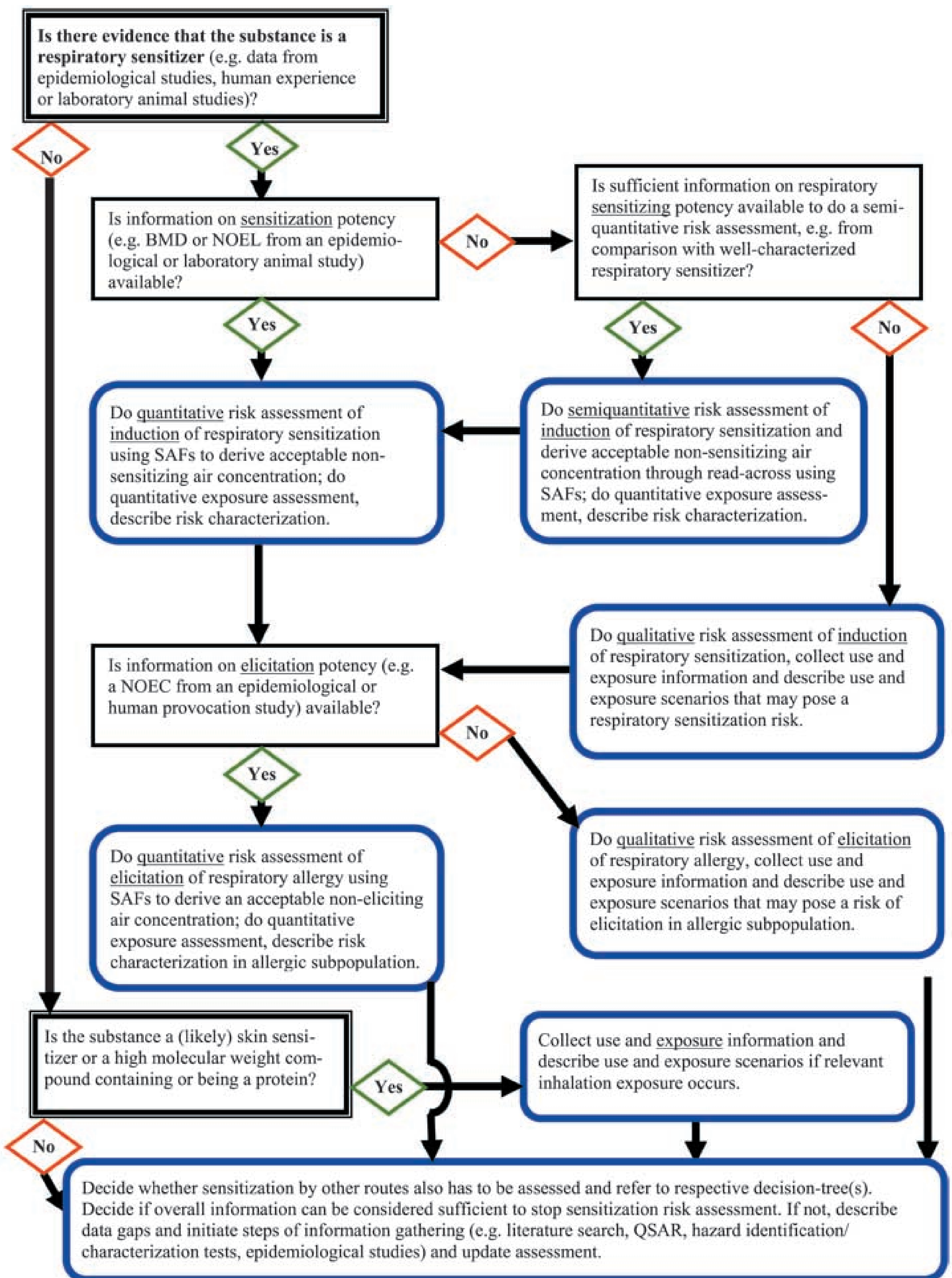

Figure 6.2B: Decision-tree for the assessment of sensitization and allergic response: respiratory sensitization.

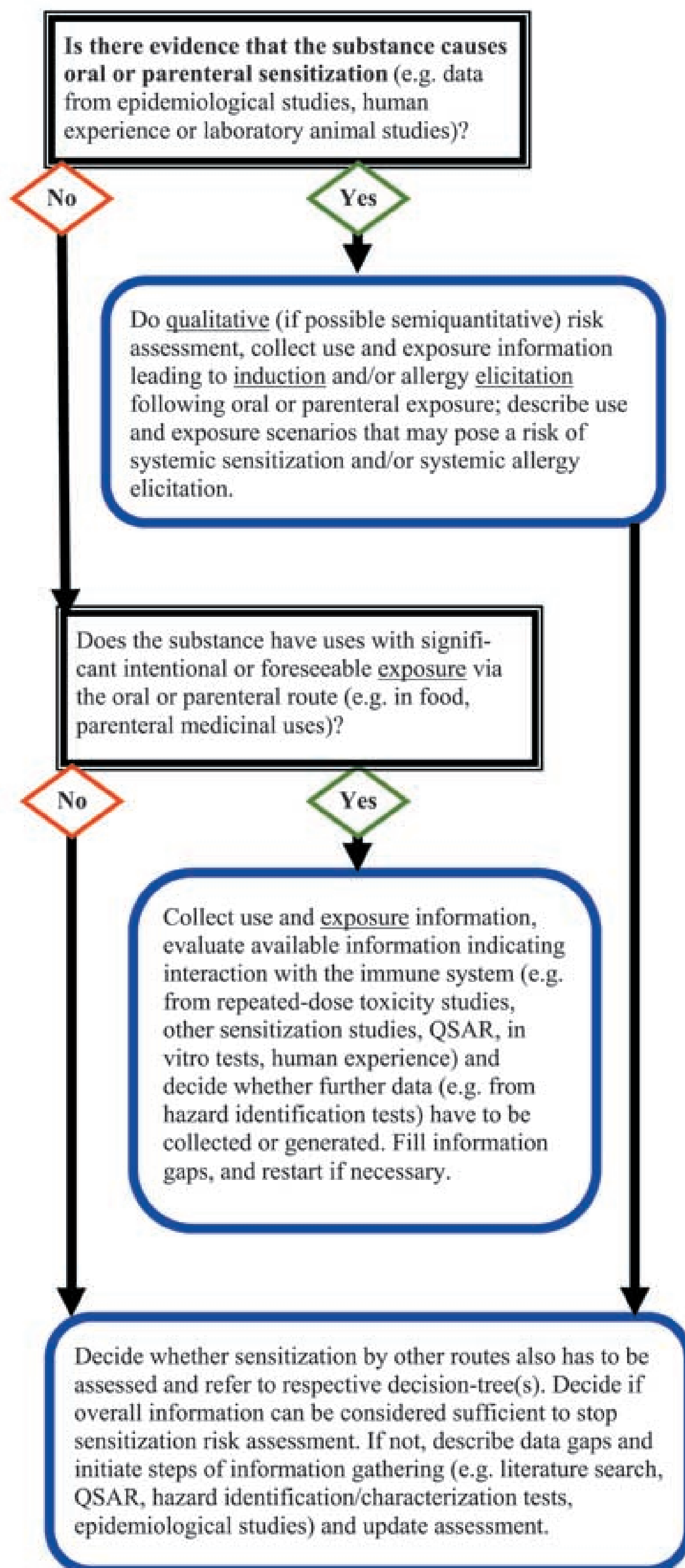

Figure 6.2C: Decision-tree for the assessment of sensitization and allergic response: systemic sensitization.

- In the decision-tree on skin sensitization (Figure 6.2A), skin sensitization is assessed if data are available from epidemiological, clinical or experimental human studies (HRIPT, HMT), from laboratory animal tests (LLNA, GPMT or Buehler test) or from in vitro tests or QSARs that indicate that the substance is a skin sensitizer.
  - ✓ If this is the case, a weight of evidence approach should consider all data on sensitizing potency (e.g. NOEL from human tests, EC_3 from an LLNA or the lower boundary of the potency category into which the substance can be grouped) in order to derive a POD for a quantitative risk assessment. In this acceptable non-sensitizing skin area, doses can be derived by applying SAFs to the POD. If quantification of sensitization potency is not possible, a qualitative risk assessment approach should be used. If possible, a quantitative exposure assessment should be performed, the results of which are then compared with the derived acceptable dose in the risk characterization. The uses of the substance and relevant human exposure scenarios should be described.
  - ✓ If a subpopulation of individuals who are already sensitized to the chemical exists, data on the elicitation potency of the chemical (e.g. as a BMD or NOEL from human elicitation patch tests or ROAT) may be available that allow a quantitative risk assessment of elicitation of skin allergy (otherwise, a qualitative risk assessment is done), again by applying an SAF to the POD, followed by quantitative exposure assessment and risk characterization as described above.
  - ✓ If no hazard identification test reporting that the substance does not have to be classified as a skin sensitizer is available, it has to be decided whether this is a data gap that needs to be filled by initiating a skin sensitization hazard identification study, taking into consideration physicochemical properties of the substance as well as information on use and exposure. Very low skin exposures might be evaluated using the dermal sensitization threshold approach that has been developed based on the TTC approach.

- The decision-tree on respiratory sensitization (Figure 6.2B) closely follows the schemes for sensitization and elicitation laid out above for skin sensitizers.
  - ✓ If the substance is a skin sensitizer or a protein-containing compound and no hazard identification test is available reporting that the substance does not have respiratory sensitization potential, it has to be decided whether a data gap exists that needs to be filled by initiating a study evaluating the possible respiratory sensitization potential, taking into consideration use and exposure information.

- In the decision-tree on systemic hypersensitivity (Figure 6.2C), it is assessed whether a substance may cause hypersensitivity reactions following oral or parenteral exposure.
  - ✓ If the substance can cause oral or parenteral sensitization/allergy, a qualitative risk assessment should be done by comparing conditions that have resulted in systemic sensitization with human exposure in order to identify uses and exposure scenarios that may pose a risk of systemic sensitization.
  - ✓ If the substance has uses with significant intentional or foreseeable oral or parenteral exposure, available information on toxic effects after repeated exposure in laboratory animals, human experience, data from in vitro studies and QSAR should be evaluated in order to decide whether a data gap exists that needs to be filled by further collection of information.

- Finally, after successively addressing skin sensitization, respiratory sensitization and/ or oral and parenteral hypersensitivity, it has to be decided whether the overall information is sufficient to stop the risk assessment for sensitization end-points or whether further information regarding sensitization potential or human exposure to the substance has to be collected. The evaluation might also purposefully be limited to only one route of sensitization, depending on the scope of the risk assessment.

6.4.2 Mode of action/mechanisms

Hypersensitivity reactions were originally divided into four types (Gell and Coombs Types I–IV) based on immunological mechanism (Murphy et al., 2008). Types I–III were transferable by serum from affected animals to a naive animal and thus are described as antibody mediated, whereas Type IV required the transfer of lymphocytes. It should be noted that a number of the disease states involve both cell-mediated and humoral components. For the purposes of this document, Types I and IV are the most relevant and will be described in more detail.

Type I hypersensitivity is mediated by antigen-specific cytophilic antibody (usually IgE) that binds to mast cells and basophils via Fc receptors. Some individuals have a genetic predisposition to develop IgE to common allergens (atopy). These individuals are more likely than the general population to develop allergic rhinitis and asthma in response to environmental allergens. The dose of antigen, route of exposure and local milieu influence the development of cytophilic antibodies. In a sensitized individual, upon subsequent exposure, the allergen binds to cytophilic antibodies on mast cells. Allergen cross-linking of the Fc receptor–bound antibody causes the release of preformed mediators such as histamine from the mast cell. In addition, immediate activation of arachidonate metabolism in the cell membranes occurs, and the generation of prostanoids (primarily prostaglandin D2) and peptidyl-leukotrienes ensues. These mast cell mediators are thought to be largely responsible for the acute symptoms of hypersensitivity seen when these reactions occur in the skin (urticaria), upper respiratory tract (allergic rhinitis or hay fever; congestion, itching, sneezing, cough) or lung (allergic asthma; bronchoconstriction). In the most severe form, a multisystem Type I hypersensitivity response (systemic anaphylaxis) can result in severe airway obstruction and cardiovascular collapse, leading to anaphylactic shock and potentially death. Type I hypersensitivity is also called immediate-type hypersensitivity because the initial reaction can occur within minutes after exposure of a previously sensitized individual to the offending antigen.

Type IV reactions are mediated by activated T cells rather than antibodies. Much has been learned about T cells since the four hypersensitivity classifications were originally proposed. As a result, the Type IV responses can now be divided into three subtypes, mediated by different populations of T cells: CD4+ Th1 and Th2 cells and CD8+ cells (Murphy et al., 2008). The CD4+ Th1 and Th2 cells recognize modified extracellular proteins presented in the context of MHC class II molecules and activate macrophages, which release a variety of cytokines and chemokines, leading to inflammation characterized by the influx of neutrophils. CD8+ T cells are cytotoxic and attack cells bearing modified intracellular proteins that are presented on the cell surface in the context of MHC class I molecules. Th1 and CD8+ reactions generally occur 24–48 hours after exposure in a previously sensitized individual and are thus referred to as DTH. Th2 cells (in addition to facilitating class switching to IgE) mobilize and activate eosinophils and mast cells. Mast cell activation results in production of Th2 cytokines, such as IL-4, IL-5 and IL-13.

As noted above, Type I hypersensitivity is the MOA associated with the most commonly studied food and drug allergies. Allergic contact dermatitis is a Type IV reaction involving several types of T cells. During the induction phase of this response, the chemical (hapten) couples to carrier proteins on dermal and epidermal cells to become fully immunogenic. The Langerhans cells take up and process the antigen and migrate to the regional draining lymph node, where they present the antigen to lymphocytes. Activation and rapid proliferation of lymph node cells ensue, resulting in the production of effector and memory T cells, which travel back to the skin. During the elicitation phase, effector Th1 and CD8+ cells are responsible for the erythema, oedema and pruritus that characteristically appear 24–72 hours post-exposure.

The application of hapten to the skin initiates a cascade of events. Epidermal keratinocytes secrete inflammatory cytokines (including IL-1β, IL-6, IL-12, TNF-α and granulocyte macrophage colony stimulating factor [GM-CSF]) that facilitate the maturation and mobilization of Langerhans cells, which also produce cytokines in an autocrine fashion. During Langerhans cell maturation, expression of cell surface molecules, including MHC class I and II and adhesion and costimulatory molecules, is enhanced, thus facilitating antigen presentation and subsequent T cell activation and clonal expansion. T cells activated in this manner express a skin homing receptor, cutaneous lymphocyte associated antigen. Although T cells are thought to be the key effector cells in the development of contact hypersensitivity, both T and B lymphocytes proliferate in response to contact sensitizers. Re-exposure to the relevant hapten triggers the same cytokine responses that occur following induction and elicits a response characterized by rapid recruitment and activation of specific T cells at the site of hapten challenge. Hapten-specific CD8+ T lymphocytes, likely the major effector population, are directly cytotoxic to chemical-exposed keratinocytes and also release cytokines that boost the inflammatory response. In addition, Th1 cells release a number of cytokines and chemokines that promote inflammation and activate mast cells and, in the presence of IFN, are also capable of killing keratinocytes. Although the hapten may persist in skin for some time, this reaction is self-limited. CD4+ regulatory T cells that secrete IL-10 appear to play an important role in this regulation.

Allergic asthma involves both Type I and Type IV responses. Sensitized individuals respond to antigen challenge with an immediate IgE-mediated Type I response. Between 2 and 8 hours after this event, a more severe and prolonged (late phase) reaction occurs, which is characterized by mucous secretion, bronchoconstriction, airway hyper-responsiveness to a variety of nonspecific stimuli (e.g. histamine, methacholine, cold air) and airway inflammation characterized by eosinophils. Late-phase responses may last up to 12 hours and do not appear to be mediated by IgE. Th2 cells and associated cytokines (particularly IL-5 and IL-13) and eosinophils are thought to play a significant role.

Protein allergens have been shown to produce sensitization and respiratory allergic responses (both early and late phase) in humans, guinea-pigs and mice. Additionally, they have been strongly associated with asthma morbidity. Of the low molecular weight chemicals that have been associated with allergic asthma, certain diisocyanates and acid anhydrides have received the most attention. Whereas protein allergens characteristically result in antigen-specific IgE, the presence of allergen-specific IgE in low molecular weight chemical respiratory allergy has not been universally demonstrated. In addition, the early-phase response does not always occur in individuals with occupational asthma triggered by these low molecular weight compounds. The mechanisms underlying low molecular weight chemical respiratory allergy are still under investigation.

Mechanisms underlying the induction of IgE have been studied extensively. There are two primary requirements for B cell immunoglobulin isotype class switching from IgM to IgE: 1) the presence of the Th2-associated cytokine IL-4 (or IL-13) and 2) direct cell to cell interaction via CD40 expressed on B cells and CD40 ligand (CD40L) expressed on T cells, basophils and mast cells. IL-4 exhibits autocrine activity in Th2 cell differentiation and promotes mast cell development. Bone marrow–derived immature mast cell precursors localize under the epithelium of mucosal areas (respiratory tract and gut) and the skin, where tissue-specific maturation and expansion occur. These mast cells contain preformed mediators and are capable of producing other effecter molecules, including IL-4 and IL-5. The IgE-armed mast cells in the respiratory tract are then set for elicitation of antigen-specific allergic events. Upon subsequent exposure, the specific allergen crosslinks the mast cell–bound IgE, resulting in the release of the preformed mediators and newly synthesized substances. Chief among the preformed mediators is histamine, which acts through receptor ligation to cause increased vascular permeability, smooth muscle contraction, vascular constriction and mucus production. In addition to histamine, there are a variety of preformed cellular chemotactic factors and enzymes. Products from the activation of two metabolic pathways of membrane-derived arachidonic acid, the lipoxygenase and cyclooxygenase pathways, provide other important mediators of allergic inflammation. The Th2-associated cytokine IL-5, secreted by mast cells, has been shown to be essential in proliferation and maturation of eosinophil precursors as well as viability and eosinophil granule protein release and chemotaxis. Eosinophils that are recruited into the lung release their own set of mediators, which are thought to be important in late-phase responses. Furthermore, cytokines (IL-5, IL-3 and GM-CSF) secreted by activated eosinophils found at the site of allergic inflammation may result in a positive feedback loop.

6.5 Uncertainty factors

The uncertainty factor approach can, in general, be applied to the POD in order to derive AELs for sensitizing substances. Levels lower than the derived AELs with regard to sensitization and elicitation are then considered without appreciable risk of sensitization of non-sensitized subjects and elicitation of allergic reactions in already sensitized subjects, respectively. The acceptability or unacceptability of the real-life exposure situation with respect to sensitization induction or allergy elicitation can then be determined accordingly (see following sections). Individual uncertainty factors that account for interspecies and intraspecies variability and, if necessary, a matrix factor and a factor for use and prolonged exposure are combined by multiplication into a total SAF. Below, the factors will be discussed individually, one after the other. It should, however, be noted that some parameters, such as vehicle effects and skin barrier, may be discussed in the derivation of more than one of these factors. Therefore, it should be ensured that the total SAF is adequate when combining the individual factors.

6.5.1 Interspecies uncertainty factor

An interspecies uncertainty factor is generally used for extrapolating results from laboratory animals to humans. An interspecies uncertainty factor of 1 is applied when the POD is derived from human data—for example, HRIPT results for induction of skin sensitization, epidemiological data for induction of respiratory sensitization and elicitation studies in humans with contact dermatitis, respiratory allergy or oral allergy.

With regard to the induction of skin sensitization, much work has been done to correlate the dose–response data obtained in the mouse LLNA with what is known about sensitizing potency in humans. The LLNA EC_3 value has been found to closely correlate with the NOEL from human sensitization tests when the skin area dose (μg/cm^2) is used as the dose metric for both (Basketter et al., 2000, 2005a; Gerberick et al., 2001a,b, 2004; Griem et al., 2003; Schneider & Akkan, 2004). For the variability in test outcomes between humans and laboratory animals, it is not possible to distinguish true interspecies differences from experimental influences, such as dose intervals and vehicle effects. Interspecies differences may, for example, be related to differences in skin penetration and metabolism. With regard to skin penetration, use of rodent data is considered conservative, because mice and rats tend to show a considerably higher skin penetration for chemicals compared with humans (a 3- to 10-fold higher penetration is often reported) (Barber et al., 1992; Boogaard et al., 2000). Metabolism may have limited relevance for the variability between humans and mice, because only local metabolism is relevant. Systemic toxicokinetic differences (e.g. allometric differences) are not considered to play an important role in skin sensitization. In line with this is the observation that sensitizing chemicals that require metabolism into "ultimate sensitizers" do not show a larger variability in terms of human NOEL and LLNA EC_3 values than do chemicals that do not need to be metabolized (Griem et al., 2003). Based on these analyses, an interspecies uncertainty factor of 3 has been proposed when the LLNA, in the absence of supporting human data, is used to derive a POD for quantitative hazard characterization (Griem et al., 2003).

When assessing inhalation toxicity, the toxicokinetic component of the interspecies uncertainty factor is usually set to 1 when exposure concentrations in air are compared between laboratory animals and humans, because respiratory parameters for the different species will implicitly contain allometric differences. For local effects on the respiratory tract, the toxicodynamic component of the interspecies uncertainty factor is also often reduced to 1. In the case of toluene diisocyanate, the same NOEC was identified from workplace epidemiological studies and from a subchronic study in guinea-pigs. The observation that for skin sensitizers the skin area doses of human NOELs and LLNA EC_3s were very similar supports the view that the underlying immunological mechanisms are comparable in qualitative and quantitative terms between laboratory animals and humans and that this could also be true for respiratory sensitization. However, too few data are available to draw general conclusions on the interspecies ratios of NOECs (or LOECs) for respiratory sensitization.

With regard to aerosols and dusts, the external exposure concentration should be corrected in order to take into account particle size limits that may be inhalable or respirable, respectively, in laboratory animals compared with humans. Further, the application of computational deposition models may sometimes support species comparison.

A weight of evidence evaluation assessing chemical-specific and other relevant information is recommended to decide on the appropriate value for the interspecies uncertainty factor.

6.5.2 Intraspecies uncertainty factor

This uncertainty factor accounts for possible variations in the sensitivity between individuals due to factors such as genetic effects, inherent barrier function, age, sex and ethnicity. For skin sensitization, some information on these contributing factors is available and is discussed below (see Felter et al., 2002; Griem et al., 2003; Api et al., 2008). Much less information is available for respiratory sensitization, and virtually nothing has been published regarding oral and parenteral sensitization. It should be noted that the following influencing

factors are usually not considered one by one, but are combined in one intraspecies uncertainty factor.

6.5.2.1 Genetic effects

With regard to skin sensitization, genetic factors, although not completely understood, are clearly relevant in determining individual susceptibility (Felter et al., 2002). It is well established that skin enzymes, predominantly located in the epidermis, can metabolize absorbed xenobiotics via reactions analogous to those determined in the liver (Smith & Hotchkiss, 2001). Thus, genetically determined differences in metabolic capabilities might be expected to influence an individual's susceptibility to the induction of allergic contact dermatitis.

Smith et al. (2000) proposed that one of the reasons for differences in individual responses to the same exposure to an allergen may be related to their susceptibility to skin irritation, such that those in whom the epidermal irritant response reaches a sufficient threshold level are more likely to be sensitized. Differences in individual susceptibility may also influence the magnitude of response by affecting other steps in the process of inducing (or eliciting) an allergic response.

Regarding the role of human leukocyte antigen (HLA) (the name for the human MHC genes) class II molecules in the development of occupational respiratory tract sensitization and asthma, particularly to low molecular weight agents, it was shown that the DQB1*0501 allele is a genetic risk factor for asthma induced by low molecular weight sensitizers such as organic acid anhydrides (Jones et al., 2004). The same allele is relevant for asthma induced by isocyanates (Mapp et al., 2000) and plicatic acid, where it has a protective role (Horne et al., 2000), suggesting various affinities of chemical sensitizers for the corresponding specific HLA class II molecules.

In the epidemiological study in bakery workers conducted by Heederik & Houba (2001), the prevalence rates of wheat allergy in atopics were about twice as high as in non-atopics over the whole exposure range.

6.5.2.2 Inherent barrier function

The inherent barrier function of the skin is one factor likely to affect individual susceptibility. The initial step in the induction and elicitation of allergic contact dermatitis requires that the allergen penetrate the stratum corneum. For example, nickel-allergic individuals showed about 10-fold lower elicitation thresholds when patch testing was performed on skin that showed slight inflammatory changes and dryness due to repeated contact with detergent solution (Allenby & Basketter, 1993, 1994).

Dermal sensitization risk assessments will usually be conducted on healthy skin and not on diseased skin. Individuals with diseased skin (e.g. psoriasis and eczema) may, on the one hand, show a considerably higher permeation of xenobiotics through the affected skin areas; on the other hand, their skin exposure may be restricted, as it can be assumed that many of these individuals are under the care of a dermatologist. In addition, age, ethnicity and sex may have an influence on inherent barrier function in healthy skin.

6.5.2.3 Sex

Although there is some indication that females are the more reactive responder population to contact allergens (Jordan & King, 1977; Rees et al., 1989), the weight of evidence suggests that females and males react similarly (Robinson, 1999; Felter et al., 2002). Those differences that have been noted in the general population for percentages of men and women with specific contact allergies can often be attributed to use and exposure differences; for example, women are more prone to contact allergies to nickel (from jewellery, especially pierced ears), whereas men are more likely to be allergic to chromium as a result of occupational exposures (Young et al., 1988).

6.5.2.4 Ethnicity

The weight of evidence indicates that there is no substantial difference in susceptibility to induction of contact allergy among individuals of different ethnic origins. In a study of induction by five common skin allergens, Kligman (1966) reported little difference in the response between individuals with highly pigmented skin and Caucasians for the strongest allergens. With less potent allergens, Kligman (1966) found that those with highly pigmented skin were increasingly resistant to the induction of sensitization compared with the response in Caucasians. Some controversy remains as to the sensitivity of Asians relative to Caucasians. In regard to percutaneous absorption of chemicals into the skin, highly pigmented skin is generally considered to be somewhat more impervious than Caucasian skin (Weigand et al., 1974).

6.5.2.5 Age

The general susceptibilities of infants and adults to contact allergens are essentially equal (Cassimos et al., 1980). The structural and functional skin barrier properties are equal from full-term infancy to late adulthood (Cunico et al., 1977; Wilson & Maibach, 1980; West et al., 1981; Fairley & Rasmussen, 1983; Harpin & Rutter, 1983). This is in terms of epidermal thickness, density of epidermal cell layers, cellular structure, functional stratum corneum and mature skin barrier function. Therefore, no adjustment of uncertainty factors for age is currently considered necessary.

6.5.2.6 Summary

In summary, several of the points discussed above argue that interindividual variability exists and thus should be covered by an intraspecies factor. For elicitation, it should also be considered that the POD used for risk assessment is already based on the occurrence of effects in the most susceptible individuals. Using a POD determined in the most susceptible subpopulation could argue for a reduced intraspecies factor.

Values that have been used in risk assessments of sensitizers are, for example, an intraspecies factor of 10 for dermal induction (Felter et al., 2002; Basketter et al., 2003; Griem et al., 2003; Api et al., 2008), factors of 3 and 10 for respiratory induction (OEHHA, 2000, 2001; NCDENR, undated) and a factor of 1 for elicitation of contact allergy (Nethercott et al., 1994; Griem et al., 2003).

A weight of evidence evaluation assessing chemical-specific and other relevant information is recommended to decide on the appropriate value for the intraspecies uncertainty factor.

6.5.3 Matrix factor

Besides the uncertainty factors routinely used in toxicological risk assessment, the use of additional factors has been proposed in some instances to reflect special circumstances with regard to sensitization. A matrix factor is sometimes applied when exposure to a sensitizer is done in pure form or in a simple vehicle in the experimental situation, whereas in the use scenarios to be assessed, the sensitizer is contained in a complex matrix that may alter the toxicokinetic behaviour or may itself promote sensitizing effects.

The matrix factor concept was originally developed for cosmetic safety assessment (Felter et al., 2002; Api et al., 2008; Basketter et al., 2008). Here, the consumer can be exposed to sensitizing ingredients in many different product forms (e.g. cream, shower gel, eau de toilette) that show varying complexity, ranging from a simple ethanol to multiphase creams. In the experimental situation, exposure to sensitizers is typically in a simple vehicle. In contrast, some consumer product formulations not only contain an inert vehicle, besides the sensitizer in question, but also may contain ingredients that are irritants or penetration enhancers. Therefore, the effect of a complex formulation/matrix on the bioavailability of a sensitizer may be substantially different from that of a simple vehicle (Felter et al., 2002).

A case-by-case evaluation has to be done to decide if the use of a matrix factor is suitable for the risk assessment that is being developed.

6.5.3.1 Irritants

Dermal irritants are known to compromise the skin barrier (Robinson et al., 2000). They are also known to serve as a promoter of dermal sensitization (Smith et al., 2000). It is apparent that some degree of direct chemical inflammation or other concurrent trauma enhances the keratinocyte activity, produced by the applied chemical itself, by some other component of the chemical delivery system or by some form of physical insult. This may account for the noted enhancing effect of primary skin irritation on the sensitization response (Kligman, 1966; Cumberbatch et al., 1993).

6.5.3.2 Penetration enhancers

Some chemicals are specifically known to affect the penetration of other chemicals through the stratum corneum (Scheuplein & Ross, 1970; Schaefer & Redelmeier, 1996). As such, it remains important to understand the experimental matrix/vehicle as to its effect on the penetration of a sensitizer, as it will affect the bioavailability of the material in the experimental situation. Typically, however, there is very little information available about the bioavailability of a sensitizer in either the experimental situation or real-life scenario.

For most cosmetic products, a matrix factor of 3 was proposed when a NOEL from an HRIPT was used for hazard characterization (Api et al., 2008).

Regarding inhalation, the terms matrix and vehicle might not be well chosen; however, it is known that both sensitization and allergy elicitation can be influenced by concomitant exposure to substances causing respiratory tract irritation and/or activation of alveolar cells. The adjuvant effect of irritant gases and particles is most likely caused by the activation of epithelial cells, pneumocytes, alveolar macrophages and dendritic cells. Alteration of the bioavailability of the sensitizing compound, as discussed above for the skin, probably does not

play a prominent role in the respiratory tract, although it has also been discussed in the literature that sorption of sensitizers to inhalable particles can influence their deposition in the respiratory tract and their toxicokinetic behaviour.

Significant increases in ovalbumin-specific IgE, IgG and IgA antibody titres were seen in rats after weekly exposure to ovalbumin (18 mg/m^3 for 0.5 hour) with additional nitrogen dioxide exposure (164 mg/m^3 for 1 hour either 1 day before or immediately after the ovalbumin exposure) compared with rats exposed only to ovalbumin. In contrast, ammonia did not influence antibody titres, although it caused irritant effects (Siegel et al., 1997). Increased IgE levels in response to ovalbumin in serum of mice were also found after co-exposure to ovalbumin (intranasal sensitization on days 0 and 14 and challenge on days 35, 38 and 41 with 50 µl of a 0.4 mg/ml ovalbumin solution) and diesel exhaust particles (3 mg/ml, mixed with ovalbumin) (Steerenberg et al., 2003). Epidemiological studies have also suggested a correlation between traffic exhaust particle exposure and the prevalence of asthma and/or sensitization to common allergens, such as pollen and house dust mites (Janssen et al., 2003). However, it is currently unclear whether all respiratory irritants exert this effect or to what extent.

6.5.4 Use and time factor

Besides the uncertainty factors routinely used in toxicological risk assessment, the use of additional factors has been proposed in some instances to reflect special circumstances with regard to sensitization. A use and time factor is sometimes applied when the real-life scenario differs from the experimental situation with regard to skin site location, skin barrier integrity, occlusion and frequency of exposure.

The use factor concept was originally developed for cosmetic safety assessment (for review, see Api et al., 2008). Whereas use conditions in experiments are well defined and controlled (e.g. site of contact, skin integrity, operator controlled, number and duration of exposures), use conditions in real-life scenarios in almost all cases involve less exaggerated exposure, are more variable and are within consumers' control. The key parameters to consider when extrapolating from the controlled experimental situation to the real-life scenario are site of contact, barrier integrity, occlusion and frequency of exposure.

A case-by-case evaluation has to be done to decide whether the use of a use and time factor is suitable for the risk assessment that is being developed.

6.5.4.1 Site of contact

Regional differences in dermal absorption can be substantial. For example, Feldmann & Maibach (1967) measured the relative regional permeability of human skin from various body sites to ^{14}C-labelled hydrocortisone. Of 11 sites evaluated, the skin of the back (where most patch studies are conducted) was intermediate in relative permeability. The plantar foot arch was correspondingly about 12-fold less permeable than the skin of the back, whereas the scalp and axillae were about 2-fold more permeable and the forehead was about 3-fold more permeable. As the permeability on the back and the arm (the sites of contact for most human experimental sensitization tests) is intermediate, it is not unlikely that some products containing a skin sensitizer will have contact with body sites that may be significantly more permeable.

6.5.4.2 Barrier integrity

As mentioned in the discussion of the intraspecies uncertainty factor, barrier integrity can be inherent, but it can also be influenced by use practices. Factors influencing dermal integrity are known to have a significant effect on dermal penetration. This might include, for example, dermatitis in an adult (Benfeldt et al., 1999), the presence of diaper rash in infants (Odio & Friedlander, 2000) and, although less dramatic, shaving (Edman, 1994).

6.5.4.3 Occlusion

Occlusion of the skin results in multiple effects, including increases in the hydration of the stratum corneum, skin temperature, microbial count, pH and dermal irritation. The increase in hydration state, in particular, has been associated with increased dermal penetration, although occlusion does not increase the absorption of all chemicals, and the relative effect of occlusion is likely to be dependent on the lipophilicity of the chemical (Zhai & Maibach, 2001).

For many cosmetic products, a use factor of either 3 or 10 was proposed when a NOEL from an HRIPT was used for hazard characterization (Api et al., 2008).

6.5.4.4 Frequency of exposure (time extrapolation)

Whereas induction and elicitation are usually determined after one exposure (e.g. for elicitation in patch test or experimental animal study) or a few exposures (e.g. for elicitation in ROAT, for induction in LLNA [3 times] and HRIPT [9 times]) in experimental situations, the question arises as to whether a lower dose would suffice for sensitization if repeated exposures over a longer time occurred. In this context, a few publications on so-called subclinical skin sensitization might be relevant. Ford et al. (1988) reported on an HRIPT using hydroxycitronellal in which groups of 66 subjects each were treated with 4200, 8400 or 12 600 $\mu g/cm^2$ during induction. One subject each of the two highest exposure groups showed a positive challenge reaction. After 6 months, 100 of the panelists who had completed the first HRIPT took part in a second HRIPT with hydroxycitronellal. During the first and second weeks of the induction phase of the second HRIPT, 29% of the subjects showed signs of allergic contact dermatitis. This result indicates that, at least for hydroxycitronellal, detectable sensitization needs longer to develop than the time between induction phase and challenge in the HRIPT (10–14 days). It is unknown whether this phenomenon occurs only at small area doses (i.e. those just beneath the sensitization threshold), whether it occurs with most or only a few sensitizing chemicals and which mechanism is involved (e.g. slow release of sensitizer initially bound to the upper skin layer [stratum corneum]). Similar observations have also been made with 2,4-dinitrochlorobenzene (Friedmann et al., 1990). In addition, Vandenberg & Epstein (1963) performed a sensitization test with nickel chloride and found that in a first sensitization test, 16 of 172 (9%) previously non-nickel-allergic subjects were sensitized, whereas upon repetition of the sensitization test 4 months later with 19 subjects who had shown a negative result in the first challenge test, 5 (19%) were successfully sensitized. Although it is currently difficult to describe this phenomenon quantitatively owing to the limited data available, it has been proposed to take time extrapolation into consideration to account for a possibly higher sensitizing chemical upon continued or repeated exposure (Griem et al., 2003).

6.5.5 Database uncertainty factor

In some cases, a limited data set may suggest the possibility of allergic effects without appropriate data to make a determination of the sensitization risk or perform a dose–response assessment. In such cases, use of a database uncertainty factor may be considered to indicate that the lack of sufficient information on this end-point may be significant. As internationally accepted test guidelines for skin sensitization hazard are available and test results for skin sensitization are in most cases available, the mentioned data gaps will more often relate to respiratory and/or oral sensitization for which sometimes anecdotal evidence may exist and which, owing to the lack of internationally accepted test guidelines, cannot be addressed in a straightforward manner.

Sometimes, for the lack of better suited studies, a POD for quantitative risk assessment has to be derived from a study in which confidence is considered low. In these cases, the database uncertainty factor may be used to address the use of a low-confidence study in the derivation of AELs. On a case-by-case basis, a justification for the application of an uncertainty factor should be provided to address database deficiency. For further discussion of the database uncertainty factor, the reader is referred to the discussion in chapter 3.

6.6 Groups at risk (developing immune system, elderly, immunocompromised)

Much information relevant under this heading has already been summarized in the discussion on the intraspecies uncertainty factor (see section 6.5.2) and will not be repeated here.

With regard to skin sensitization, several studies address the importance of including subpopulations, such as those with multiple allergies, who may be more susceptible (Friedmann & Moss, 1985; Moss et al., 1985; Felter et al., 2002). However, the differences in terms of thresholds for induction and elicitation seem to be well below 1 order of magnitude.

Similar to skin sensitization, existing respiratory sensitization (e.g. allergic asthma) seems to exert some limited influence on the risk of developing respiratory allergy to additional respiratory sensitizers. In their review, Mapp et al. (2005) wrote that atopy (skin reactivity to common inhalants) is a predisposing factor in workers exposed to high molecular weight agents, but it is a weak predictor of sensitization and development of occupational asthma. Atopy is not a risk factor for asthma induced by low molecular weight agents such as western red cedar or diisocyanates. For exposure to high molecular weight work-related allergens, subjects with new occupational sensitization are at greater risk of developing sensitization to common aeroallergens than are subjects without sensitization. However, after removal or diminution of exposure to both low and high molecular weight agents causing occupational asthma, subjects are not at increased risk for developing IgE-mediated sensitization to common allergens, indicating that atopic status does not increase even years after the diagnosis of occupational asthma.

There is no compelling evidence to suggest that the elderly are more susceptible to the induction and elicitation of skin, respiratory or systemic sensitization (Bakos et al., 2006). In general and as discussed above, there are no indications that children are much more susceptible to developing allergies. However, it is difficult to evaluate this point because, once acquired, allergies tend to persist throughout life. Therefore, higher incidence rates for skin and respiratory allergies during childhood do not necessarily indicate a higher susceptibility. The

question as to whether a susceptible person would be less likely to get a sensitization if not exposed before adulthood cannot be answered from the currently available data. Currently, no adjustment of uncertainty factors for age is considered necessary.

6.7 Derivation of acceptable exposure level

The total SAF is a combination of the uncertainty and other factors defined in section 6.5 above and is calculated by multiplying the interspecies, intraspecies, matrix and use and time factors. As discussed above, this approach is currently considered applicable for skin and respiratory sensitization. Dividing the exposure level that was defined as the POD for risk assessment by the total SAF will result in a daily exposure dose at which it is considered unlikely that induction of sensitization or elicitation of allergic reactions will occur, or the AEL. The AEL is also termed the "acceptable non-sensitizing dose/concentration" or "acceptable non-eliciting dose/concentration".

Another approach that has been applied to sensitizing detergent enzymes is to use relative potency data from experimental tests to derive safe levels relative to a well-characterized reference allergen. Induction potency information, including thresholds, for detergent enzymes may be determined in guinea-pigs and mice (Kawabata et al., 1996; Sarlo et al., 1997; Robinson et al., 1998). Subtilisin (trade name Alcalase) was chosen as the reference allergen because the American Conference of Governmental and Industrial Hygienists developed a threshold limit value in the workplace for subtilisin A of 60 ng of protein per cubic metre based on historical human data. The dose–response relationships of new enzymes were compared with that of subtilisin and used to determine the potency factor difference between subtilisin and the new enzymes. For less potent and equivalent enzymes, the occupational exposure guidelines used for subtilisin were assumed to be safe for the new enzyme. For more potent enzymes, the occupational exposure guideline was lowered according to the potency factor derived by comparing the two dose–response curves. Workers exposed to new enzymes at these levels were monitored for new sensitization via annual or semiannual SPTs, and results were similar to that observed for subtilisin (i.e. no more than 0–3% new sensitizations per year, and, as with subtilisin, allergic symptoms were not observed; Sarlo et al., 1997).

6.8 Exposure assessment

Exposure assessment comprises the qualitative and quantitative description of the contact of an individual with a chemical for specific durations of time (IPCS, 2009). For exposure to occur, an individual must be present and must come into contact with the pure chemical or the medium containing the chemical. Exposure usually results in absorbed dose when chemicals enter the body. Exposure is described in terms of the intensity (concentration), frequency and duration of contact (USEPA, 1992).

General methods for experimental measurement of exposure as well as for modelling exposure using computational models and for describing exposure have been reviewed elsewhere (USEPA, 1992; IPCS, 2006b, 2009; ECHA, 2010a,b). Further information on aggregate exposure can be found in USEPA (2001). It is beyond the scope of this guidance document to summarize the complexity of and various approaches to exposure assessment or even to provide detailed guidance on how exposure assessment should be done and documented in the context of sensitization risk assessment. When characterizing exposure to sensitizing chemicals, the relevant route (dermal, inhalation or systemic, or combinations of these),

general use, exposure context (e.g. single, intermittent, daily, continuous), regulatory frame-work (e.g. workplace, cosmetics, household products, biocides, artists' paints, medicinal products) and exposed (sub)population will influence the selection of experimental and computational tools used to generate numerical exposure descriptors and the format in which these are documented.

The case-study on citral (see Case-study 4) provides an example for an abbreviated exposure assessment for skin sensitizers in cosmetic and household products based largely on the pro-posed method for sensitizing fragrance ingredients by the International Fragrance Association (IFRA)/Research Institute for Fragrance Materials (RIFM). The case-study on soluble platinum salts (see Case-study 3) briefly discusses the occupational inhalation exposure to this group of respiratory sensitizers.

As discussed above in section 6.3, the most adequate dose metric for describing exposure to skin sensitizing substances is the daily substance dose per area of skin (µg/cm^2 per day). This represents the aggregate exposure, defined as the total area dose of a chemical to one skin site from different sources over 1 day (cf. Cowan-Ellsberry & Robinson, 2009). It should be noted that this represents a pragmatic approach because, although aggregating exposure from different sources/products contacted within a short period of time is certainly required, there are at present no data available that would allow defining the adequate length of the time period. This uncertainty, however, is covered by applying the use and time factor.

Exposure to respiratory sensitizing substances can be described as a TWA concentration (mg/m^3 during a day or an 8-hour workshift); however, sometimes peak exposure concentra-tions or aggregate exposure values may also be useful exposure metrics. Although there is some uncertainty as to which methods should be used to determine dose–response relation-ships for systemic hypersensitivity reactions, currently reporting the exposure as a body weight–related dose seems adequate until more information suggesting a better alternative is available.

Using a deterministic approach in exposure assessment means that point estimates, such as a maximum (or average) use concentration and a maximum (average) exposure dose, are used. Therefore, the result reflects a "high end", "upper bound" or "worst case" (or a "central tendency" when average values are used). In contrast to the deterministic approach, a prob-abilistic approach uses the full range of the data and produces a distribution of values as an output. Depending on the method and the availability, either all or at least several input parameters are given as probability distributions. From the input parameters, a probability distribution for the exposure of interest is calculated using specialized computer software. Often a population-based distribution is calculated from which the 50th, 90th and 99th per-centiles can be reported. Although no examples of probabilistic exposure assessment for a sensitizing chemical have been published so far, the approach has successfully been used for food allergens, such as peanut protein (Crevel et al., 2007, 2008; Spanjersberg et al., 2007; Kruizinga et al., 2008).

6.9 Risk characterization

Risk characterization is the integration of information from hazard identification, dose–response assessment and exposure assessment into a coherent picture. According to IPCS (1999a):

> Risk characterization aims to provide a synthesis of estimates of exposure levels and health risks; it also summarizes sources of uncertainty in scientific data [(regarding both hazard characterization and exposure characterization), indicates the confidence in the risk assessment conclusions, suggests what additional data would be necessary to strengthen the risk assessment] and provides the primary basis for making risk management decisions [e.g. on personal protective measures at the workplace]. The results of a risk assessment (as summarized in the [risk] characterization) are the basis of identification of chemical exposures that pose no significant health threat and those that present significant risks. Additionally, to the extent permitted by available data, risk characterization indicates how risk varies with exposure, [describes the number of people exposed and whether levels are of public health concern, and identifies susceptible subpopulations,] to help risk managers evaluate a range of options. It assists risk management officials and decision makers in allocating scarce resources and money to the most important resolvable uncertainties and reduction of risks.

The evaluation of the real-life exposure situation with respect to sensitization induction or allergy elicitation can then be determined accordingly. To this end, the POD for risk assessment (for either induction or elicitation), expressed as area dose, is divided by the total SAF to derive an AEL. An estimated/determined exposure level, expressed as area dose, below this AEL is then considered without appreciable risk of, respectively, sensitization of non-sensitized subjects and elicitation of allergic symptoms in already sensitized subjects (see also section 6.7).

For induction and elicitation of skin sensitization and respiratory sensitization against chemicals, a quantitative risk characterization currently seems feasible in cases for which adequate hazard characterization and exposure data can be provided. This will more often be the case for skin sensitizers. Otherwise, and in most cases of systemic hypersensitivity reactions, only a qualitative (or semiquantitative) risk characterization can be done. Then, uses and exposure information leading to or posing a risk of systemic sensitization and/or systemic allergy elicitation can be described.

Instead of a deterministic risk assessment approach, a probabilistic risk characterization approach has been proposed for food allergy and has been successfully applied in some cases (Spanjersberg et al., 2007; Kruizinga et al., 2008; Madsen et al., 2009). The model predicts the most likely number of allergic reactions that might result from the accidental presence of an allergenic constituent in a food product. This calculation factors in a statistical distribution of the minimum eliciting dose in the sensitized subpopulation, also including the proportion of the population that is allergic, and statistical distribution data for variables determining the intake of the allergenic constituent (presence and concentration in foods, likelihood that an allergic person consumes the food and amount of the food consumed per eating occasion).

The probabilistic risk assessment approach may also be applied to predict the likelihood of allergic skin or allergic respiratory tract reactions in the general population and may constitute an interesting instrument for risk characterization in the future.

As mentioned above, the risk characterization serves to inform risk management decisions. Comparison of an exposure with the derived AEL informs whether or not a risk of an immune reaction is likely for the exposure scenario evaluated. It should be noted, however, that this does not constitute a conclusion on whether the exposure situation itself is acceptable or unacceptable; that is, even if a risk cannot be ruled out, it does not mean that the use of the chemical has to be limited or banned by regulatory measures. Especially for elicitation, the risk is relevant only for the subgroup of already sensitized individuals and not for the general

population. Here, it is generally more useful for affected individuals to avoid contact with the chemical. This can be achieved by, for example, avoidance of contact with the chemical at the workplace or adequate product labelling of food and cosmetic products. For the latter, the elicitation risk assessment may be useful to define elicitation-based threshold concentrations for product labelling.

Examples of risk assessments, including risk characterization, are given in the case-studies on halogenated platinum salts (see Case-study 3) and the fragrance ingredient citral (see Case-study 4) at the end of this guidance document.

7. ASSESSMENT OF AUTOIMMUNITY AND AUTOIMMUNE DISEASE

7.1 Introduction

Autoimmunity and autoimmune diseases result from immune responses against self-molecules. The immunological effectors and mechanisms involved in autoimmune reactions are the same as those associated with responses to foreign antigens, including activation of the innate and adaptive immune systems, production of inflammatory mediators and activation of T lymphocytes or the generation of antibodies with specificity for self-antigens. Thus, chemicals that induce immunosuppression (e.g. mercury(II) chloride) or hypersensitivity (e.g. antibiotics) may also have an impact on autoimmunity. In many instances, the events that initiate the immune response to self are unknown; however, intrinsic factors (e.g. specific gene polymorphisms, sex-related hormones and age) and extrinsic factors (e.g. lifestyle, exposures to certain drugs, chemicals and infectious agents) have been shown to be associated with the induction, development or exacerbation of autoimmunity.

7.2 Hazard identification

A large number of chemicals and therapeutic agents have been identified through epidemiological or laboratory studies as potential triggers for expression of autoimmunity and have been suggested to both induce onset and modulate the severity of autoimmune disease (Table 7.1) (IPCS, 2006a; Rose & Mackay, 2006). However, it is important to note that spontaneous autoimmune disease may occur in the absence of specific chemical exposures and that the strongest associations of predisposing factors for autoimmune diseases are with specific genetic loci. Hazard assessment for the potential to modulate autoimmunity (as with other alterations in immune function) will likely best be accomplished using a tiered approach and utilization of multiple methods. Occupational epidemiological studies often provide the best opportunity for identifying chemical-induced modulation of the immune system in human populations, as exposure levels tend to be higher than those found outside the workplace. Work-related exposures to compounds such as crystalline silica, heavy metals and solvents have been associated with a number of systemic autoimmune diseases. Individuals with high-level exposures to silica-containing mineral dusts have been shown to demonstrate elevated risk for a number of systemic autoimmune diseases, including rheumatoid arthritis, scleroderma antineutrophil cytoplasmic antibody–related vasculitis and systemic lupus erythematosus. Exposures to tobacco smoke and iron particles have been shown to modify disease incidence and severity in workers exposed to silica, stressing the need to identify potentially hazardous co-exposures to accurately assess the risk for development of disease.

Vinyl chloride has been linked to the development of a scleroderma-like disease characterized by skin thickening, Raynaud phenomenon, acro-osteolysis (shortening of the terminal digital phalanges due to bone resorption) and pulmonary involvement. The linkage between vinyl chloride and autoimmunity stimulated research into associations between systemic autoimmune diseases and other solvents (e.g. trichloroethylene, trichloroethane and xylenes), predominantly in occupational settings. An increased risk for systemic sclerosis was reported in several studies, but the risk is not consistent for all systemic autoimmune diseases (reviewed in IPCS, 2006a).

Table 7.1: Environmental exposures and therapeutics associated with autoimmunity.[a]

Autoimmune syndrome	*Compound*
Haemolytic anaemia	Chlorpromazine
	α-Methyldopa
	Penicillins
	Sulfa drugs
Hepatitis	Ethanol
	Halothane
	IFN-α
	Lipid-lowering drugs
Myositis	Estrogens
	L-Tryptophan
	Ultraviolet radiation
Rheumatoid arthritis	IFN-γ
	Organochlorine pesticides
	PCBs
	Quinidine
	Silica
	Tetracyclines
	Tobacco smoke
Scleroderma/systemic sclerosis	Diphenylhydantoin
	IL-2
	Silicone
	Spanish toxic oil
	Trichloroethylene
	Tryptophan
	Vinyl chloride
Systemic lupus erythematosus or lupus-like syndrome	Aromatic amines
	Chlorpromazine
	Formaldehyde
	Hydralazine
	IFN-γ
	Isoniazid
	Procanimide
	Silica
	Trichloroethylene
Thrombocytopenia	IFN-α
	Iodine
	Quinidine
	Rifampicin
Thyroiditis	Iodine
	Lithium
	PCBs, PBBs

Table 7.1 (continued)

Autoimmune syndrome	*Compound*
Vasculitis	Allopurinol
	Silica
	Tetracyclines

PBBs, polybrominated biphenyls

[a] Adapted from Miller (2006). This is not intended to be a comprehensive review, but is meant to provide illustrative examples of the types of compounds associated with autoimmunity.

7.3 Hazard characterization

A basic understanding of the typical methodologies used to evaluate the induction or exacerbation of autoimmunity in animal models is necessary to evaluate the database of studies for hazard characterization of a given chemical as the first step in risk assessment. Detailed discussions of end-points and methods utilized in characterizing autoimmunity are provided in EHC 236: *Principles and methods for assessing autoimmunity associated with exposure to chemicals* (IPCS, 2006a). The data set for most chemicals is unlikely to contain data on more than one or two animal models of autoimmune disease. The risk assessor should refer to chapter 3 for guidance on conducting risk assessments when the immune system is a proposed target and to the assay descriptions in EHC 236 (IPCS, 2006a) for end-points contained in the data set for the chemical in question to provide specific context, cautions and information that may assist in the interpretation of autoimmunity data for risk assessment.

7.4 Clinical and epidemiological data

Autoimmune disorders can affect virtually any site in the body and present as a spectrum of diseases ranging from organ specific, in which antibodies and T cells react to self-antigens localized in a specific tissue, to systemic, characterized by reactivity against a specific antigen or antigens present in various tissues. Recent estimates suggest that 3–5% of the general population suffers from autoimmune diseases, and there is epidemiological evidence that the prevalence of certain autoimmune diseases is increasing in industrialized countries (reviewed in IPCS, 2006a; Cooper et al., 2009). In addition, there is evidence that a number of common health problems, such as atherosclerosis, inflammatory bowel disease and aspects of male and female infertility, may have an autoimmune component. Women have a significantly higher risk of developing an autoimmune disease compared with men, and in a significant number of the most common autoimmune diseases (thyroiditis, scleroderma, systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis), a female predominance is observed. However, for some autoimmune diseases, such as ankylosing spondylitis and adult-onset diabetes, there appears to be a higher risk among men.

Lifestyle factors such as diet, smoking, therapeutic and recreational drug use, infection with certain bacteria and viruses, and exposure to ultraviolet radiation and environmental chemicals have all been implicated in the pathogenesis of autoimmune diseases (Heindel et al., 1999; IPCS, 2006a). Tobacco use demonstrates the complexity of potential interactions between environmental factors with regard to autoimmune disease. Smoking has been confirmed as a risk factor for Graves hyperthyroidism and the development and prognosis of Graves ophthalmopathy (Vestergaard, 2002). However, several studies have demonstrated that tobacco use is associated with a reduced prevalence of thyroid peroxidase antibodies and

elevated levels of thyroid stimulating hormone, suggesting that smokers may be protected against certain autoimmune thyroid disorders, such as Hashimoto thyroiditis (Vestergaard et al., 2002; Belin et al., 2004). Similarly, an inverse association has been observed between tobacco use and the risk of ulcerative colitis (Loftus, 2004), whereas a number of studies have shown an increased risk of Crohn disease in smokers. It has been suggested that, at least for autoimmune thyroid disease, tobacco use (or lack thereof) in genetically susceptible individuals may skew the immune response and determine, to some extent, the type of disease that will develop (Krassas & Wiersinga, 2006). Smoking has also been associated with Goodpasture disease, as has heavy exposure to hydrocarbons, such as automobile exhaust, solvents and gasoline (Bombassei & Kaplan, 1992).

For some diseases, the causal link between factors such as bacterial or viral infection and autoimmunity has been fairly well established. Many peptide fragments of microbial agents are homologous with host proteins, and the induction of an immune response to these antigens can result in cross-reactivity with self-antigens and the induction of autoimmunity. The best example of this "molecular mimicry" is a membrane protein on the β-haemolytic streptococcus bacterium, which has a high degree of homology with cardiac myosin. Antibodies that target the bacterium also cross-react with cardiac muscle and induce rheumatic fever. Antibodies to *Yersinia enterocolitica*, a bacterium normally associated with food poisoning outbreaks, cross-react with a variety of thyroid antigens, and increased levels of antibodies to *Yersinia* have been demonstrated in patients with Graves disease or autoimmune thyroiditis.

7.5 Laboratory animal data

Animal models of autoimmunity have been used to explore both molecular mechanisms and therapeutic interventions for a variety of autoimmune diseases (Germolec, 2005). However, there are currently no validated models to assess or identify chemicals that induce or exacerbate autoimmune diseases. The popliteal lymph node assay (PLNA), which measures nonspecific stimulation and proliferation in the lymph nodes draining chemically exposed tissues, has been shown to be a useful tool for screening for immunostimulating compounds (Pieters et al., 2002). Rodents that are genetically predisposed to develop autoimmune disease, such as the lupus-prone MRL mouse and the non-obese diabetic (NOD) mouse, a model for insulin-dependent diabetes, have been used to elucidate the role of specific genetic loci in the disease process and can be used as tools to evaluate whether a chemical has the potential to modify disease severity in genetically susceptible individuals. In some of these genetically prone models, autoimmunity is induced by exposure to chemical or biological agents. Additional animal models utilize immunization with purified self-antigens, often in the presence of adjuvants, to elicit autoimmune responses.

7.6 (Ir)reversibility of effects

In general, the biological mechanisms of autoimmune disease preclude the ability to reverse the disease process. Once a cascade of self-reactivity is initiated, the persistence of adaptive immune cells that recognize native antigens or neoantigens and the generation of chronic inflammation perpetuate the disease. A unique exception may be compounds that induce autoimmunity via the haptenization of native proteins. In some instances, such as occur with drug-induced anaemias, removal of the chemical allows resolution of disease.

7.7 Biological plausibility

7.7.1 Weight of evidence approach to assessment of risk for autoimmunity

Epidemiological studies, animal models and an assessment of the most appropriate methods and end-points with which to assess autoimmunity are critical factors in reducing the uncertainty in the determination of human risk for autoimmunity following exposure to chemicals. A number of general (e.g. changes in leukocyte counts or cytokine levels) or specific (increased autoantibody levels) immune effects would suggest the potential for increased risk to develop autoimmune disease and as such would "trigger" the risk assessment process. A comprehensive listing of these effects is presented in Table 3.1 in chapter 3. Hazard characterization of the potential for a chemical to modulate the immune system and promote self-reactivity can be accomplished with a weight of evidence approach that evaluates the available epidemiological and laboratory animal data for that chemical.

Hazard identification for autoimmunity should result in weight of evidence conclusions based on the available human and laboratory animal data for a given chemical. The risk assessor should consider the entire database of effects, including data that support induction or exacerbation of autoimmunity as well as data that do not support chemical-associated autoimmunity. Data are evaluated within the same or similar assays, as well as across divergent measures of the immune system and across multiple species. For each assay, a dose–response relationship for chemical exposure in the absence of generalized overt toxicity is a necessary criterion in demonstrating autoimmunity.

The weight of evidence conclusions are strengthened by consistency (particularly across species, sexes or related end-points), biological plausibility and breadth (range of effects) of the evidence for immunotoxicity. A lack of consistency among specific assays or types of immunotoxicity across species, strains or sexes does not necessarily represent conflicting data and often represents species, strain or sex differences. Conflicting data should be evaluated by the strengths and weaknesses (e.g. sample size and exposure duration) of the individual studies, as well as in the context of the remainder of the immunotoxicity database for a given chemical. Additional information with which to interpret species, strain or sex differences may be gained by considering toxicokinetic data (when available) or the likelihood of sex differences resulting from hormonally active chemicals, such as endocrine disrupting chemicals. As with other non-cancer end-points, the weight of evidence evaluation should represent an expert judgement of the database to determine the potential for autoimmunity associated with a given compound in accordance with the following key considerations (Hill, 1965; IPCS, 1999a; Weed, 2005): experimental evidence, dose–response relationship, consistency of the association, strength of the association, temporal association, biological plausibility, specificity, coherence and analogy.

A series of questions similar to those used to organize immunosuppression data (see chapter 4) can be applied to the evidence of autoimmunity to help develop weight of evidence conclusions. The questions are arranged to evaluate the available data from the strongest and most predictive data (human data) through the least predictive (immune organ weight). A brief description of the relative strength and predictability of different assays is presented below for major types of immunotoxicity data. The process is summarized in Figure 7.1. The risk assessor should refer to the detailed text below for important considerations for identifying key strengths and weaknesses for particular types of data and utility of data for

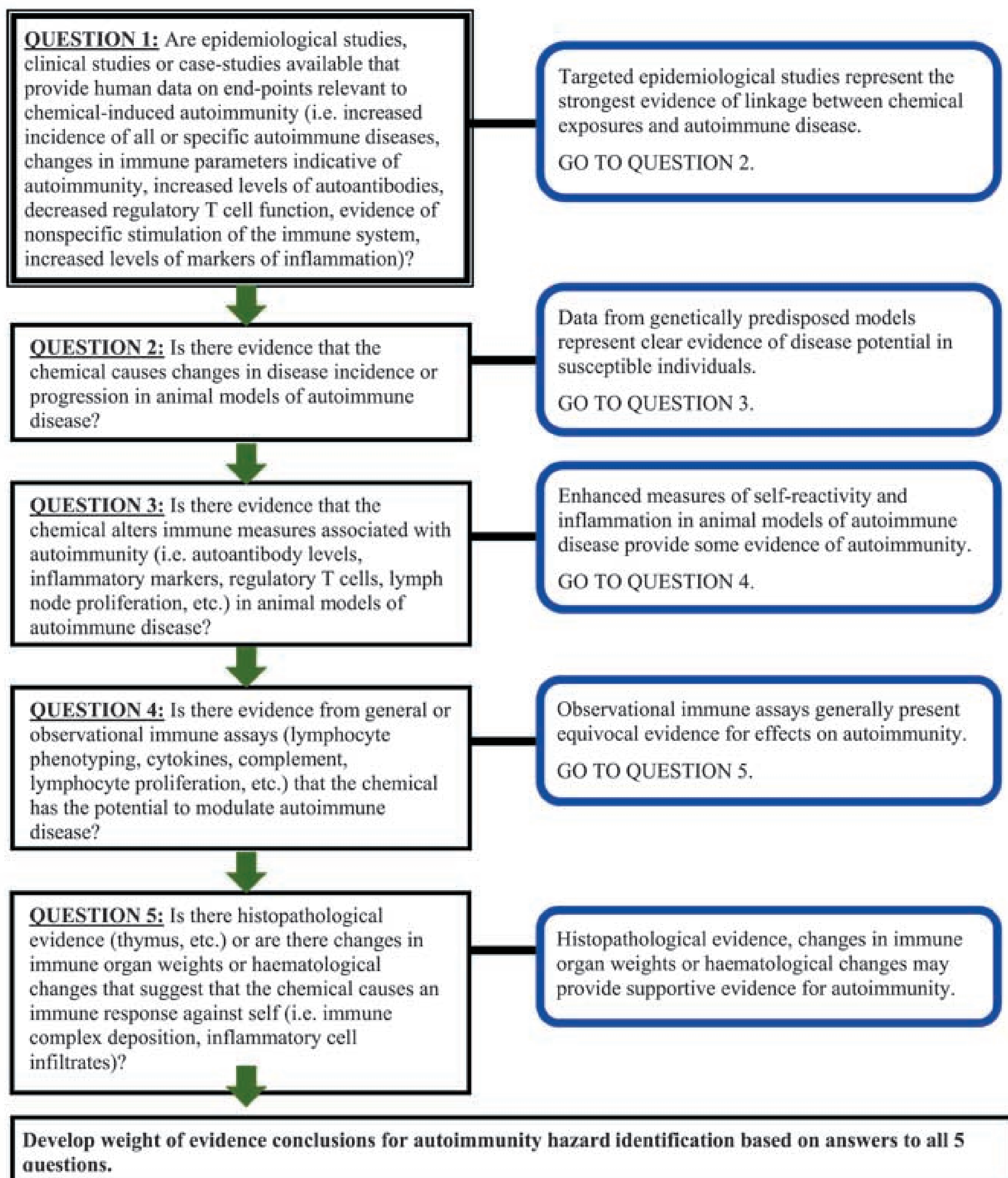

Figure 7.1: Schematic for organizing all available data for a weight of evidence approach for assessment of chemical-induced autoimmunity. The figure presents a summary of categorical data binning, from the most to least predictive, as described in section 7.7.1, rather than a decision-tree. Note: If there are immunotoxicological data relevant to end-points other than autoimmunity, evaluate those data in the appropriate chapter and include in weight of evidence evaluation for immunotoxicity.

derivation of effect levels. Weight of evidence conclusions for autoimmunity hazard for a given chemical should be developed by considering all five of the following questions:

1) *Human data*: Are epidemiological studies, clinical studies or case-studies available that provide human data on end-points relevant to chemical-induced autoimmunity (i.e. increased incidence of all or specific autoimmune diseases, changes in immune parameters indicative of autoimmunity, increased levels of autoantibodies, decreased regulatory T cell function, evidence of nonspecific stimulation of the immune system, increased levels of markers of inflammation)?
   - ✓ Controlled clinical studies with quantitative evaluation of diagnostic criteria for the relevant diseases and documented exposure levels represent the strongest data and provide clear evidence of autoimmunity appropriate for derivation of effect level(s). However, human data are more likely to be an enumeration of immune system components or evaluation of burden of disease following exposures.

2) *Modulation of disease incidence or progression (laboratory animal data)*: Is there evidence that the chemical causes changes in disease incidence or progression in animal models of autoimmune disease?
   - ✓ Modulation of disease incidence or progression in genetically predisposed animal models of autoimmunity would be considered clear evidence for an effect on autoimmunity and appropriate data for derivation of effect level(s). Modulation of a biologically relevant immune function such as lymph node reactions to local application of the chemical of interest (e.g. PLNA) would increase the strength of the data and support an MOA. Modulation of disease in genetically predisposed animal models of autoimmunity with additional evidence of immunotoxicity (e.g. changes in inflammatory cell populations, cytokines, altered histology, immune organ weight) would increase the strength of the data and support biological plausibility. Modulation of disease incidence or progression in multiple species or multiple animal models of autoimmune disease with concordance among end-points increases the support for clear evidence of effects on autoimmunity.

3) *Immune function (laboratory animal data)*: Is there evidence that the chemical alters immune measures associated with autoimmunity (i.e. autoantibody levels, inflammatory markers, regulatory T cells, lymph node proliferation, etc.) in animal models of autoimmune disease?
   - ✓ Positive results in lymph node cell proliferation assays such as the PLNA would be considered some evidence for potential effects on autoimmunity and provide data appropriate for derivation of effect level(s) when supported by additional evidence that the chemical affects autoimmunity.
   - ✓ Increased levels of autoantibodies in non-autoimmune disease–prone strains of mice or changes in immune measures associated with autoimmunity would be considered some evidence that a chemical has the potential to modulate autoimmune disease and provide data appropriate for derivation of effect level(s) when supported by additional evidence that the chemical affects autoimmunity. For example, when the effect is observed in multiple rodent strains or multiple species, there is greater support for use of the end-point in the derivation of an effect level. Modulation of autoantibody levels or lymph node cell proliferation assays in combination with additional evidence of inflammation (e.g. immunophenotyping, cytokines, altered histology, immune organ weight) increases the weight of evidence for an effect on autoimmunity.

- ✓ Modulation of measures associated with autoimmunity in combination with additional evidence of immune system dysregulation that supports an MOA or biologically plausible mechanism increases the weight of evidence for an effect on autoimmunity.

4) *General immune assays (laboratory animal data)*: Is there evidence from general or observational immune assays (lymphocyte phenotyping, cytokines, complement, lymphocyte proliferation, etc.) that the chemical has the potential to modulate autoimmune disease?
   - ✓ Lymphocyte phenotyping, cytokines and other assays may add MOA information to present a biologically plausible suggestion of autoimmune effects.
   - ✓ The predictive value of changes in immune cell populations and altered soluble mediator concentrations to indicate inflammation is well established; however, the linkage with autoimmune disease is less clear, and therefore these data should generally not be used to derive an effect level for autoimmunity.

5) *Histopathology and haematology (laboratory animal data)*: Is there histopathological evidence (thymus, etc.) or are there changes in immune organ weights or haematological changes that suggest that the chemical causes an immune response against self (i.e. immune complex deposition, inflammatory cell infiltrates)?
   - ✓ Descriptive histopathological evidence from specific target organs may indicate self-reactivity and support the concept of chemical-induced modulation of autoimmune disease, but should not be used to derive an effect level for autoimmunity.
   - ✓ Limited histopathological evidence alone is equivocal.
   - ✓ Haematology may reveal inflammatory conditions.

The risk assessor should develop the weight of evidence for autoimmunity hazard identification based on answers to all five questions. The weight of evidence conclusions for autoimmunity should also describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps. A small database with negative data is equivocal. Just as positive data on a range of assays strengthen the weight of evidence for immunotoxicity, negative data on a range of more predictive assays such as immune function data increase confidence to support a lack of immunotoxicity. The strength of the immune database will determine whether additional evidence is necessary to determine immunotoxicity. Incomplete or questionable data sets and high usage or high risk of exposure should trigger a request for additional data, if regulatory mandate allows.

When induction or exacerbation of autoimmunity is indicated by the weight of evidence for a particular chemical, these conclusions are then prepared to be brought forward to perform a dose–response assessment. That process begins with the selection of the most appropriate end-point(s) or critical effect(s) and the development of POD(s) for autoimmunity. Health-based guidance values or reference values are then calculated by dividing the POD(s) by the total uncertainty factor (see sections 3.3.7 and 4.9 for a detailed discussion of dose–response assessment and derivation of reference values). Data from human exposures (e.g. occupational exposure studies and case reports) are preferred for the critical effect, because fewer assumptions are required to determine the relative risk of immunotoxicity for the general population from human data compared with experimental animal data. Therefore, when human data are used for the critical effect and the POD, smaller uncertainty factors are generally utilized to derive the reference values. Nevertheless, all available data are considered

for the critical effect. The quantitative risk assessment may be based on laboratory animal data even if there are human data for a given chemical in cases such as inadequate information on dose levels, no information on effects at low doses or absence of a NOEL in the human data set.

Dose-related changes in two principal types of data provide clear evidence of induction or exacerbation of autoimmunity appropriate for use as the critical effect for chemical-related autoimmunity: 1) human data on end-points relevant to chemical-induced autoimmunity and 2) changes in disease incidence or progression in animal models of autoimmune disease. Data on immune measures associated with autoimmunity from animal models of autoimmune disease can also be used to derive effect levels when the data are supported by additional evidence that the chemical induces or exacerbates autoimmunity. It is particularly recommended that the risk assessor consult an expert in immunotoxicology or clinical immunology to help interpret the biological plausibility and adversity of these less predictive assays. In general, PODs are developed from the most sensitive adverse immune end-point(s) from the most appropriate species (or the most sensitive mammalian species, in the absence of information to determine the most appropriate species). Data from general immune assays, haematology, histopathology and immune organ weight changes may indicate potential immunotoxicity and are useful to support biological plausibility and a potential MOA for autoimmunity observed in more predictive data (e.g. disease progression in animal models of autoimmune disease). Observational end-points, such as phenotyping, lymphocyte proliferation and altered soluble mediator (cytokines or complement) concentrations, should generally not be used to derive an effect level for autoimmunity, because they are not considered to be reliable predictors of adverse autoimmunity. Changes in immune organ weights and general histopathology may indicate potential immunotoxicity and can be used to support more predictive data; however, these data should not be used to derive an effect level for autoimmunity because of the low predictive value of these end-points when considered alone.

7.7.2 Mode of action/mechanisms

Lifestyle and environmental factors may alter self-recognition by inducing genetic mutations, creating novel antigens through binding to self-proteins or modifying regulatory factors that control immune and inflammatory responses. Lymphocytes that recognize self-antigens with high affinity undergo negative selection in the bone marrow (B cells) and thymus (T cells) and are eliminated via apoptosis. However, autoreactive B and T cells constitute a normal part of the immune cell pool, and natural autoantibodies are observed in sera from “normal, healthy” individuals. The presence of these cells presents a low-grade risk for autoimmune disease in most individuals, because recognition of self-antigens occurs with low affinity and can be controlled by peripheral tolerance, a post-thymic control mechanism that limits antigen-specific activity via the regulatory interactions of a variety of cell types and soluble mediators. The requirement for two signals from antigen-presenting cells, one antigen-specific and a second nonspecific signal, for lymphocyte proliferation is a good example of one such regulatory check. In the absence of a co-stimulatory signal, self-reactive cells are rendered unresponsive (anergic). In some instances, however, these autoreactive cells can recognize self-antigens in situations that overcome anergy and make the antigens appear immunogenic. One such example is cytokine-mediated polyclonal activation following infection or chemical-induced inflammation. Microbial pathogens nonspecifically stimulate innate immunity, inducing the production of soluble mediators and co-stimulatory molecules important in the perpetuation of the immune response. In these cases, the inflammatory stimulus or a microbial protein may serve as an adjuvant, and tolerance can be broken. The purported

association between vaccination and autoimmunity postulates a similar mechanism, where immunization with an antigen in the presence of adjuvant may break tolerance and promote reactivity to self-proteins. It has also been suggested that HCB may act as an adjuvant, directly activating macrophages and other inflammatory cells and generating an inflammatory signal that polyclonally stimulates T lymphocytes (Ezendam et al., 2005) (see detailed Case-study 2 below).

Following activation, regulation of beneficial immune responses is mediated by a number of inhibitory pathways that balance the positive and negative aspects of immune system activation. Intrinsic defects or chemical agents that modify these regulatory pathways may lead to failure in restoring normal immune homeostasis and contribute to the pathogenesis of autoimmune disease. Changes in apoptotic pathways leading to inappropriate cell death or survival or disturbances in the clearance of apoptotic cells have been suggested as the underlying mechanisms for several autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus and Hashimoto thyroiditis. It is easy to see how these types of changes can lead to a self-perpetuating pathology. Phagocytosis of particulate materials such as silica by alveolar macrophages results in lipid peroxidation, increased proinflammatory cytokine production and secretion of reactive oxygen species and proteolytic enzymes, eventually leading to cell death. As silica is released by dying cells, it may be reingested by other macrophages, creating a cyclical process of inflammation and necrotic cell death. Dysfunction of regulatory NK T cell or T cell activity through altered cytokine production and/or deletion or mutation of cell surface molecules have been described for several autoimmune diseases. DNA methylation plays an important role in the regulation and expression of a number of inflammatory mediators. There is a growing body of literature that suggests that epigenetic changes resulting in altered DNA methylation patterns can modify immune function, contributing to the development of autoimmune diseases such as systemic lupus erythematosus (Strickland & Richardson, 2008). Organ-specific autoimmune diseases are typically characterized by cell-mediated responses directly affected by autoreactive CD8+ (cytotoxic) T cells or indirectly via release of proinflammatory cytokines and other soluble mediators by activated CD4+ T cells and macrophages. In contrast, systemic autoimmune diseases are frequently characterized by specific autoantibodies that can cause injury via activation of complement, blocking or stimulating cell surface receptors or aggregation into immune complexes that activate nonspecific inflammatory responses. It has been suggested that metals such as mercury induce autoimmune disease via the creation of new high-affinity binding sites for MHC molecules (see detailed Case-study 5 below). Drugs such as penicillin and halothane are proposed to induce reactions in which antigen-specific T cells provide help to antibody-producing B cells that recognize chemically modified proteins, but not the native form of the self-protein.

Oxidative damage has been implicated as a mechanism in a number of autoimmune diseases. Free radical–induced damage, lipid peroxidation and autoantibodies against oxidatively modified proteins and DNA have been observed in patients with systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes and autoimmune hepatitis. Oxidatively modified proteins may act as neoantigens, promoting the breakage of tolerance. The generation of free radicals and the induction of lipid peroxidation have been implicated in halothane-induced hepatitis and trichloroethylene-induced autoimmune disease. The anaesthetic halothane is metabolized via two major cytochrome P450 (CYP)–dependent pathways, both of which are involved in halothane-induced liver injury. At normal oxygen concentrations, halothane is oxidatively metabolized to trifluoroacetyl chloride, which may covalently modify hepatic proteins such as CYP2E1, leading to immune-mediated liver injury. In contrast, under

hypoxic conditions, halothane is metabolized via a reductive pathway to yield 1,1,1-trifluoro-2-chloroethyl free radical, which then reacts with cellular proteins and lipids, resulting in an immune-mediated hepatotoxicity (reviewed in Masubuchi & Horie, 2007).

As discussed in detail in chapter 3 (sections 3.3.6 and 3.3.7), when data are available to develop an MOA from laboratory animal studies, the MOA can be evaluated using a weight of evidence approach to establish human relevance (Boobis et al., 2008). During the characterization of the hazard database, the weight of evidence evaluation of a proposed animal model for autoimmunity can be used to determine the human relevance and therefore to address the potential for the development of autoimmunity following chemical exposure in humans.

7.8 Life stage considerations and groups at risk

It has been demonstrated for a number of environmental chemicals (e.g. TCDD, mercury, lead) that the same toxicant may disrupt different immune processes, depending upon the specific timing of exposure and the target organ dose (Dietert, 2009a). With regard to the specific timing of exposure, there is evidence from laboratory animal studies and human epidemiology that a single toxicant may promote different immune-associated diseases, depending upon the specific window of exposure (Holladay, 1999; Dietert & Piepenbrink, 2006b). As we better understand the consequences of immune dysregulation, there is increasing suspicion that early-life exposures may lead to increased risk for autoimmune diseases later in life. A number of health concerns have been raised with regard to the children of women who received DES during pregnancy to prevent preterm delivery or pregnancy loss. As part of the follow-up to a large multicentre epidemiological study that examined the incidence of cancer and other diseases in DES-exposed and unexposed cohorts, Noller et al. (1988) examined the self-reported prevalence of autoimmune diseases in 1711 exposed women and 922 controls. The overall frequency of autoimmune diseases was significantly elevated in exposed women when compared with the control group (28.6 per 1000 versus 16.3 per 1000, $P = 0.02$). Of the 14 autoimmune diseases reported in the cohort, only Hashimoto thyroiditis had a higher prevalence in exposed women (Noller et al., 1988). A number of additional studies have suggested that the offspring of DES-treated women exhibit a variety of immune system perturbations, including enhanced T cell proliferation and elevated NK cell activity, that could contribute to immune dysregulation (Ford et al., 1983; Ways et al., 1987; Burke et al., 2001) and an elevated risk for autoimmune disease.

Multiple sclerosis is an autoimmune disease characterized by self-reactivity to myelin basic protein, inappropriate activation of microglial cells and a T cell–mediated lymphocytic infiltration of the nervous system with destruction of the myelin sheath. Although the disease has been commonly diagnosed in young adults, it has been suggested that the disease is now increasingly diagnosed in children and adolescents (Thomas & Banwell, 2008). The excess risk of multiple sclerosis in dizygotic twins compared with non-twin siblings, along with evidence for maternal effects on disease rates, suggests that gestational or early-life exposures may contribute to susceptibility (Ebers, 2008). Although we currently do not understand what environmental factors may contribute to the disease process, exposure to xenobiotics that induce inappropriate myelomonocytic cell activation, inappropriate regulation of autoreactive cells or altered Th function could play a role in the increased risk of multiple sclerosis (Dietert, 2008).

Several investigators have examined immunological effects in inbred and autoimmune disease–prone mouse strains following prenatal or perinatal exposure (reviewed in Holladay, 1999). The prototypical immunotoxicant TCDD has been shown to induce thymic atrophy, alter thymocyte maturation and expression of MHC molecules and increase the number of extrathymic autoreactive T cells (Holladay et al., 1991; Blaylock et al., 1992; Silverstone et al., 1994), suggesting that the compound may promote autoimmunity. In utero exposure to TCDD has been reported to alter the time to disease onset in mice prone to autoimmune glomerulonephritis (Silverstone et al., 1998; Smith & Germolec, 2000). In C57BL/6 mice, a strain that is not genetically predisposed to the development of autoimmune disease, gestational exposure to TCDD altered T cell populations in the spleen and thymus (Mustafa et al., 2008). Increased immune complex and complement C3 deposition in the glomeruli and elevated titres of autoantibodies indicate that these mice may be at risk for the development of autoimmunity (Mustafa et al., 2008; Holladay et al., 2011). Similar findings were observed in the SNF1 (SWR × NZB:F1) lupus-prone mouse following a single dose of TCDD administered on day 12 of gestation. Increased autoantibody production and immune complex deposition suggest that prenatal exposure to TCDD may exacerbate autoimmune nephritis in females and induce early disease onset in male SNF1 mice (Holladay et al., 2011). As discussed above, prenatal exposure to DES has been associated with immune dysregulation in humans. In laboratory rodents, DES is a potent immunotoxicant, and in utero exposure results in thymic atrophy and suppression of cell- and humoral-mediated immunity in a sex-specific fashion (reviewed in Luebke et al., 2006a); however, there is only limited evidence that these alterations in immune function may affect disease in autoimmune disease–prone mice following developmental exposure (Stoll & Gavalchin, 2000).

With few exceptions (e.g. type 1 diabetes and myocarditis), autoimmune diseases are not common in children and adolescents, and the usual age of diagnosis for many autoimmune diseases is above 40 years (Jacobson et al., 1997). Because immunosenescence is associated with a decline in adaptive immunity, it is somewhat paradoxical that ageing is linked to an increased frequency of autoantibody production, chronic inflammatory disease and autoimmune disease (Hakim & Gress, 2007). It has been suggested that rather than considering age-related changes in the immune system as a general decline in immune responses, it may be more appropriate to view the process as immune remodelling and dysregulation (Huang et al., 2005). Although it is unclear if the number of regulatory T cells decreases with age, studies in both rodents and humans suggest that there is a decrease in functional activity of regulatory T cells that may contribute to the development and progression of autoimmune diseases (Tsaknaridis et al., 2003; Zhao et al., 2007). Reduced B cell lymphopoiesis and the lack of competition from naive B cells may lead to retention of self-reactive B cells in lymphoid follicles (Johnson & Cambier, 2004). In addition, although the number of B cells in the periphery remains fairly constant, it has been suggested that the immune response in aged individuals is skewed towards utilization of long-lived antigen-experienced B cells that produce low-affinity autoantibodies (Yung & Julius, 2008). Cytokine production is altered in a number of T cell populations in the elderly, and elevated levels of proinflammatory cytokines may contribute to altered function of both B and T cells and nonspecific immune activation resulting in a breakdown in tolerance, allowing autoimmunity to proceed (Huang et al., 2005).

7.9 Dose–response relationships and thresholds

As discussed in detail in chapter 3 (section 3.3.7), the determination and evaluation of a dose–response relationship are important factors in establishing the potential for many

compounds to induce immune system toxicity. It is somewhat problematical for autoimmunity, however, as the same chemical may have differing effects on the immune system, depending on the level of exposure and the target organ dose. The prototypical immunotoxicant cyclophosphamide provides an interesting example of this atypical dose–response relationship. Cyclophosphamide is routinely used as a positive control in immunotoxicology studies. When used at cumulative doses of 100–200 mg/kg body weight, it suppresses a number of immune parameters, including host resistance to tumour cell challenge and bacterial infection, induction of antigen-specific antibody responses, tumour cell killing via cytotoxic T lymphocytes and lymphoproliferative responses (Luster et al., 1993). Surprisingly, certain immune measures have been shown to be enhanced following treatment with lower doses of cyclophosphamide (Luster et al., 1993; Brode & Cooke, 2008). Administration of cyclophosphamide has been shown to increase DTH responses, enhance antitumour responses and augment the progression of type 1 diabetes in NOD mice (reviewed in Brode & Cooke, 2008). Two proposed mechanisms underlying these augmented immune responses include skewing of Th2/Th1 responses and the removal or inhibition of regulatory or suppressor cell populations. Although the relationship between enhanced immune responses and breakdown of self-tolerance is unclear, there is increasing evidence that suppression of regulatory factors may increase the risk for autoimmune diseases.

A similar effect of suppression of some immune parameters and stimulation of others has been shown for HCB, a pesticide associated with immune effects in humans following accidental and occupational exposures. In mice, HCB has been shown to be a potent immunosuppressive agent, decreasing antibody responses and resistance to infectious disease and neoplasia. In contrast, studies in the Brown Norway and other genetically susceptible rat strains have demonstrated that HCB exposure enhances production of serum and antigen-specific immunoglobulins and stimulates lymphocyte proliferative responses. For additional information on the epidemiology, pathology and mechanisms of HCB-induced modulation of the immune system, the reader is referred to the HCB case-study (see Case-study 2 below) and IPCS (2006a).

Autoimmune end-points in the Brown Norway rat have been used as the basis for current oral RfDs in the risk assessment for mercury(II) chloride in the USA (USEPA, 1995b). Although a weight of evidence approach was used and multiple studies were considered, three studies using the Brown Norway rat as the test strain demonstrated that the development of mercury-induced autoimmune glomerulonephritis was the most sensitive end-point for adverse effects following mercury exposure and were used to set a recommended safe drinking-water exposure level of 0.010 mg/l for inorganic mercury (Druet et al., 1978; Bernaudin et al., 1981; Andres, 1984). The production and deposition of IgG antibodies to the glomerular basement membrane were considered the first step in the formation of mercury-induced autoimmune disease. Because of the similarities between the disease processes in the Brown Norway rat and in sensitive humans, a combined uncertainty factor of 10 was used to account for both laboratory animal to human extrapolation and sensitive human populations (i.e. intra-individual variability) (USEPA, 1995b). In effect, the uncertainty factor used to calculate criteria and health advisories was reduced 10-fold because the animal model is considered a good surrogate for the study of mercury-induced kidney damage in sensitive humans (USEPA, 1995b).

In addition to identifying the shape of the dose–response curve and the effective dose range for autoimmune effects, factors such as exposure (route, timing and duration) and toxicokinetics that might affect comparisons with human exposure scenarios are identified and

discussed as part of the dose–response evaluation (see chapter 3, sections 3.3.7–3.3.9). Other factors, such as the development of tolerance, may complicate the assessment of dose–response relationships in autoimmunity. For example, low-level exposures to some agents that promote autoimmunity, such as mercury(II) chloride and penicillamine, have been shown to induce tolerance in some rodent models, such that autoimmune pathology is not induced following subsequent exposure to higher doses of these compounds. As discussed in chapter 3, the interpretation of dose–response data should identify doses associated with the adverse effect as well as doses associated with no adverse effects, to determine the most appropriate end-points or critical effects.

7.10 Uncertainty factors

The reader should consult chapter 3 (section 3.3.10) for a general discussion of the application of uncertainty factors in the characterization of the risk for a given chemical to induce immune system toxicity. However, because of unique concerns with regard to genetic variability of the human population and the observed predisposition of some genotypes to exhibit specific autoimmune diseases, as well as differential susceptibilities associated with age and sex, the risk assessor may consider the addition of uncertainty factors in order to derive AELs.

7.10.1 Genetic susceptibility

Familial aggregation and laboratory animal studies suggest a strong association between genetics and most autoimmune diseases. Concordance rates between identical twins range from approximately 9% to 40%, depending on the disease. Limited concordance may be explained by non-identity in immune repertoires due to T cell receptor and immunoglobulin gene recombination, variations in receptor assembly and somatic mutation, although evidence indicates that environmental factors can contribute to disease etiology. Many susceptibility genes have been identified in transgenic animal models; research on these monogenic autoimmune diseases has shown the importance of mutations in proteins associated with fas-mediated T cell apoptosis, negative selection in the thymus and the development and activation of regulatory T cells. However, any gene coding for products that are involved in the induction and maintenance of self-tolerance and in regulating immune effector functions as well as organ-specific functions may be involved in defining individual susceptibility. In the majority of autoimmune diseases, a multigenic process with multiple susceptibility loci working in concert has been suggested.

The most clearly established genetic association is with specific alleles within the MHC gene complex (Rose & Mackay, 2006). Functionally polymorphic genes encoding FcγRIIA, FcγRIIIA and FcγRIIIB have also been implicated as genetic factors in determining the pathogenesis and course of many autoimmune diseases. Polymorphisms in regulatory regions of genes coding for immunoinhibitory receptors, such as CTLA-4, that regulate T cell activation have also been shown to be important in disease susceptibility. Gene polymorphisms that affect the function or the level of expression of regulatory or effector molecules of inflammation, fibrosis or other pathological processes involved in autoimmune disease development have also been observed. Examples of these include systemic sclerosis (TGF-β1, TGF-β2, TGF-β3), juvenile idiopathic arthritis (IL-1α), rheumatoid arthritis (IL-4), systemic lupus erythematosus (IL-10), Sjögren syndrome (IL-10), juvenile idiopathic inflammatory myopathies (IL-1RA) and Wegener granulomatosis (IL-10). TNF polymorphisms have been implicated as independent susceptibility factors for rheumatoid arthritis and systemic lupus

erythematosus. These polymorphisms may be directly involved in disease pathogenesis, as TNF-α is known to be a strong inflammatory factor and has been a successful target for therapeutic intervention and long-lasting immune response modification. Finally, polymorphisms in genes associated with non-immune parameters, such as drug-metabolizing enzymes, may result in differential susceptibilities to drug- or chemical-induced autoimmunity. This is likely the result of changes in the generation of protein adducts and covalently modified antigens through the use of alternative metabolic pathways.

Genetic defects that lead to primary immunodeficiencies are now recognized as the basis of susceptibility to specific autoimmune syndromes; for a large number of primary immunodeficiencies, autoimmunity is the primary disease and is present in most individuals with the specific genetic defect (Carneiro-Sampaio & Coutinho, 2007; Torgerson, 2008). Defects in critical steps in the process of establishing tolerance and immune regulation are systematically associated with clinical manifestations of autoimmunity early in life and have been identified in genes important in T cell regulation, somatic recombination of T and B cell surface receptors, apoptosis and the production of complement components (Carneiro-Sampaio & Coutinho, 2007). The two most common antibody deficiencies, selective IgA deficiency and common variable immunodeficiency, are associated with self-reactivity to a broad group of target tissues, and clinical manifestations of autoimmunity may appear in as many as 35% of individuals with primary immunodeficiencies. The fact that not all individuals with a particular primary immunodeficiency develop any or the same manifestations of autoimmunity is further support for the influence of environmental factors on the development and progression of these diseases. As discussed above, transgenic and knockout mice are frequently used to elucidate the molecular mechanisms through which genetically based immune dysregulation may alter the development, maintenance and function of regulatory T cells, leading to abnormal immune tolerance.

While it is believed that a genetic predisposition to self-reactivity exists in all individuals with autoimmune diseases, differing susceptibility factors may govern the timing of the disease or the specific disease that an individual develops. As with other multifactorial diseases, such as cancer, it is suggested that both genetic and environmental factors interact to determine disease outcome and progression; however, we have little knowledge with regard to whether they result in cumulative and sequential changes or are the sequelae of mixtures of exposures. This would imply that the timing of evaluation of immune effects may have an impact on the perceived risk for specific diseases. For example, mercury-containing compounds have potent immunosuppressive effects in many rodent strains (reviewed in Havarinasab & Hultman, 2005). However, in genetically susceptible strains such as the Brown Norway rat, the pattern of effects changes in a relatively short time frame to reflect immunostimulation, as characterized by polyclonal B cell activation, increased serum immunoglobulin levels and increases in circulating autoantibodies. Interestingly, the LOELs for immunostimulation and immunosuppression for methylmercury are similar (Havarinasab et al., 2007).

7.11 Exposure assessment

As with other toxicological end-points and complicated biological processes, assessing the relative contributions that environmental exposures may have in the risk for development of disease is problematical. Miller et al. (2000) proposed a structured set of criteria to define environmentally associated autoimmune diseases in the human population. The five primary elements of these criteria are temporal plausibility, exclusion of other causative agents,

dechallenge (resolution or improvement of the condition after removal of the agent), rechallenge (recurrence or worsening of the condition after re-exposure to the agent) and biological plausibility. Identification of analogous cases or nearly identical cases and evidence for a dose–response effect are also considered as supportive of a proposed association. The proposed tiered approach provides a framework upon which to assess the level of evidence for associations between exposures to exogenous agents and autoimmune diseases. In relatively rare instances, there is epidemiological evidence for temporal associations between specific environmental exposures and the onset of autoimmunity. For example, the development of eosinophilia-myalgia syndrome and toxic oil syndrome, autoimmune disorders similar to diffuse fasciitis with eosinophilia and systemic sclerosis, has been associated with the ingestion of impure L-tryptophan-containing dietary supplements (eosinophilia-myalgia syndrome) and the consumption of contaminated rapeseed oil produced by a particular refinery (toxic oil syndrome; Kaufman & Krupp, 1995). A number of studies suggested that the degree of illness correlated with the amount and frequency of intake (Tabuenca, 1981; Kamb et al., 1992; Back et al., 1993), suggesting a potential dose–response relationship. However, there is often a long latency period between exposure and the development of disease, and for many compounds, the weight of evidence from human studies remains only suggestive. Laboratory animal models of chemical-induced autoimmunity must then provide important information on the shape of the dose–response curve, effective dose range, sensitive end-points and biomarkers of exposure and effect.

7.12 Risk characterization

As summarized in chapter 3, risk characterization is the portion of the risk assessment process in which the hazard characterization, quantitative dose–response assessment and exposure assessment are combined to provide a synthesis of estimates of exposure levels and health risks. It also considers sources of uncertainty in the scientific data (regarding both hazard characterization and exposure), indicates the confidence in the risk assessment conclusions, suggests what additional data would be necessary to strengthen the risk assessment and provides the information used in making risk management decisions. The results of a risk assessment (as summarized in the risk characterization) are the basis for identifying levels of chemical exposure that are believed to represent those at which there are no significant risks to human health and those at which health effects may occur. Additionally, to the extent permitted by the available data, risk characterization indicates how risk varies with exposure, describes the number of people exposed, discusses whether levels are of public health concern and identifies susceptible subpopulations. It assists risk management officials and decision-makers in identifying issues of concern for the allocation of resources and reduction of risks (IPCS, 1999a).

As is true for all forms of immunotoxicity, ideally, a quantitative risk assessment is performed for autoimmunity associated with chemical exposure. In the case where the available data do not allow for this, a qualitative risk assessment may be possible.

Two examples of the conduct of a risk assessment for autoimmunity, including risk characterization, are given in the case-studies on mercury and trichloroethylene (see Case-studies 5 and 6, respectively).

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GLOSSARY[1]

Adverse effect: Any change in the morphology, physiology, growth, development, reproduction or lifespan of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences.

Aggregate exposure: Exposure to a single chemical from multiple sources and by all exposure routes over a given period of time.

Allergen: An antigen that induces an allergic or hypersensitivity reaction, resulting in immune-mediated or non-immune-mediated tissue damage; restricted mainly to immediate hypersensitivity or anaphylactic reactions.

Allergenicity: The capacity to induce allergy.

Allergic contact dermatitis: An inflammatory skin disease resulting from allergic sensitization.

Allergic response: Adverse response of an allergic individual to the specific allergen.

Allergy: Hypersensitivity caused by exposure to an exogenous antigen (allergen) resulting in a marked increase in reactivity and responsiveness to that antigen on subsequent exposure, resulting in adverse health effects.

Anaphylaxis: Local or systemic immediate hypersensitivity reaction initiated by mediators released after immunological stimulation.

Antibody: Immunoglobulin molecule produced in response to immunization or sensitization, which specifically reacts with antigen.

Antigen: Any substance that induces a specific immunological response.

Antinuclear antibody: Antibody directed to a nuclear antigen; can have various specificities (e.g. to single- or double-stranded DNA or histone proteins).

Antinucleolar antibody: Antibody specifically recognizing nucleolar elements.

Apoptosis: Programmed cell death.

Atopy: In general terms, “unwanted reactivity”; used mostly to describe the state of general systemic or local hypersensitivity reactions related to genetic predisposition.

Autoantibody: Immunoglobulin (antibody) that is directed against the organism’s own antigens (autoantigens).

Autoantigen: Antigen to which an autoimmune reaction is directed.

Autoimmune disease: A disease involving immune responses against self-antigens, resulting in pathological change.

Autoimmunity: Inappropriate reaction of the immune system against the organism’s own antigens (autoantigens) that may be either destructive or non-destructive. Destructive autoimmunity is associated with the development of autoimmune diseases.

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[1] This glossary contains brief definitions of selected terms used in this guidance document. The definitions have been taken from the guidance document itself or from one of several WHO/IPCS documents (see end of glossary for sources). The reader should refer to these documents for expanded definitions or for definitions of terms not included here.

Biomarker: Indicator of changes or events in biological systems. Biological markers of exposure refer to cellular, biochemical, analytical or molecular measures that are obtained from biological media such as tissues, cells or fluids and are indicative of exposure to an agent. Biomarkers of effect refer to biological changes that represent an alteration in endogenous body constituents (e.g. depression of cholinesterase levels as an indicator of exposure to pesticides).

Chemical-specific adjustment factor: A modified default 10-fold uncertainty factor that incorporates appropriate data on species differences or human variability in either toxicokinetics (fate of the chemical in the body) or toxicodynamics (actions of the chemical on the body).

Cross-reactivity: Reactivity of antigen-specific elements 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.

Delayed-type hypersensitivity: Inflammatory lesion mediated by effector T lymphocytes or their products, with attraction mainly of macrophages towards the inflammatory lesion.

Dose–response relationship: Relationship between the amount of an agent administered to, taken up by or absorbed by an organism, system or (sub)population and the change developed in that organism, system or (sub)population in reaction to the agent.

Elicitation: Production of a cell-mediated or antibody-mediated allergic response by exposure of a sensitized individual to an allergen.

Exposure assessment: Evaluation of the exposure of an organism, system or (sub)population to an agent (and its derivatives). Exposure assessment is the third step in the process of risk assessment.

Hazard: The inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub)population is exposed to that agent.

Hazard characterization: The qualitative and, wherever possible, quantitative description of the inherent property of an agent or situation having the potential to cause adverse effects. This should, where possible, include a dose–response assessment and its attendant uncertainties. Hazard characterization is the second of four steps in risk assessment.

Hazard identification: The identification of the type and nature of adverse effects that an agent has an inherent capacity to cause in an organism, system or (sub)population. Hazard identification is the first of four steps in risk assessment.

Health-based guidance value: A numerical value derived by dividing a point of departure (e.g. a no-observed-adverse-effect level or benchmark dose lower confidence limit) by a composite uncertainty factor to determine a level that can be ingested over a defined time period (e.g. lifetime or 24 hours) without appreciable health risk.

Hypersensitivity: Increased reactivity or sensitivity; in immunological reactions, often associated with tissue destruction.

Immunocompetence: Capacity of B or T lymphocytes to specifically recognize antigen, resulting in a specific immunological reaction.

Immunomodulation: Immunosuppression, immunostimulation and promotion of markers of autoimmune disease.

Immunostimulation: Unintended stimulation of the immune system.

Immunosuppression: Dominant immunological tolerance, a phenomenon that plays an active role in regulating T and B cell responses to both foreign antigens and autoantigens (suppressor T lymphocyte). The downregulation of responses to autoantigens is a major regulatory mechanism involved in the induction and maintenance of self-tolerance.

Immunotoxicity: Any adverse effect on the immune system that can result from exposure to a range of environmental agents, including chemicals.

Inflammation: Process whereby blood proteins or leukocytes enter tissue in response to or in association with infection or tissue injury.

Margin of exposure: Ratio of the no-observed-adverse-effect level or benchmark dose lower confidence limit for the critical effect to the theoretical, predicted or estimated exposure dose or concentration.

Mechanism of action: The specific biochemical interaction through which a substance produces an effect on a living organism or in a biochemical system.

Mode of action: A biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data.

Non-threshold effect: An adverse effect for which there is no dose or exposure concentration below which the stated effect is not observed or expected to occur.

Risk: The probability of an adverse effect in an organism, system or (sub)population caused under specified circumstances by exposure to an agent.

Risk analysis: A process for controlling situations where an organism, system or (sub)population could be exposed to a hazard. The risk analysis process consists of three components: risk assessment, risk management and risk communication.

Risk assessment: A process intended to calculate or estimate the risk to a given target organism, system or (sub)population, including the identification of attendant uncertainties, following exposure to a particular agent, taking into account the inherent characteristics of the agent of concern as well as the characteristics of the specific target system. The risk assessment process includes four steps: hazard identification, hazard characterization (dose–response assessment), exposure assessment and risk characterization.

Risk characterization: The qualitative and, wherever possible, quantitative determination, including attendant uncertainties, of the probability of occurrence of known and potential adverse effects of an agent in a given organism, system or (sub)population, under defined exposure conditions. Risk characterization is the fourth step in the risk assessment process.

Risk management: The process of weighing policy alternatives, decision-making and action taking.

Self-tolerance: Specific immunological unresponsiveness to a defined autoantigen.

Sensitization: Induction of specialized immunological memory in an individual by exposure to antigen.

Threshold: Dose or exposure concentration of an agent below which a stated effect is not observed or expected to occur.

Threshold effect: An adverse effect for which there is a dose or exposure concentration below which the stated effect is not observed or expected to occur.

Tolerance: Persistent condition of specific immunological unresponsiveness, resulting from previous non-sensitizing exposure to the antigen.

Uncertainty factor: Reductive factor by which an observed or estimated no-observed-adverse-effect level or other reference point, such as the benchmark dose or benchmark dose lower confidence limit, is divided to arrive at a reference dose or standard that is considered safe or without appreciable risk.

Main sources of definitions in Glossary:

FAO/WHO (2009) *Principles and methods for the risk assessment of chemicals in food*. Rome, Food and Agriculture Organization of the United Nations, and Geneva, World Health Organization (Environmental Health Criteria 240; http://www.who.int/entity/foodsafety/chem/principles/en/index.html).

IPCS (1996) *Principles and methods for assessing direct immunotoxicity associated with exposure to chemicals.* Geneva, World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 180; http://www.inchem.org/documents/ehc/ehc/ehc180.htm).

IPCS (1999) *Principles and methods for assessing allergic hypersensitization associated with exposure to chemicals*. Geneva, World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 212; http://www.inchem.org/documents/ehc/ehc/ehc212.htm).

IPCS (2004) *IPCS risk assessment terminology. Part 1: IPCS/OECD key generic terms used in chemical hazard/risk assessment; Part 2: IPCS glossary of key exposure assessment terminology.* Geneva, World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 1; http://www.who.int/entity/ipcs/methods/harmonization/areas/ipcsterminologyparts1and2.pdf).

IPCS (2006) *Principles and methods for assessing autoimmunity associated with exposure to chemicals.* Geneva, World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 236; http://www.who.int/ipcs/publications/ehc/ehc236.pdf).

ANNEXES

ANNEX 1: SELECTION OF NATIONAL AND EUROPEAN REQUIREMENTS AND GUIDELINES

European Union (European Commission)

The European Commission regulation on chemicals and their safe use (EC 1907/2006), which entered into force on 1 June 2007, deals with the Registration, Evaluation, Authorisation and Restriction of Chemical Substances (REACH). Annex I of the regulation sets out the details of how to carry out a chemical safety assessment and document it in a chemical safety report. The annex has been supplemented by a technical guidance document on information requirements and chemical safety assessment. These guidance documents can be obtained via the web site of the European Chemicals Agency (http://echa.europa.eu/reach_en.asp). In general, REACH regulations require that quantitative exposure estimates for relevant exposure scenarios are compared with the derived no-effect level (DNEL), which in turn is obtained by application of assessment (or uncertainty) factors to NOAEL (or LOAEL) values. The result is expressed as the risk characterization ratio, which is then used to assess the safety of an exposure scenario. For skin sensitizers, a qualitative risk characterization is usually required, but the guidance document also provides the possibility of performing a quantitative risk assessment, as discussed in chapter 6 of this document. Regarding direct toxic effects on (parts of) the immune system, the risk assessment steps are not different from those for other systemic toxic effects affecting other organs and include derivation of a NOAEL, application of assessment factors to derive a DNEL, quantitative exposure estimates for relevant exposure scenarios and derivation of the risk characterization ratio. The reader is referred to the technical guidance document for further information.

United States of America (USEPA)

At the time of preparation of this WHO/IPCS guidance document, the USEPA was in the process of developing guidance for immunosuppression risk assessment. The development of guidance by the USEPA was intended to provide tools for USEPA scientists to objectively evaluate the potential for immunosuppression in experimental and clinical models following exposure to environmental chemicals. The USEPA document under development focuses predominantly on unintended immunosuppression, because there is wide acceptance of the relevance of immunosuppression end-points in humans and experimental animals for the determination of human risk. When completed, the USEPA guidance document will also supply basic information to the public about USEPA's risk assessment methods for immunosuppression risk assessment.

ANNEX 2: SELECTION OF WHO/IPCS GUIDANCE ON CHEMICAL RISK ASSESSMENT

The following Environmental Health Criteria monographs and Harmonization Project Documents are useful references for the risk assessor:

Environmental Health Criteria monographs

IPCS (1999) *Principles for the assessment of risks to human health from exposure to chemicals*. Geneva, World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 210; http://www.inchem.org/documents/ehc/ehc/ehc210.htm).

IPCS (2006) *Principles for evaluating health risks in children associated with exposure to chemicals*. Geneva, World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 237; http://www.who.int/entity/ipcs/publications/ehc/ehc237.pdf).

IPCS (2009) *Principles for modelling dose–response for the risk assessment of chemicals*. Geneva, World Health Organization, International Programme on Chemical Safety (Environmental Health Criteria 239; http://whqlibdoc.who.int/publications/2009/9789241572392_eng.pdf).

Harmonization Project Documents

IPCS (2004) *IPCS risk assessment terminology. Part 1: IPCS/OECD key generic terms used in chemical hazard/risk assessment; Part 2: IPCS glossary of key exposure assessment terminology.* Geneva, World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 1; http://www.who.int/entity/ipcs/methods/harmonization/areas/ipcsterminologyparts1and2.pdf).

IPCS (2005) *Chemical-specific adjustment factors for interspecies differences and human variability: guidance document for use in dose/concentration–response assessment.* Geneva, World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 2; http://whqlibdoc.who.int/publications/2005/9241546786_eng.pdf).

IPCS (2007) *Part 1: IPCS framework for analysing the relevance of a cancer mode of action for humans and case-studies; Part 2: IPCS framework for analysing the relevance of a non-cancer mode of action for humans.* Geneva, World Health Organization, International Programme on Chemical Safety (Harmonization Project Document No. 4; http://www.who.int/ipcs/methods/harmonization/areas/non_cancer/en/index.html).

CASE-STUDIES

CASE-STUDY 1: ASSESSMENT OF IMMUNOSUPPRESSION CAUSED BY LEAD EXPOSURE

C1.1 Introduction

Lead (Pb) toxicity is most often associated with children's health issues, particularly those related to learning disorders (Shen et al., 2001). Blood lead levels (BLLs) as low as 10–15 µg/dl have been associated with cognitive and behavioural deficits (Bellinger, 1995; Garavan et al., 2000). The United States Centers for Disease Control and Prevention (CDC) define elevated BLLs, or the threshold for intervention, to be 10 µg/dl or greater in children 6 years of age or younger. The main sources of lead exposure in children are deteriorating paint and paint dust found in old homes, and differences in individual exposure levels are often associated with social and economic factors. Although BLLs are decreasing in children due to efforts to restrict lead use, CDC estimates that approximately 11% of children 6 years of age or younger in the USA still have BLLs in excess of 10 µg/dl (Binns et al., 2001).

In adults, elevated BLLs that result from occupational exposure, although significantly reduced over the last several decades, are still a concern in almost all countries and regions, including Western Europe and the USA. The United States National Health and Nutrition Examination Survey indicates that 700 000 adults in the USA have BLLs greater than 5 µg/dl, with many thousands having BLLs greater than 25 µg/dl from occupational exposure. Health effects from lead exposure have been reported in multiple organ systems, including the nervous, haematological and reproductive systems. Lead's effect on multiple organ systems may be due to its ability to compete for calcium, iron and zinc binding sites. Effects related to low-level lead exposure, including immunological effects, are not as well understood, but are still of concern. Several published studies in which the immunological effects from lead exposure were studied, particularly in occupational settings, are available, but some ambiguity still exists regarding the effect of lead on the human immune system. The data available from laboratory animal studies using lead are more robust, more homogeneous and clearer regarding the effect of lead on the immune system.

The present case-study was conducted to evaluate the guidance provided for the assessment of immunosuppression, as presented in chapter 4 of this document. Lead was selected for the case-study because it is typical of many putative immunotoxic chemicals, for which considerable experimental and human data have been collected, in that only moderate effects have been reported in exposed humans. As with other areas of toxicology, human data are preferable for the risk assessment of lead immunotoxicity, because less extrapolation is needed, and thus the data provide a more accurate estimate of risk to the general population. Statistically significant differences in immune system end-points have been demonstrated between exposed and control populations, although in some cases laboratory values in the exposed populations are within normal reference ranges. Nevertheless, human health effects in exposed populations, including reduced resistance to infection, have been reported when laboratory values cluster at the lower end of the reference range (Luebke et al., 2004). Thus, there is evidence that statistically significant changes in immune end-points may predict adverse health effects, although acceptance of the evidence as predictive is not universal. Regarding lead, the vast majority of the human studies were collected from adults with occupational exposure, although some recent attention has focused on environmental exposure in children. Nonetheless, data evaluated for the current lead case-study presented here support the conclusion that lead is immunotoxic.

This case-study is not a formal risk assessment of lead as an immunotoxicant. Furthermore, only immunotoxicity data were evaluated as part of this exercise; therefore, other forms of toxicity (e.g. developmental neurotoxicity) were not considered, even though adverse effects in other organ systems may occur at doses lower than those associated with lead immunotoxicity. This exercise begins with a brief summary of the available evidence for lead-induced immunosuppression, followed by application of a weight of evidence approach for the assessment of immunosuppression (chapter 4, Figure 4.1).

C1.2 Background: immunotoxicity data for lead

Heavy metals, in general, exert a variety of effects on the immune system. Lead is one of the most studied environmental and occupational chemicals for effects on the immune system, with publications dating back to the 1950s (Belli & Giuliani, 1955). Both experimental animal and occupational worker studies, as well as several children's studies, exist in which immunological effects following lead exposure were observed. Tables C1.1–C1.4 list the key publications, summarize results and serve as a focal point for all later discussions. These tables include adult experimental animal studies (Table C1.1), prenatal and postnatal experimental animal studies (Table C1.2), adult human studies (Table C1.3) and studies in children (Table C1.4), few of which have been done. It is important to note that the tables do not include every published lead immunotoxicity study. Rather, the selected publications relating to immunosuppression were considered useful for illustrating the risk assessment framework presented in chapter 4. Publications included in these tables generally included BLLs determined at or proximal to the time at which immune testing was conducted. This allows direct exposure comparisons to be made between humans and experimental animals by an internal dose metric, substantially increasing confidence in the quantitative evaluation of immunosuppressive effects. Mechanistic studies were not included in the tables unless they were useful in supporting the risk assessment. Experimental animal studies that employed prenatal or early postnatal exposure were included to assess whether individuals exposed during immune system development might represent a uniquely susceptible population. Experimental animal studies that failed to report BLLs or presented only negative findings were generally excluded from the tables, as they do not contribute to the assessment process, given the number of studies that reported both BLLs and positive immunotoxicity findings.

Human studies relevant to immunosuppression were exclusively epidemiological and retrospective in nature. No case-studies or prospective studies were identified. With the exception of several studies conducted in schoolchildren, in which relatively low BLLs were reported from environmental exposure, human data were derived from occupationally exposed cohorts. These epidemiological studies generally provided minimal exposure histories, often limited to a mean or range of years on the job, but did include current BLLs. In addition, as a whole, the data in human studies are inconclusive as to the effect of lead on the immune system. It appears that lead may be immunotoxic; however, some human studies suggest an effect on the immune system, and others suggest that lead has no effect. The lack of study reproducibility and the variability of many of the data points limit the utility of the human data in a risk assessment of lead's immunotoxicity.

More recently, the spectrum of immunopathologies potentially associated with lead exposure has expanded to include evidence that exposure may increase serum IgE and the incidence of atopic diseases. For example, experimental animal studies have demonstrated that lead affects CD4+ cells and B cells, resulting in increased production of Th2 cytokines and IgE (Dietert

Table C1.1: Effects of lead exposure on the immune system of adult experimental animals.

Species/strain	*Exposure*	*Dose*	*BLL (µg/dl)*[a]	*Immunological effects*[b,c]	*Notes*	*Reference*
CBA/J mice	Lead acetate in drinking-water for up to 4 weeks	0 mg/l 82.9 mg/l 2072 mg/l	— 18 >100	↓ antigen presentation (2-week lead exposure); 47% (82.9 mg/l), 92% (2072 mg/l)	No effect on phagocytic function or IL-1 production	Kowolenko et al. (1988)
BDF1 mice	Lead acetate in drinking-water for 3 weeks	0 mg/l 50 mg/l 200 mg/l 1000 mg/l	0.7 ± 0.4 (± SE) 25.4 ± 1.3 38.6 ± 3.2 82.6 ± 5.9	↓ T cell–dependent immune response to SRBCs; 45% (50 mg/l), 35% (200 mg/l), 36% (1000 mg/l)	—	Blakley & Archer (1981)
CBA/J mice	Lead acetate in drinking-water for 18 months	0 mg/l 13 mg/l 1300 mg/l	ND	No clear dose–response effects; 13 mg/l induced an increase in lymphocyte stimulation, but 1300 mg/l resulted in no change	Lead also found in chow diet at ≤1.12 mg/kg	Koller et al. (1977)
BALB/cByJ mice	Lead acetate in drinking-water for 8 weeks	0 mg/l 414.4 mg/l	ND ~45	↑ splenic bacterial burden and serum IFN-γ after infection with *Listeria monocytogenes*; ↓ splenic weight	No effect on serum IL-6	Kim & Lawrence (2000)
CBA/J and C57BL/6 mice	Lead acetate in drinking-water for up to 10 weeks	0 mg/l 16.6 mg/l 82.9 mg/l 414.4 mg/l 2072 mg/l	ND	↑ splenic bacterial burden after infection with *L. monocytogenes* at 2072 mg/l; ↑ mortality rate at ≥82.9 mg/l	No effect on cell-mediated immune response to SRBCs; statistics not reported	Lawrence (1981)
Swiss albino mice	Lead acetate by intraperitoneal injection once daily for 15 days	10 mg/kg body weight	ND	↓ clearance of *Staphylococcus aureus* from blood; ↓ migration and cell adhesion of splenic macrophages following infection	—	Bishayi & Sengupta (2003)

Table C1.1 (continued)

Species/strain	*Exposure*	*Dose*	*BLL (μg/dl)*[a]	*Immunological effects*[b,c]	*Notes*	*Reference*
Swiss-Webster CFW, CBA/J, SJL/J, DBA/1J, C57BL/6J, A/J, BALB/c and NZBWF1 mice	Lead acetate in drinking-water for up to 8 weeks	0 mg/l	2.3–4.3 (mean range)	No effect in any mouse strain on the SRBC plaque-forming assay	—	Mudzinski et al. (1986)
		2072 mg/l	59.2–132 (mean range)			
C3H/HeN	Lead acetate in drinking-water for up to 18 weeks	0 mg/l	2.9 ± 1.1	↑ susceptibility to *Salmonella typhimurium* strain SL1344 infection; ↓ survival post-infection (80% at 0 mg/l, 40% at 1036 mg/l and 0% at 2072 mg/l); ↑ IL-4; ↓ IFN-γ, IL-12	No change in IL-2 or TNF-α	Fernandez-Cabezudo et al. (2007)
		1036 mg/l	20.5 ± 1.1			
		2072 mg/l	106.2 ± 8.9			
BALB/c	Lead acetate in drinking-water for 3 weeks	0 mg/l	<2	↓ DTH response to SRBCs (dose dependent)	—	McCabe et al. (1999)
		32 mg/l	9 ± 1			
		128 mg/l	49 ± 15			
		512 mg/l	87 ± 7			
		2048 mg/l	169 ± 23			

ND, not determined; SE, standard error

[a] Mean values, ± standard deviation when available, unless indicated otherwise.
[b] Effects occurred in all lead-exposed groups unless indicated otherwise.
[c] Effects are significantly different from controls ($P < 0.05$).

Table C1.2: Effects of lead on the immune system of prenatally and/or postnatally exposed animals.

Species/strain	*Exposure*	*Dose*	*BLL (μg/dl)*[a]	*Immunological effects*[b,c]	*Notes*	*Reference*
Sprague-Dawley rats	Dams exposed to lead acetate in drinking-water for 7 weeks (premating) and throughout mating, gestation and lactation; offspring weaned at PND 21 and continued on direct lead exposure at same doses as dams until PNDs 35–45	0 mg/l 25 mg/l 50 mg/l	5.5 ± 1.0 29.3 ± 14.1 52.8 ± 10.0	Offspring: ↓ absolute and relative thymus weights; ↑ absolute and relative spleen weights (50 mg/l males only); ↓ IgG; dose-dependent ↓ in cell-mediated immune response to SRBCs	No histopathological evidence of toxicity in thymus or spleen; no effect on serum IgA or IgM levels	Luster et al. (1978)
Sprague-Dawley rats	Dams exposed to lead acetate in drinking-water for 7 weeks (premating) and throughout mating, gestation and lactation; offspring weaned at PND 21 and continued on direct lead exposure at same doses as dams until PNDs 35–45	0 mg/l 25 mg/l 50 mg/l	5.5 ± 1.0 29.3 ± 14.1 52.8 ± 10.0	Offspring: ↓ absolute and relative thymus weights; ↑ absolute and relative spleen weights (50 mg/l males only); ↓ mitogenic response to antigen in splenic lymphocytes; ↓ DTH response to purified protein antigen	No histopathological difference in thymus or spleen	Faith et al. (1979)
F344 rats	Lead acetate in drinking-water during mating and pregnancy; only female offspring were assessed at 13 weeks of age	0 mg/l 100 mg/l 250 mg/l 500 mg/l	0.0 (± SE) 39.4 ± 6.7 70.8 ± 8.2 112.0 ± 19.9 (BLLs of dams during pregnancy)	Offspring: ↓ total WBC count at ≥250 mg/l; ↓ IFN-γ (500 mg/l only)	Neither offspring nor dams were exposed after parturition; many immune phenotype and functional parameters for offspring exhibited irregular dose–response trends	Miller et al. (1998)

Table C1.2 (continued)

Species/strain	*Exposure*	*Dose*	*BLL (µg/dl)[a]*	*Immunological effects[b,c]*	*Notes*	*Reference*
F344 rats	Lead acetate in drinking-water during mating and pregnancy	0 mg/l 250 mg/l	~5.5 66.2 ± 2.2[d] (dam) 49.0 ± 7.4[e] (dam) 8.0 ± 0.6[f] (offspring) 6.8 ± 1.2[g] (offspring)	No effect on immune phenotype or functional parameters in dams; ↓ DTH response to KLH antigen; ↓ IFN-γ; ↑ IL-4 and TNF-α in offspring of the high-protein diet/lead group; ↓ IL-4 in the low-protein diet/lead group	Lead exposure ceased at parturition for both dams and offspring; immune parameters examined in dams at 7–8 weeks post-treatment and in female offspring 12–13 weeks post-treatment; high- and low-protein diet is a known covariate for this study	Chen et al. (2004)
F344 rats	Dams exposed to lead acetate in drinking-water throughout gestation	0 mg/l 50 mg/l 100 mg/l 250 mg/l	≤3.0 in all male and female offspring at weeks 5 and 13 postpartum	↓ DTH response in females of the high-dose group (250 mg/l) only	Lead exposure ceased at parturition for dams and offspring; differences in BLL observed only on PND 1; however, immune parameters assayed at weeks 5 and 13	Bunn et al. (2001a)
BALB/c mice	Dams exposed to lead acetate in drinking-water at ~GD 15 through 4 weeks postpartum; offspring continued on direct exposure to lead acetate in drinking-water at same doses as dams for an additional 2 weeks	0 mg/l 16.6 mg/l 82.9 mg/l 207.2 mg/l	Significantly increased BLL in all exposed neonate mice compared with controls; graphical representation of data only	Offspring: ↑ IgE; ↓ splenic WBC counts in animals at ≥2 weeks postpartum	BLLs of 5 µg/dl = ↑ IgE	Snyder et al. (2000)

GD, gestational day; PND, postnatal day; SE, standard error

[a] Mean values, ± standard deviation when available, unless indicated otherwise.
[b] Effects occurred in all lead-exposed groups unless indicated otherwise.
[c] Effects are significantly different from controls ($P < 0.05$).
[d] BLL in dams on high-protein diet.
[e] BLL in dams on low-protein diet.
[f] BLL in female offspring on high-protein diet.
[g] BLL in female offspring on low-protein diet.

Table C1.3: Effects of lead exposure on the immune system of adult humans.

Occupation/exposure	*Sample number*[a]	*BLL (µg/dl)*[b]	*Immunological effects*[c,d]	*Notes*	*Reference*
Battery plant/lead smelter workers; average exposure of 10 years	Reference (53) Exposed (72)	12.0 55.4 (SD not reported)	↓ C3 complement, ↓ IgM, ↓ IgG, ↓ IgA	—	Ewers et al. (1982)
Firearms instructors	Reference (36) Low (36) High (15)	— 14.6 ± 4.6 31.4 ± 4.3	↓ % of CD3+ and CD4+, ↓ T cell and B cell mitogenic response (PHA or PWM), ↓ % of HLA-DR+ cells	—	Fischbein (1993)
Plant workers; average exposure of 10 years	Reference (21) Exposed (39)	11.8 ± 2.2 38.4 ± 5.6	No effects	Serum Ig levels and functional immunity unchanged	Kimber et al. (1986)
Lead smelter workers; average exposure of 5.3 years	Reference (84) Exposed (145)	<2–12 39 (15–55)	↓ % of monocytes, CD4+/CD8+ and CD8+/CD56+ cells	Significance achieved only after adjusting for variables (e.g. smoking)	Pinkerton et al. (1998)
Variable occupations; average exposure of 141 months	Reference (25) Exposed (38)	— 62.3 ± 21.6	↑ lymphocytes and C4 complement, ↓ IgM	—	Coscia et al. (1987)
Lead storage battery plant; average exposure of ~5.8 years	Exposed (606)	~23 ± 10	↑ IgE	No control population used for reference	Heo et al. (2004)
Lead acid battery workers; average exposure of 5.8 years	Reference (20) Exposed (33)	<10.0 12.0–80.0	↓ neutrophil migration, activity	Serum Ig levels and mitogenic response to antigen unchanged	Queiroz et al. (1993, 1994)
Lead stearate workers	Reference (29) Low (19) High (10)	7 <20 >20	↓ CD16+ (NK) cells	—	Sata et al. (1998)
Storage battery plant workers; average exposure of 6 years	Reference (25) Exposed (25)	16.7 ± 5.0 74.8 ± 17.8	↓ CD4+, IgG, IgM and C3 and C4 complement	IgA also decreased but did not reach statistical significance	Undeger et al. (1996)

Table C1.3 (continued)

Occupation/exposure	*Sample number*[a]	*BLL (μg/dl)*[b]	*Immunological effects*[c,d]	*Notes*	*Reference*
Reference population	(30)	4.5	↓ lymphocyte proliferation, ↑ IFN-γ	Average exposure duration for all three lead groups approximated at 11–12 years	Mishra et al. (2003)
Three-wheel drivers	(30)	6.5 ± 4.7			
Battery workers	(34)	128.1 ± 104.7			
Silver jewellery makers	(20)	17.8 ± 18.5			

PWM, pokeweed mitogen; SD, standard deviation

[a] Number in parentheses equals *N* for that group; reference = control group selected as reference for comparison with lead-exposed population.
[b] Mean values, ± SD when available, unless otherwise indicated.
[c] Serum levels.
[d] Effects occurred in all lead-exposed groups unless indicated otherwise.

Table C1.4. Effects of lead exposure on the immune system of children.

Age group	*Sample group/number*[a]	*BLL (μg/dl)*[b]	*Immunological effects*[c,d]	*Notes*	*Reference*
Preschool-aged (4–6 years old) children	Reference (7)	22.6	No effects on serum Ig, C3 levels or immune response to soluble antigen	—	Reigart & Graber (1976)
	Exposed (12)	45.3			
Children aged 9 months – 6 years	Reference (~179)	<9	↑ IgE levels	Comprehensive panel of immune parameters examined, but no change associated with increased lead exposure	Lutz et al. (1999)
	Exposed (~100)	≥10			
Children aged 3–6 years	Reference (35)	6.4	↓ CD4+ and ↑ CD8+ cells	—	Li et al. (2005)
	Exposed (35)	14.1			
Children aged 6 months – 15 years	6–35 months	7	↑ IgA, IgG and IgM levels in children under 3 years of age with BLLs ≥15 μg/dl	No effects in children over 3 years of age; results are confounded by the presence of cadmium	Sarasua et al. (2000)
	36–71 months	6			
	6–15 years	4			

[a] Number in parentheses equals *N* for that group, when available; reference = control group selected as reference for comparison with lead-exposed population.
[b] Mean values, ± standard deviation when available, unless indicated otherwise.
[c] Serum levels.
[d] Effects occurred in all lead-exposed groups unless indicated otherwise.

& Piepenbrink, 2006). Similarly, Boscolo et al. (1999) reported that elevated B cell numbers and serum IgE concentrations were associated with increased BLLs. Elevated serum IgE levels have also been detected in children exposed to environmental lead (Lutz et al., 1999). In a prospective study of the children of 224 women in Poland recruited in the second trimester, maternal and cord BLLs below 2 µg/dl were significantly associated with the frequency of allergic sensitization in the children, determined by skin prick testing to common allergens at age 5 (Jedrychowski et al., 2011). The data are not further reviewed here, because the case-study is on the potential immunosuppression associated with lead. However, it is useful to note that for a full evaluation of the immunotoxicity of lead, the risk assessor would complete an evaluation of the data on immunosuppression following guidance presented in chapter 4 and Figure 4.1. Then, as directed in Figure 4.1, the risk assessor would evaluate immunotoxicological data relevant to end-points other than immunosuppression in the appropriate chapter and include that in a weight of evidence evaluation for immunotoxicity. Given the data described above, the risk assessor would evaluate the data on IgE and hypersensitivity following the guidance in chapter 6 on sensitization and allergic response.

C1.3 Assessment of lead-induced immunosuppression

C1.3.1 Exposure assessment for lead

C1.3.1.1 Transience of immunotoxicity observed in adults

Generally speaking, the immune system has a high capacity to adapt and rebound following an insult. Insufficient data are available to clearly establish whether the immunotoxic effects of lead are transient or persistent; no adult human or experimental animal studies were identified that assessed immunotoxicity over time after lead exposure ceased. Circumstantial evidence, including the lack of effect on stem cells and BLLs decreasing upon cessation of exposure, suggests that the effects of lead are not persistent, particularly following adult exposure. The types of immune effects observed suggest alterations in immune regulatory events (e.g. altered CD4/CD8 cells or regulatory cytokines), and there is little evidence that there are significant effects on haematopoietic stem cells, at least at relevant exposure levels that would result in permanent damage. One should, however, note that the human elimination half-life of inorganic lead is 30 days in blood and approximately 27 years in bone. Therefore, the potential exists for lead bioaccumulation in soft tissues, such as liver and kidney, and bone over time. Furthermore, in adult humans, bone lead accounts for approximately 94% of the total body burden. Whereas lead in bone appears to serve as a source of bioavailable lead in blood long after exposure has ceased and has been linked to non-immune end-points such as cardiovascular and renal effects, it is currently not clear whether bone lead contributes to suppression of immunity over a long period of time. Lead exposure increases immature immune cell types (progenitor cells), indicating developmental inhibition, which may suggest that the effects will be longer lasting; however, there is no substantial evidence that this is the case.

C1.3.1.2 Sensitivity of developing immune system to lead-induced immunotoxicity

It is generally believed that the developing immune system is more susceptible than the mature immune system to chemicals. The developing immune system generally has a greater risk of toxicity than the adult system as a result of two factors—lower doses can cause adverse effects and adverse effects may be longer lasting—or a combination of the two (Luebke et al., 2006). However, available evidence indicates that immune effects occur in

children and adults at similar BLLs. BLLs at and below 10 µg/dl prenatally or in infants can result in cognitive and behavioural deficits (Goyer, 1993; Bellinger et al., 2004; Hu et al., 2006; USEPA, 2006; Jedrychowski et al., 2008), whereas studies across several animal species suggest that perinatal BLLs of approximately 10 µg/dl are also associated with juvenile immunotoxicity (Dietert et al., 2004). In a large study conducted in children, Sarasua et al. (2000) found BLLs greater than 15 µg/dl to be positively associated with changes in serum IgG, IgA and IgM levels and peripheral B cell counts. In adults, changes observed in immunoglobulin levels have been inconclusive (see Table C1.3). Thus, available data do not suggest age-related differences in immunosuppressive effects of lead in humans. In neonatal mice, immunotoxic changes can be observed at BLLs less than 20 µg/dl, whereas in adult rodents, immune alterations have been observed at similar BLLs (≤40 µg/dl) (Dietert et al., 2004). Experimental animal data do suggest that there are windows of vulnerability during development in which lead-induced effects on the immune system may be particularly detrimental (Dietert et al., 2004). The developing fetus and child may also represent life stages with higher exposure because of physiological factors or behaviour, such as ingestion of dust and paint chips. The increased mobilization of lead from bone during pregnancy is likely to represent an increased exposure for the developing fetus relative to lower maternal BLLs before and after pregnancy.

C1.3.1.3 Persistence of immunotoxicity following developmental exposure

In aggregate, published data suggest that immunotoxic effects are likely to persist longer when exposure occurs during development. There have been no immunotoxicity studies conducted in children following the removal of lead from their environment. In experimental animals, Bunn et al. (2001b) and Miller et al. (1998) administered lead in the drinking-water to female F344 rats either from days 2 to 21 of gestation or from 2 weeks preceding mating throughout pregnancy. Numerous immune alterations were observed, particularly in the female pups, including a pronounced reduction in the DTH response (LOEL = 250 mg/l) and IFN-γ production, whereas production of IL-4 and total serum IgE were elevated (LOEL = 100 mg/l). Bunn et al. (2001a) reported that suppression of the DTH response was associated with a BLL of 38 µg/dl (immediately post-exposure); the BLL for the 100 mg/l dose in females at birth was 7.6 µg/dl. In both of these studies, BLLs at the time of immune assessment (5 and 13 weeks of age) were at background levels, suggesting that the lead-induced immunotoxicity may persist following exposure during development.

C1.3.2 Application of the weight of evidence approach

A series of questions is presented in chapter 4, section 4.8.1, “Weight of evidence approach for assessment of immunosuppression”, that is intended to aid in organizing and characterizing immunotoxicity data from strong to weak evidence of significant immunosuppression. The questions are reproduced and answered below, followed by a discussion of the supporting immunotoxicity data.

C1.3.2.1 Are there epidemiological studies, clinical studies or case-studies available that provide human data on end-points relevant to immunosuppression (i.e. incidence of infections, response to vaccination, DTH, lymphocyte proliferation, other data)?

Yes. There have been quite a few retrospective studies in humans exposed to lead. The data are inconclusive as to whether lead is immunosuppressive due to the variability of the data; however, a number of studies do suggest that lead has some effect on the immune system.

Several studies suggest that lead exposure affects resistance to infections in humans. Ewers et al. (1982) reported a "slight tendency" (no statistical analysis presented) for an increase in the annual incidence of colds and influenza in men exposed occupationally for at least 2 years. The range of BLLs was 21.3–85.2 µg/dl in exposed workers and 6.6–20.8 µg/dl in controls. A study of Japanese lead workers determined that individuals with BLLs greater than 60 µg/dl were significantly more likely to have two or more colds annually compared with individuals with BLLs below 60 µg/dl (Horiguchi et al., 1992). Rabinowitz et al. (1990) reported increased risk of respiratory tract illness, severe ear infections and illness other than colds or influenza (OR 1.2–1.5; 95% confidence interval [CI] 1–2.4) in children of lead industry workers.

Six human studies were identified in which functional testing was conducted. The first study did not detect an association between BLLs and antibody responses to tetanus toxoid vaccination in children (Reigart & Graber, 1976). However, in this study, only 7 children with BLLs below 30 µg/dl (group mean BLL = 22.6 µg/dl, range = 14–30 µg/dl, considered "normal" at the time) were compared with a group of 12 children with "elevated" BLLs (mean = 45.3 µg/dl, range = 41–51 µg/dl). It should be noted that in this study, the BLLs in the control group exceeded the current CDC action limit.

A series of five manuscripts reported inhibitory effects of lead on PMNLs and macrophages. Governa et al. (1988) reported that chemotaxis of PMNLs was significantly impaired in Italian lead workers at a mean BLL of 63.2 µg/dl compared with 19.2 µg/dl in the reference population. In this study, haematological and metabolic parameters were similar between controls and lead workers. Increased BLLs were also reported to be directly correlated with decreased PMNL chemotactic indices in lead workers (Valentino et al., 1991). Queiroz et al. (1993, 1994) reported decreased PMNL chemotaxis and lytic activity in workers with BLLs of 12–90 µg/dl. Bergeret et al. (1990) reported similar effects at BLLs of 71 µg/dl in workers compared with 9 µg/dl in the control group. Effects of lead on PMNLs and macrophages were also reported by Pineda-Zavaleta et al. (2004), in that an association was found between BLLs in children (ranging from 4 to 50 µg/dl) and activation of macrophages and PMNLs. However, it appears that at least two studies reported results from the same test population, and in some studies the effects were not dose related.

It does appear that PMNLs are one of the targets of lead's toxicity. One study assessed intracellular killing of *Candida albicans* and *C. pseudotropicalis* by neutrophils in workers exposed to lead (Queiroz et al., 1994). Phagocytosis of both antigens and phagocytic splenic function were normal in all workers; however, the lytic activity of *C. albicans* was impaired. The average BLL of the 33 workers examined was 43.2 µg/dl. These data may be suitable for a quantitative risk assessment, as BLLs were recorded and the effect on PMNLs seems reproducible. Uncertainty factors as described in section 3.3.10 of chapter 3 of the guidance should be applied as follows:

- The interspecies uncertainty factor would be 1, as this study was conducted in humans.
- The intraspecies uncertainty factor to account for interindividual variability would be 10 in the absence of more definitive data. In addition, this study did not examine dose–response relationships, so the minimum dose at which an effect on PMNLs would be observed is not clear.
- The LOAEL to NOAEL uncertainty factor would be 10.

- The subchronic to chronic uncertainty factor would be 3. The mean exposure period in this study was 4 years, but some workers were exposed for only 6 months.
- The database for lead toxicity is comprehensive, and it includes a substantial number of data on the immune effects caused by lead. Thus, the database uncertainty factor would be 1.

To complete the derivation of an AEL, the guidance recommends consideration of groups at risk (i.e. children and elderly) and then dividing the POD by the total uncertainty factor. Using the above uncertainty factors for a risk assessment of immunosuppression, the total uncertainty factor applied would be 300 (1 for interspecies, 10 for intraspecies, 10 for LOAEL to NOAEL, 3 for subchronic to chronic and 1 for database). Application of this uncertainty factor to the BLL obtained from the study (i.e. the POD) results in a BLL of 0.144 µg/dl (i.e. 43.2/300) as the AEL.

There are a number of studies that report lead effects on general immune assays in young and adult humans (see Tables C1.3–C1.4). In children, environmental exposure to lead has been positively associated with serum immunoglobulin levels (i.e. IgG, IgM, IgA and IgE) as well as the number of CD8+ and B cells and negatively associated with CD4+ cell numbers (see Table C1.4). In adults, lead exposure has been negatively associated with NK and B cell numbers, C3 and C4 complement concentrations, and serum IgG, IgM and IgA levels. Two studies of occupational lead exposure reported reduced mitogen responses in lead-exposed individuals (Fischbein et al., 1993; Mishra et al., 2003). Mishra et al. (2003) reported significantly reduced mitogen responses in workers with BLLs as low as 6.5 µg/dl, although non-specific mitogen stimulation, as a test for immune function, is rarely used due to an apparent lack of sensitivity. In adult humans, decreased monocytes were observed in a study of occupational exposure to lead over 5 years in which BLLs ranged from 15 to 55 µg/dl (Pinkerton et al., 1998). There were no histopathological or organ weight data available for humans.

Despite the seemingly large number of human studies in lead-exposed individuals, most data in humans were considered inadequate to establish an accurate POD (see Tables C1.3–C1.4). None of the studies established a causal association, and all were retrospective in nature. In addition, there is little information on immune functional end-points in humans with low BLLs. Furthermore, a biologically plausible immunotoxic profile could not be established, nor were the reported effects between populations with similar BLLs consistent. For example, several studies showed decreased immunoglobulin levels in lead-exposed workers; however, several studies also showed no effect on immunoglobulin levels. Other studies suggested that lead may be immunostimulatory in children (see Table C1.4). Sarasua et al. (2000) found that in children under 3 years of age with BLLs greater than or equal to 15 µg/dl, IgA, IgG and IgM levels were increased. In addition, Lutz et al. (1999) found that IgE levels were increased in children with lead exposure greater than or equal to 10 µg/dl; however, in this study, no effect was observed on other general immune parameters. One study suggested that lead may cause a shift in the T cell responses (Li et al., 2005), but yet another study suggested that lead has no effect on the immune system in children (Reigart & Graber, 1976). The weight of evidence in humans suggests that lead exposure produces changes in the immune system, but there are not enough data to determine the precise effect and/or mechanism of immunotoxicity; in fact, there appears to be evidence that lead acts (especially at lower levels) as an immunostimulating agent rather than an immunosuppressive agent. The PMNL data described above were determined to be suitable for a quantitative risk assessment because of the number of studies in which an effect was observed. The AEL for these data was determined to be 0.144 µg/dl (see calculations above).

Note: The following questions from the weight of evidence approach refer to experimental animal data only.

C1.3.2.2 Is there evidence that the chemical reduces resistance to infections and/or tumours?

Yes. There is clear evidence from multiple animal studies that host resistance to bacterial infection is compromised following lead exposure as low as 82.9 mg/l or at a BLL as low as 20.5 µg/dl. A USEPA document on air quality criteria (USEPA, 2006) reported multiple rodent host resistance studies in which mortality was increased in animals exposed to lead and a variety of pathogens. A lead dose of 20 mg/kg body weight administered intravenously in rats led to 80–96% mortality from *Staphylococcus epidermidis* and *Escherichia coli*, compared with 0% mortality in non-lead-exposed animals. In mice, lead exposures of 2000 mg/l orally for 2 weeks led to 100% mortality from EMC virus, compared with 19% mortality in non-lead-exposed mice.

Multiple studies in adult mice reported increased susceptibility to bacterial challenge following exposure to lead (see Table C1.1). Susceptibility was monitored by either mortality or bacterial counts in organs (e.g. the spleen). The results showed increased susceptibility to *Listeria monocytogenes* at drinking-water concentrations of greater than or equal to 82.9 mg/l for 4 weeks (Lawrence, 1981), *Staphylococcus aureus* by daily intraperitoneal lead injections of 10 mg/kg body weight for 15 days (Bishayi & Sengupta, 2003) and *Salmonella typhimurium* following exposure to drinking-water containing lead at 1036 or 2072 mg/l for 16 weeks (Fernandez-Cabezudo et al., 2007). The most sensitive model appears to be reduced resistance to *Listeria* infection in mice, where increased bacterial counts in the spleen (at exposure of 2072 mg/l) and increased mortality (at exposure above 82.9 mg/l) were observed following exposure to lead in drinking-water for 4 weeks (Lawrence, 1981). This exposure level could have been selected as the LOAEL. However, BLLs were not determined in this study, and direct exposure comparisons could not be made between humans and experimental animals using an internal dose metric, substantially decreasing confidence in the quantitative evaluation of immunosuppressive effects.

Lead exposure in animals leads to decreased host resistance to other pathogens. These data appear to contain strong evidence that lead may be immunosuppressive, in particular the Fernandez-Cabezudo et al. (2007) study, in which a dose-related decrease in survival was observed following lead exposure. These data also include BLL values for each dose group (20.5 and 106 µg/dl at 1036 and 2072 mg/l, respectively), increasing the utility of the host resistance data in determining a POD. Host resistance assays, when designed and conducted well and related to immune system defects, provide the most direct evidence of an adverse health effect. In addition, two epidemiological studies were identified that suggest decreased resistance to respiratory infection in lead workers, and changes were also observed in general immune assays in rodents. The combination of host resistance, functional immune measures and general immune assays makes the rodent immunotoxicity data for lead a very strong data set. The oral route of exposure in this study is extremely relevant to human exposure.

In the Fernandez-Cabezudo et al. (2007) study, C3H/HeN mice were exposed to a range of concentrations (0, 1036 and 2072 mg/l) of lead acetate in the drinking-water for approximately 16 weeks. The average BLLs were 2.9 ± 1.1, 20.5 ± 1.1 and 106.2 ± 8.9 µg/dl in the 0, 1036 and 2072 mg/l exposure groups, respectively. Deliberate lead exposure increased susceptibility to *Salmonella* infection in mice, as demonstrated by increased bacterial burden

in target organs at the higher dose and increased mortality at both doses; bacterial burdens were not assessed in the 1036 mg/l exposure group. No changes in numbers or function of B and T cells were observed in this study. Ex vivo–cultured splenocytes showed a marked decrease in IFN-γ and IL-12p40 production. Increased secretion of IL-4 by splenocytes was observed in lead-exposed mice as well, suggesting a plausible explanation of the observed shift in the in vivo anti-*Salmonella* antibody response from the protective IgG2a isotype to the non-protective Th2-induced IgG1 isotype. BLLs correlated with the dose of lead exposure in the 1036 and 2072 mg/l lead acetate groups, respectively. The BLL in control mice was similar to background levels in the USA population. Mice were infected with a sublethal dose of a virulent strain of *Salmonella typhimurium* at week 16 of treatment and observed for mortality for up to 60 days. Overall survival in control mice was 80%, with a median survival time of 60 days. Mice exposed to lead acetate at a concentration of 1036 mg/l had a survival rate of 40%, with a median survival time of 26 days. None of the mice treated with lead acetate at 2072 mg/l survived the infection, with a median survival time of 16 days. The increased mortality at the high dose correlated with increased bacterial burden in mesenteric lymph nodes, spleen and liver. Cytokine production (IL-4, IL-12, TNF-α and IFN-γ) was assessed using ex vivo–cultured spleen cells. Reductions in levels of IL-12p40 of 22–25% without stimulation and 42–45% with stimulation were observed in lead-exposed splenocytes. Without stimulation, splenocytes did not secrete any detectable levels of TNF-α, and levels of IFN-γ following stimulation were reduced 27–35% in the 1036 and 2072 mg/l groups, respectively. In contrast, levels of IL-4 were increased in splenocyte cultures of lead-exposed mice. The LOAEL for lead acetate in this study was 1036 mg/l, with a corresponding BLL of 20.5 ± 1.1 µg/dl. The use of this LOAEL is somewhat less than ideal owing to the lack of a no-effect level; however, this was one of the few host resistance studies that included BLLs, making these data more suitable for a lead risk assessment. This study was conducted in adult animals; therefore, it likely underestimates the risk to children, whose immune system has yet to fully develop, as well as the elderly, whose immune systems are undergoing senescence.

To continue the risk assessment using the Fernandez-Cabezudo et al. (2007) LOAEL, uncertainty factors as described in section 3.3.10 of chapter 3 of the guidance should be applied:

- The interspecies uncertainty factor would be 3. This study was not conducted in humans; however, the metric being used is an internal concentration, not a dose level or dose concentration. Changes in host resistance have also been observed in humans.
- The intraspecies uncertainty factor to account for interindividual variability would be 10 in the absence of more definitive data.
- The LOAEL to NOAEL uncertainty factor would be 10.
- The subchronic to chronic uncertainty factor would be 10.
- The database for lead toxicity is comprehensive, and it includes a substantial number of data on the immune effects caused by lead. Thus, the database uncertainty factor would be 1.

To complete the derivation of an AEL, the guidance recommends consideration of groups at risk (i.e. children and elderly) and then dividing the POD by the total uncertainty factor. As discussed above, there is evidence that the immune systems in children and the elderly are more susceptible to toxicity. Using the above uncertainty factors for a risk assessment of immunosuppression, the total uncertainty factor applied would be 3000 (3 for interspecies, 10 for intraspecies, 10 for LOAEL to NOAEL, 10 for subchronic to chronic and 1 for database).

Application of this uncertainty factor to the BLL obtained from the LOAEL (i.e. the POD) results in a BLL of 0.0068 µg/dl (i.e. 20.5/3000) as the AEL.

C1.3.2.3 Is there evidence that the chemical reduces immune function (antibody production, NK cell function, DTH, MLR, CTL, phagocytosis or bacterial killing by monocytes, etc.)?

Yes. Considerable evidence demonstrates the effect of lead on functional immune responses in both the adult and developing experimental animals. Effects included both suppression of the antibody PFC response, an indicator of humoral immune function, and suppression of DTH, a classical measure of cell-mediated immunity. However, the effects on DTH appear much more consistent between investigations than the effects on the PFC response. For example, Luster et al. (1978) reported decreased PFC responses following prenatal/postnatal exposure at BLLs as low as 29 µg/dl in Sprague-Dawley rats, and Blakley & Archer (1981) reported suppressed PFC responses in vitro at a BLL of 25 µg/dl. However, Mudzinski et al. (1986) and Lawrence (1981) failed to show effects on the PFC response following testing in rodents and under various exposure paradigms. The DTH response is dependent upon T cell priming and recruitment to a site of antigen localization, where interference with this Th1-mediated process would lead to a decreased immune response to pathogenic challenge. In contrast to the PFC data, consistent suppression of the DTH response has been observed across multiple test species and at various exposure paradigms, including prenatal, neonatal or adult exposure (Table C1.5). BLLs as low as 6.8 µg/dl in 4-week-old pups were associated with suppression of the DTH response (Chen et al., 2004). A decreased DTH response was also observed in pups exposed to lead until postnatal day 45 (BLLs greater than or equal to 29.3 µg/dl), whose dams were exposed from premating to weaning (Faith et al., 1979).

A study by McCabe et al. (1999) also included BLL values for each dose group, making these data desirable for obtaining a POD. The route of exposure in this study, which was drinking-water, is extremely relevant to human exposure. The weaknesses of the data are a lack of correlating human data. DTH testing is used in children to help diagnose primary T cell deficiencies; however, less is known about the correlation between minor/moderate changes in DTH responses and corresponding human health. In this study, adult BALB/c mice were sensitized intravenously with 10^8 SRBCs followed by a subcutaneous challenge 4 days later in the right foot pad with SRBCs. The DTH response (foot pad swelling) was measured by comparing the size of the challenged foot pad before and after (24 hours) antigen challenge. In control mice, a 0.48 mm increase in foot pad size occurred; however, in mice exposed to 512 mg/l lead orally, only a 0.11 mm increase in foot pad size occurred. The lowest effective dose in this study was 512 mg/l, with a corresponding BLL of 87 µg/dl. This study was conducted in adult animals; therefore, it likely underestimates the risk to children, whose immune system has yet to fully develop, as well as the elderly, whose immune systems are undergoing senescence.

Lead has been shown to be immunotoxic to macrophages, not only by interfering with macrophage development such as the response to colony stimulating factor–1 (CSF-1) (Kowolenko et al., 1989), but also by altering mature tissue macrophage function. Antigen presentation and T lymphocyte stimulation in mouse macrophages are decreased following lead exposure as low as 82.9 mg/l for up to 4 weeks (Kowolenko et al., 1988). Nitric oxide production is decreased in macrophages from multiple species, and one in vitro study showed reduced nitric oxide production at BLLs equivalent to 10 µg/dl (Tian & Lawrence, 1996). Nitric oxide is important to the function of macrophages, as it is, in part, responsible for the antimicrobial and cytotoxic activity of this leukocyte.

Table C1.5: Lead-induced suppression of delayed-type hypersensitivity and related responses.[a]

Species/ strain	*Age*	*Route[a]*	*LOEL*	*Duration*	*BLL (µg/dl)*	*Reference*
Mice						
BALB/c	Adult	Oral	512 mg/l	3 weeks	87	McCabe et al. (1999)
Swiss	Adult	Subcutaneous	0.5 mg/kg body weight per day	3 days	NM	Laschi-Loquerie et al. (1984)
BALB/c	Adult	Intraperitoneal	0.025 mg/day	30 days	NM	Müller et al. (1977)
Rats						
SD	Prenatal	Maternal	250 mg/kg	5 weeks	6.8 at 4 weeks	Chen et al. (2004)
CD	Embryo/fetal	Maternal	500 mg/kg	6 days	NM	Bunn et al. (2001c)
F344/CD	Embryo/fetal	Maternal	250 mg/kg	3 weeks	NM	Bunn et al. (2001b)
F344	Embryo/fetal	Maternal	250 mg/kg	3 weeks	34.8 at birth	Bunn et al. (2001a)
F344	Embryo/fetal	Maternal	250 mg/kg	5 weeks	NM	Chen et al. (1999)
F344	Embryo/fetal	Maternal	250 mg/kg	5 weeks	NM	Miller et al. (1998)
Wistar	Adult	Oral	25 mg/kg	16 weeks	29.3	Faith et al. (1979)
Chickens						
Cornell K	Embryo	*In ovo*	200 µg	Acute, embryonic day 12	NM	Lee et al. (2002)
Cornell K	Embryo	*In ovo*	200 µg	Acute, embryonic day 12	87	Lee et al. (2001)
Goats						
Outbred	Adult	Oral	50 mg/kg body weight per day	6 weeks	NM	Haneef et al. (1995)

NM, not measured

The data on macrophages appear to be consistent, in that macrophage function seems to be suppressed upon lead exposure. In addition, in vitro human data suggest that lead exposure leads to decreased chemotaxis of PMNLs, which may be relevant to the increased respiratory infections in humans. Both of these cell types are very important to innate as well as adaptive immunity. Cell-mediated immunity suppression data are consistently seen with lead exposure; however, the data on lead's effect on humoral immunity are less conclusive. The data on DTH suppression in animals may be the most complete and reproducible data set on lead as an immunosuppressant. Therefore, these data are suitable for a quantitative risk assessment. Although it is clear that the DTH response was suppressed at 512 mg/l (BLL of 87 µg/dl), the McCabe et al. (1999) study also tested lower doses to establish a dose–response

relationship. Mice were exposed to 0, 32, 128, 512 or 2048 mg/l; however, the authors do not report the statistics for individual comparisons of the DTH relative to control except for the 512 mg/l dose. It is unknown if mice exposed to lead doses of 128 and 32 mg/l with corresponding BLLs of 49 and 9 µg/dl had a suppressed DTH response. Therefore, the 512 mg/l dose with the corresponding BLL of 87 µg/dl was used as a LOAEL.

To continue the risk assessment using the McCabe et al. (1999) lowest effective dose or LOAEL, uncertainty factors as described in section 3.3.10 of chapter 3 of the guidance should be applied:

- The interspecies uncertainty factor would be 3. This study was not conducted in humans; however, the metric being used is an internal concentration, not a dose level or dose concentration.
- The intraspecies uncertainty factor to account for interindividual variability would be 10 in the absence of more definitive data.
- The LOAEL to NOAEL uncertainty factor would be 10.
- The subchronic to chronic uncertainty factor would be 10.
- The database for lead toxicity is comprehensive, and it includes a substantial number of data on the immune effects caused by lead. Thus, the database uncertainty factor would be 1.

To complete the derivation of an AEL, the guidance recommends consideration of groups at risk (i.e. children and elderly) using the intraspecies uncertainty factor and then dividing the POD by the total uncertainty factor. As discussed above, there is evidence that the immune systems in children and the elderly are more susceptible to toxicity. Using the above uncertainty factors for a risk assessment of immunosuppression, the total uncertainty factor applied would be 3000 (3 for interspecies, 10 for intraspecies, 10 for LOAEL to NOAEL, 10 for subchronic to chronic and 1 for database). When one applies this uncertainty factor to the BLL obtained from the LOAEL (i.e. the POD), the AEL is 0.029 µg/dl (i.e. 87/3000).

C1.3.2.4 Is there evidence from general or observational immune assays (lymphocyte phenotyping, cytokines, complement, lymphocyte proliferation, etc.) that the chemical is immunosuppressive?

Yes. There is evidence that lead affects general markers of immune system health in experimental animals. Lead exposure in animals causes a shift in immune cells to immature cell types (progenitor cells) (Burchiel et al., 1987). Whereas Fernandez-Cabezudo et al. (2007) reported no gross alteration in the ratio of B to T lymphocytes in mice administered lead in drinking-water at 2072 mg/l for 16 weeks (BLL = 106 ± 9 µg/dl), concentrations of the cytokines IFN-γ and IL-12p40 were reduced and secretion of IL-4 was increased in ex vivo–cultured splenocytes. This increased production of IL-4 correlated with a shift in the in vivo anti-*Salmonella* antibody response from the protective IgG2a isotype to the Th2-induced IgG1 isotype. The authors suggested that rather than a cytotoxic process, lead causes a shift in immune responsiveness to Th2-type reactions. Chen et al. (2004) also reported that maternal lead exposure (250 mg/l in drinking-water) during early development reduced IFN-γ production in adult offspring. They also reported that offspring from lead-exposed dams fed a high-protein diet had elevated production of both IL-4 and TNF-α compared with offspring from lead-exposed rats fed a normal diet, although the relevance of these findings in terms of this risk assessment is unclear.

Decreases in temporal levels of cytokines are not necessarily reliable evidence of immunosuppression, and the predictive value of immunophenotyping has not been established. Several studies demonstrated no difference in immune parameters. All of these factors call into question the use of these data in a quantitative or qualitative risk assessment; however, the observed immunosuppressive effects do add to the weight of evidence evaluation of lead and its suppression of the immune system.

C1.3.2.5 Is there evidence that the chemical causes haematological changes (e.g. altered WBC counts) suggestive of immune effects?

Yes. Very few immunotoxicological studies reported significant haematological effects from lead exposure. Total peripheral WBC counts were significantly decreased in offspring of dams that received lead at either 250 or 500 mg/l (BLL = 70.8 and 112 μg/dl) in their drinking-water during breeding and pregnancy (Miller et al., 1998). Although peripheral WBCs were not enumerated, Snyder et al. (2000) reported similar decreases in splenic lymphoid cells in mice at 2–3 weeks of age exposed in utero and postnatally to doses of lead acetate as low as 16.6 mg/l. At greater than or equal to 4 weeks of age, only the mice that were treated during gestation and lactation maintained this decrease in splenocytes. In these studies, dams were given lead at 16.6–207.2 mg/l in the drinking-water starting on day 15 of gestation. Bunn et al. (2001a) reported decreased relative and absolute peripheral monocyte numbers (74% decrease) and increased relative neutrophil numbers in 13-week-old female offspring of dams exposed to lead during gestation (100 mg/l in the drinking-water). Male offspring had increased relative neutrophil numbers and decreased relative lymphocytes at 5, but not 13, weeks of age.

Associations between moderate changes in WBC counts of this nature and health effects are often difficult to establish and cannot be justified in view of the fact that both functional immune and host resistance data for lead are available. Many of these studies did not report BLLs, and the data are not consistent. A quantitative or qualitative risk assessment using these data would be of questionable utility.

C1.3.2.6 Is there histopathological evidence (thymus, spleen, lymph nodes, etc.) that suggests that the chemical causes immunotoxicity?

No. Faith et al. (1979) reported that prenatal/postnatal exposure to lead altered spleen and thymus weights (see section C1.3.2.7), but that there were no histopathological differences between the organs in the control and exposed groups.

C1.3.2.7 Is there evidence that the chemical reduces immune organ weight (thymus, spleen, lymph nodes, etc.)?

Yes. Although very few animal studies included examination of lymphoid organs, changes in lymphoid organ weights were noted primarily following prenatal/postnatal lead exposure. Faith et al. (1979) reported significant decreases in thymus weights in rats following prenatal and postnatal lead exposure (25 mg/l in drinking-water) at BLLs as low as 29 μg/dl; however, spleen weights increased, only in males, at a reported BLL of 52.8 μg/dl (50 mg/l in drinking-water). Bunn et al. (2001a) determined relative splenic weights in male and female rats at 5 and 13 weeks of age following gestational exposure, with the following results. Female offspring of rats administered lead at 250 mg/l in drinking-water had significantly increased relative spleen weights at 13 weeks of age, but this effect could not be detected at 5 weeks of age. The relative spleen weights of all female treatment groups were significantly higher than

those of all male treatment groups (except males at 250 mg/l compared with female controls) at 13 weeks of age. In the study by Kim & Lawrence (2000), adult mice had decreased spleen weights following lead exposure (BLL = 45 µg/dl) and infection with *L. monocytogenes*, further supporting a role of lead in the reduction of host resistance.

As immune organ weight data are limited in animals and contradictory for the spleen, these findings are equivocal towards the weight of evidence for lead-induced immunosuppression.

C1.3.3 Weight of evidence review of risk assessments of lead as an immunosuppressant

Lead is found in a myriad of environmental sources, including landfills, hazardous waste sites, mining areas, older domestic structures and even soil around old fruit orchards. As such, normal human activity patterns present potential exposures to this ubiquitous metal via ingestion of food and water, inhalation of air or swallowing particulate matter that contains lead. Furthermore, it should be noted that lead exists in several forms (e.g. lead salts, tetra-ethyl lead), and the physiological effects of lead and lead compounds via all possible routes of exposure are numerous. However, this case-study is not intended to be a comprehensive review of the toxic effects of lead in general; rather, the hazard characterization presented here is focused specifically on the immunosuppressive effects of inorganic lead via the oral route.

As noted in the studies highlighted in this case-study, the effects of lead on the immune system vary across similar internal dose ranges (i.e. BLLs) and exposure durations/life stages (e.g. Tables C1.1–C1.4). Several human studies are available illustrating associations between increased BLLs and alterations in immune phenotype. Results from these human studies, including occupationally exposed adult populations and environmental exposures in children, were almost always limited to non-functional tests of immunity. Lead-induced decreases in host resistance are a primary effect of concern for the immune system because of the public health implications, and there were few functional testing or incidence data available on infectious diseases in humans. Ewers et al. (1982) reported suggestive anecdotal evidence of reduced resistance to infection in lead workers with BLLs 2-fold or more greater than those considered "normal" at that time. The effects (when measured) most commonly observed in human epidemiological studies included changes in serum immunoglobulin levels, particularly IgG and IgA, and immunophenotypic profiles; however, the changes were not consistent and varied greatly between adults and children. There was little evidence of changes in standard haematological parameters, such as WBC counts or differentials. One major weakness of the human studies in workers was that the epidemiological evaluations were retrospective in nature and contained minimal occupational exposure histories. There was also evidence of data inconsistencies in human studies. For example, whereas decreased numbers of CD4+ cells and normal numbers of CD8+ cells were a common observation, several studies reported normal numbers of CD4+ cells but reduced numbers of CD8+ cells. Inconsistencies in the human epidemiological studies may be due to type 1 errors from multiple testing modalities, faulty study design or simply human variability. Furthermore, it should be noted that the test methods initially adopted by immunotoxicologists to assess immune function in humans (and experimental animals) prior to 1990 were not standardized. Some assays are still not standardized; therefore, the methods must be assessed fully, as well as the results. The tests that were commonly performed and the experimental design by which they were conducted were established ad hoc, often resulting in inconsistent findings.

Therefore, empirical observations of immunosuppressive effects of lead in humans are not considered a reliable basis for quantitative assessment.

The immunosuppressive effects of lead exposure in adult and neonatal rodents include alterations in antigen presentation, decreased cytokine production, decreased antibody and DTH responses and increased susceptibility following challenge with infectious agents. Host resistance data are available in animals following challenge with different infectious agents, including both intracellular and extracellular bacteria (see Table C1.1). It should be cautioned, however, that in several instances, increased susceptibility to infection occurred at BLLs below those reported to affect markers of immune function (Lawrence, 1981). This may be due to variances in duration of exposure or non-immunological mechanisms (Luster et al., 1978). It can be argued that the host resistance data provide better insight into immune and general health as a whole. As discussed in chapter 4, the host resistance assays present the best animal data for a quantitative risk assessment, especially in those studies in which BLLs were measured. However, the POD should be considered in the context of the larger picture of all immune effects; that is, lead may not be strictly immunosuppressive but rather immunomodulatory, in that its effects on the immune system are not always suppressive. One of the most consistent findings in animals following lead exposure was a reduced DTH response (see Table C1.5). However, although significant decreases in the antibody response following immunization were commonly reported, the effect was not consistent across studies or species. Human data on DTH seemed to suggest an upregulation of the cell-mediated immune response in lead-exposed workers and children, contradictory to the effects seen in animals. A general weakness of the rodent studies was that they represented subchronic exposures and were conducted in only one sex. The strengths of the animal database for lead-induced immunosuppression include broader coverage of decreased host resistance, ample number of developmental immunotoxicity studies and greater confidence in the exposure–response relationships (see Tables C1.1 and C1.2).

Mechanistic data applicable to risk assessment are limited. For example, the MOA for lead-induced developmental effects is believed to be related to changes in regulatory events, most notably a shift in the balance of Th1/Th2 cell activity, evidenced by changes in regulatory cytokine levels, including IL-4 and IFN-γ (Dietert & Piepenbrink, 2006). The biological plausibility of persistent chemical-induced altered immunoregulation following developmental exposure to lead is supported by similar outcomes following exposure to a variety of xenobiotics (Wang & Pinkerton, 2007). Dietert & Zelikoff (2009) suggested that lead exposure during immune system development causes a persistent shift in the ratio of Th1 to Th2 cytokine production, even after BLLs fall to control values. The shift results in a predominance of an allergic phenotype, reducing Th1-driven responses that protect the host against infections. However, the data, while interesting, are observational and are not discussed here in detail, because the underlying defect responsible for shifting Th1 and Th2 cytokine production has yet to be identified.

C1.4 Conclusions

The data in experimental animals and humans, although variable, suggest that lead suppresses defence mechanisms and induces hypersensitivity. Numerous studies in adult rodents have shown that oral lead exposure induces changes in functional and general immune assays. Although comparative data are limited, it would appear that cell-mediated immunity, rather than humoral immunity, is the primary target, as evidenced by multiple studies revealing decreased host resistance to pathogens that are cleared by cell-mediated immune responses.

Epidemiological studies in environmentally exposed children and occupationally exposed adults also show immune effects, but in many of the studies, only non-functional immune tests were conducted (e.g. serum immunoglobulin levels and immunophenotypic analyses). Importantly, the immune effects seen in humans occur at BLLs similar to those associated with immunotoxicity in many animal studies. Although the effects of lead on infectious disease incidence were studied infrequently in humans, a number of susceptibility studies conducted in animals indicated that lead decreases resistance to both Gram-positive and Gram-negative bacterial infection at lead concentrations similar to, if not lower than, levels that affected functional immunity. Immune changes have also been repeatedly observed in developmental immunotoxicity studies in rodents as well as in studies of children with environmental exposure, although the latter are somewhat limited in number and scope. Immune effects in children occurred at BLLs similar to those associated with effects in occupationally exposed adults, with LOAELs in the range of 10–20 µg/dl. Although immune effects may be more persistent if exposure occurs during immune system development, supporting data are limited.

PMNLs are critical cells in nonspecific immunity and play a pivotal role in host defence against extracellular pathogens. These cells usually act in concert with numerous humoral factors to increase the effectiveness of a host's defence. In addition, the macrophage, another leukocyte important to innate immunity and pathogen clearance, is also negatively impacted by exposure to lead. Therefore, lead appears to inhibit chemotaxis of PMNLs, suppresses macrophage function and decreases overall cell-mediated immunity; these effects may possibly explain the increased incidence of infection in humans and experimental animals.

The three most consistent findings are decreased PMNL function in occupationally exposed humans (AEL = 0.144 µg/dl), decreased host resistance in C3H/HeN mice following exposure to lead in drinking-water for 16 weeks (AEL = 0.0068 µg/dl) and suppressed DTH responses in BALB/c mice following a 3-week exposure to lead in drinking-water (AEL = 0.029 µg/dl). All three of the guidance values are based on LOAELs, increasing the uncertainty in identifying the lowest dose associated with immunosuppression. Furthermore, both animal studies employed subchronic exposure, resulting in additional uncertainty in extrapolating from these data to the development of a reference value for chronic human exposure. Thus, even though there is a relatively large number of lead immunotoxicity studies, the reference values from the animal data include total uncertainty adjustments of 3000, the largest total uncertainty factor allowed by many organizations that conduct risk assessments. Total uncertainty factors greater than 3000 are not considered appropriate for derivation of a reference value due to the unacceptable level of uncertainty involved.

The guidance states that suppression of human or rodent host resistance and functional immune measures are considered strong support for immunosuppression. The data therefore provide very convincing evidence that lead is immunosuppressive. Although human data (PMNL) were available to derive an AEL, functional data from exposed populations are rare and generally do not include a NOAEL (see Tables C1.3 and C1.4), contributing to uncertainty on effects in the lower dose range. Therefore, the animal data should be considered for deriving the reference value, as they provide considerable support for immunosuppression, and reference values based on the animal data would be protective of lower dose ranges not covered by the human data.

The reference value for host resistance was based on mortality (60% at a lead concentration of 1036 mg/l in drinking-water; Fernandez-Cabezudo et al., 2007), a crude, binary outcome

assessed in a single study. Increased bacterial counts were reported at the higher (2072 mg/l) exposure level. Although mortality is a dramatic outcome, the absence of bacterial counts at the lower exposure level prevents a conclusion that differences in mortality were due to colonization of lymphoid organs. There is a 4-fold difference between the reference value derived from the host resistance data (AEL = 0.0068 µg/dl) and the reference value derived from the DTH data (AEL = 0.029 µg/dl). The conservative approach for the risk assessor would be to use data that result in the lowest reference value.

However, the absence of bacterial counts creates greater uncertainty in the host resistance assay discussed above, and closer consideration of the DTH data is recommended. The reference value based on the DTH data (AEL = 0.029 µg/dl) is well supported by multiple independent studies from different laboratories and investigators that report similar lead-associated suppression of the DTH response, increasing confidence in this end-point. Furthermore, multiple investigators reported reduced resistance to infection with *Listeria monocytogenes* (Table C1.1); these studies were not chosen to determine a POD in this case-study because BLLs were not determined. Nevertheless, resistance to this organism is correlated with DTH responses (North et al., 1997), increasing confidence in the biological plausibility of altered DTH as an indication of immunosuppression and our choice of DTH as the appropriate AEL.

Please note that this case-study on lead is provided to illustrate how the risk assessment guidance can be used for assessing the risk of immunosuppression. It does not represent a comprehensive risk assessment or a final regulatory position.

C1.5 References

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CASE-STUDY 2: ASSESSMENT OF IMMUNOSTIMULATION INDUCED BY HEXACHLOROBENZENE

C2.1 Introduction

HCB is a persistent organic pollutant (POP) that has been used in the past as a fungicide for seed grains. In the 1970s, such use was prohibited in most countries. However, HCB is still used as an industrial chemical and is an unintended by-product of several processes (Bailey, 2001). HCB exposure has been associated with several toxic effects in humans, rodents and other species. The toxicity of HCB became apparent in Turkey during the 1950s, when a part of the population was accidentally exposed to high levels after seed grain treated with HCB was used to prepare bread. Approximately 3000–5000 people developed a syndrome that was called Porphyria Turcica, because the main feature was hepatic porphyria. Other symptoms observed in these victims, such as enlarged lymph nodes and arthritis, could be indicative of effects on the immune system (Cam, 1958). It was estimated that the people were exposed to 50–200 mg/day for a number of months, but the basis for this estimate was not presented, and therefore the exposure levels in all of the studies described below are unknown (IPCS, 1997).

The immunotoxic effects of HCB have been extensively studied in rodents, with studies demonstrating immunostimulation as well as immunosuppression. Interestingly, there is a strong species specificity of effects: in mice, HCB suppressed most parameters for immune function, whereas in rats, HCB stimulated most parameters for immune function (Vos, 1986; Michielsen et al., 1999b).

In this case-study, immunostimulation induced by HCB will be assessed as described in chapter 5. The focus of this case-study is unintended stimulation of the immune response and the potential implications for risk assessment. As discussed in chapter 5, deliberate stimulation of the immune system is typically beneficial and an intended therapeutic outcome, although unintended stimulation may signal changes in other immune system functions that may be deleterious. HCB was selected for this case-study because it has been shown that oral exposure can induce immunostimulation in certain species. Furthermore, data obtained in humans also suggest that this chemical can stimulate the immune system.

It is important to note that this case-study is not intended to be a full risk assessment of the health effects associated with exposure to HCB or a comprehensive assessment of all immune effects of HCB. This compound was selected to illustrate the application of the weight of evidence approach depicted in Figure 5.1 of chapter 5 to performing a risk assessment on the immunostimulatory effects induced by chemicals.

C2.2 Background on immune effects induced by HCB

HCB induces adverse immune effects in different species. In humans, immunotoxic effects are seen among the victims of the poisoning in Turkey and also in workers exposed occupationally to HCB in a chemical plant in Brazil. In these workers, impaired functions of neutrophilic granulocytes and increased serum IgM and IgG levels were observed (Queiroz et al., 1998a,b).

Studies in laboratory animals revealed that HCB has opposite immunotoxic effects in mice and rats. HCB exposure in mice suppresses the immune system (Loose et al., 1978; Barnett et al., 1987), whereas stimulation is observed in rats (Vos et al., 1979a,b, 1983; Schielen et al., 1993; Michielsen et al., 1997). Oral exposure of Wistar rats to HCB results in a dose-dependent increase of the number of peripheral neutrophilic and basophilic granulocytes and monocytes and of spleen and lymph node weights. Histopathology shows increased marginal zones and follicles and extramedullary haematopoiesis in the spleen and increased numbers of high endothelial venules in mesenteric lymph nodes and popliteal lymph nodes. The primary and secondary IgM and IgG responses to tetanus toxoid, a thymus-dependent antigen, are elevated in HCB-exposed rats (Vos et al., 1979a). Schielen et al. (1993) showed that HCB increases the number of CD3+ macrophages in the spleens of Wistar rats. These macrophages are associated with experimentally induced autoimmune diseases such as rheumatoid arthritis (Dijkstra et al., 1987, 1992) and are thought to be capable of activating B-1 cells (Damoiseaux et al., 1991). B-1 cells are known to produce natural antibodies, such as anti-DNA antibodies. HCB exposure has been shown to increase the number of splenic B-1 cells and serum IgM levels against autoantigens (Schielen et al., 1993, 1995b), indicative of an autoimmune component in HCB-induced immunostimulation.

A study comparing three different rat strains (Wistar, Brown Norway and Lewis) showed that the Brown Norway rat appears to be the most susceptible strain for HCB-induced immunotoxicity (Michielsen et al., 1997). Brown Norway rats are known to be more prone to develop type 2–dependent autoimmunity, whereas Lewis rats are more susceptible to developing type 1–mediated autoimmune diseases (Donker et al., 1984). Immune effects induced by HCB in Brown Norway rats are summarized in Table C2.1.

Table C2.1: Summary of immunotoxic effects of HCB in the Brown Norway rat.[a]

Parameter	*Doses (mg/kg body weight per day)*	*References*
Increased spleen weight	7.5, 22.5	Michielsen et al. (1997)
Increased popliteal, axillary and mandibular lymph node weight	22.5	Michielsen et al. (1997, 2002)
Increased number of high endothelial venules in popliteal lymph nodes	7.5, 22.5	Michielsen et al. (1997)
Granuloma formation in the mesenteric lymph nodes	22.5	Michielsen et al. (1997)
Inflammatory skin lesions: hyperplasia epidermis, activated dermal vessels, infiltrates of neutrophils, macrophages and eosinophils	7.5, 22.5	Michielsen et al. (1997, 1999a)
Inflammatory lung lesions: focal accumulations of macrophages, granuloma formation, perivascular eosinophilic infiltrates, high endothelial-like venules	7.5, 22.5	Michielsen et al. (1997)
Increased total serum IgM and IgE levels	22.5	Michielsen et al. (1997)
Increased total serum IgG levels	7.5, 22.5	Michielsen et al. (1997)
Increased serum IgM levels against single-stranded DNA	7.5, 22.5	Michielsen et al. (1997)
Increased in vitro and in vivo airway hyper-responsiveness	22.5	Michielsen et al. (2001, 2002)

[a] Brown Norway rats were exposed to HCB via the diet for 3 or 4 weeks. The table contains only significant changes. Dietary exposure to HCB was converted to dose in mg/kg body weight per day based on the standard assumption that rats consume 5% of their body weight per day; therefore, mg/kg body weight per day = mg/kg feed × 0.05.

The immunotoxic effects of HCB in Brown Norway rats were further investigated in a microarray study. Gene expression profiles were assessed in messenger ribonucleic acid (mRNA) isolated from the spleen, mesenteric lymph nodes, blood, liver and kidney after 28 days of exposure to HCB at a dose of 7.5 or 22.5 mg/kg body weight per day. It was shown that after HCB exposure, upregulation of genes encoding proinflammatory cytokines, antioxidants, acute-phase proteins, mast cell markers, complement, chemokines and cell adhesion molecules was induced. These gene expression data demonstrate that the innate immune system plays an important role and that HCB induces a systemic inflammatory response that was not confined only to the immune organs, but also occurred in liver and kidney (Ezendam et al., 2004b).

C2.3 Assessment of immunostimulation induced by HCB

C2.3.1 Application of the weight of evidence approach for assessment of immunostimulation

A series of questions is presented in chapter 5, section 5.8.1, “Weight of evidence approach for assessment of immunostimulation”, that is intended to aid in organizing and characterizing immunotoxicity data from strong to weak evidence of significant immunostimulation. The questions are reproduced and answered below, followed by a discussion of the supporting immunotoxicity data.

C2.3.1.1 Are there epidemiological studies, clinical studies or case-studies that provide human data on end-points relevant to immunostimulation (i.e. unintended stimulation of cellular or humoral immune function, autoimmunity or allergy)?

Yes. There are indications that HCB has immunotoxic effects in humans exposed to high levels.

The first evidence for toxic effects of HCB was noted during the 1950s, when a part of the population in Turkey was accidentally exposed to HCB. The most important toxic manifestation was porphyria cutanea tarda, which developed predominantly in children aged 4–14 years. In these victims, a few symptoms may be indicative of an effect on the immune system, such as the development of arthritis in 50% of the cases (Dogramaci, 1964; Peters et al., 1982). Furthermore, lymph node enlargement was reported, which might be an indication of immunostimulation (Cam, 1958; Peters, 1976; Gocmen et al., 1986). In breastfed children from mothers exposed to HCB, a different syndrome developed. These children did not develop hepatic porphyria, but instead developed a very severe syndrome called pembe yara (pink sore), manifested as rose-red skin lesions. This disease was fatal in approximately 95% of the victims, and the cause of death in most cases was a secondary pulmonary infection, suggesting impaired host resistance (Cam, 1960).

Additional human evidence for HCB-induced immune effects was found in workers exposed to HCB in a Brazilian chemical plant that produced carbon tetrachloride and perchloroethylene (tetrachloroethylene). In the production process, solid residues were generated, particularly HCB, which accounted for 55–85% of the residues, and HCB levels were in the range of 0.1–16.0 μg/dl in the workers (Queiroz et al., 1998a). Serum concentrations of IgM and IgG were elevated, compared with non-exposed healthy controls, and there was a direct relationship between IgM concentrations and length of HCB exposure (Queiroz et al., 1998a). Furthermore, neutrophilic granulocytes isolated from these exposed workers were less

efficient in killing yeast, which was probably a result of interference with oxidative burst (Queiroz et al., 1998b). A German study of subjects occupationally exposed to HCB or PCBs for more than 6 months evaluated dose–response relationships between blood levels of HCB or PCBs and cellular (numbers of lymphocyte subpopulations, in vitro lymphocyte response) or humoral (plasma cytokine levels, immunoglobulin autoantibodies) immunological dysfunctions. A strong negative association was shown between high HCB blood levels and IFN-γ blood levels, suggesting that HCB may have an impact on Th1 immunity (Daniel et al., 2001). However, in both the Brazilian and German studies, the study populations were exposed to other chemicals in addition to HCB, making it impossible to draw conclusions on the specific immune effects of HCB.

Human data provide limited evidence for HCB-induced immune effects. Some effects, such as the enlarged lymph nodes and the development of arthritis identified in the Turkish incident and the observed increase in serum IgM and IgG levels in the Brazilian plant workers, point towards immunostimulation caused by HCB. Other symptoms, however, such as impaired host resistance in the Turkish food contamination incident and impaired function of neutrophilic granulocytes in the Brazilian workers, are indicative of immunosuppression. It could be argued that suppression of specific immune resistance to infections could have been the result of a nonspecific immunostimulation induced by HCB. However, it is not possible to use the human data for a hazard characterization of HCB; the lack of reliable quantitative exposure information and the likelihood of simultaneous exposure to other immunotoxic chemicals preclude the derivation of a dose–response relationship for any effect on the immune system.

Note: The remaining questions refer to experimental animal data. Many of the animal studies employed dietary exposure to HCB instead of direct dosing, and HCB exposure levels were expressed as mg/kg feed in the publications. For the sake of consistency across other case-studies, HCB exposures in this case-study are expressed as mg/kg body weight per day. Dietary concentrations were converted to mg/kg body weight per day based on the standard assumption that rats consume 5% of their body weight per day; therefore, mg/kg body weight per day = mg/kg feed × 0.05.

C2.3.1.2 Is there evidence that exposure to the chemical is associated with exacerbation of hypersensitivity responses or induction or exacerbation of autoimmune disease or alters the outcome of host resistance assays?

Yes. HCB exposure in rodents has effects on experimental autoimmune diseases and host resistance.

(a) Effects on autoimmune diseases

The effects of HCB on experimental autoimmunity have been studied in Lewis rats in two different models: adjuvant arthritis (AA) and experimental allergic encephalomyelitis (EAE). Male Lewis rats were orally exposed to diets containing HCB at 0, 50, 150 or 450 mg/kg diet (equivalent to 0, 2.5, 7.5 and 22.5 mg/kg body weight per day). After 6 weeks, AA or EAE was induced in these rats. A dose-dependent suppression of AA was observed in all exposure groups, and rats exposed to the highest dose of HCB did not develop AA. In contrast, the highest dose of HCB increased the severity of EAE. EAE is normally reversible, and clinical signs resolve within a few days after onset. However, rats receiving the 22.5 mg/kg body weight per day dose developed a progressive form of the disease and died (Van Loveren et

al., 1990; Michielsen et al., 1999b). The differential effects of HCB on these disease models could possibly be explained by specific effects of HCB on macrophage functioning (Ezendam et al., 2005). Macrophages are important effector cells in EAE, and activation by HCB might lead to a more progressive form of the disease.

These data demonstrate that HCB can modulate autoimmune diseases, but they do not demonstrate that HCB itself is capable of inducing autoimmunity. In chapter 7, guidance is provided for the risk assessment of autoimmunity. At the moment, there are no validated animal models to predict if a chemical can cause autoimmunity. One assay that is used in this field is the PLNA with reporter antigens. In this animal model, which is used to screen the immunostimulatory effects of chemicals, the chemical of interest is injected in the foot pad together with a reporter antigen, and the response in the draining lymph node is used as a read-out. The assay does not evaluate the effects of oral exposure, but simply assesses the intrinsic capacity of the injected chemical to induce an immune response in the draining popliteal lymph node. The addition of reporter antigens makes it possible to assess whether chemicals are capable of inducing neoantigens, which could lead to autoimmunity (Pieters, 2000). The effects of HCB and two of its oxidative metabolites (tetrachlorohydroquinone and tetrachlorobenzoquinone) were tested in the PLNA with reporter antigens. HCB was negative in this assay, but its metabolites tetrachlorohydroquinone and tetrachlorobenzoquinone increased the number of cells and increased the number of IgM and IgG1 antibody secreting cells to the T cell–independent antigen TNP-Ficoll, indicative of formation of neoantigens and T cell activation (Ezendam et al., 2003). These data suggest that in the PLNA, these oxidative metabolites of HCB are capable of forming neoantigens. However, it is unclear if after oral exposure these metabolites are involved in the immunotoxic effects of HCB. The finding of Schielen et al. (1995a) that the CYP pathway is not involved in HCB-induced effects argues against the involvement of these oxidative metabolites, calling into question the utility of these data for risk assessment.

HCB exposure increased IgM levels against several autoantigens, including single- and double-stranded DNA, IgG and phosphatidylcholine. This was observed in Wistar rats exposed to HCB for 3 weeks at 25 or 50 mg/kg body weight per day; IgG antibodies recognizing these autoantigens were not increased. It has been proposed that the increased levels of autoantibodies were the result of stimulatory effects of HCB on B-1 cells, which are committed to produce these autoantibodies (Schielen et al., 1993; Michielsen et al., 1997). In chapter 7, which deals with chemical-induced autoimmunity, it is stated that increased levels of autoantibodies in non-autoimmune disease–prone strains would be considered as some evidence that a chemical has the potential to modulate autoimmune diseases, which is also described in section C2.3.1.2a. However, IgM autoantibodies are less compelling evidence for autoimmunity than are IgG autoantibodies.

It is important to note that HCB behaves differently from other chemicals that are capable of inducing autoimmune diseases, such as D-penicillamine and mercury(II) chloride. The effects of these chemicals are strongly genetically determined; for example, autoimmunity is induced in Brown Norway rats, whereas Lewis rats are resistant. For these chemicals, it has been shown that the disease could be adoptively transferred to naive recipients (Fournié et al., 2001, 2002). In the case of HCB, there is no strong strain dependency, and it was not possible to transfer the disease with cells, indicating a lack of specific T cell sensitization (Ezendam et al., 2005).

In summary, HCB exposure can worsen experimental autoimmunity, apparently by stimulating the immune system, although aggravation of EAE was observed only at the highest dose (Van Loveren et al., 1990). The EAE data constitute evidence of exacerbated autoimmune disease, but confidence in the data as a predictor of disease is low given that the data come from a single study that was published as an abstract and therefore not subject to peer review, and effects were observed only at the highest dose tested. As a result, an AEL was not determined using these data.

(b) Effects on host resistance

As noted above, unintended stimulation may be accompanied by suppression of other functions, resulting in a net negative effect on immune system health. For example, Vos et al. (1979b) assessed immune function and resistance to infection in rats exposed to HCB before and after birth. Dams and weaned offspring received HCB at a dose equivalent to 2.5 or 7.5 mg/kg body weight per day, based on maternal or offspring body weights; exposure spanned early gestation until 5 weeks of age. Exposure to HCB at a dose of 7.5 mg/kg body weight per day increased the antibody response to *Trichinella spiralis* in deliberately infected rats and doubled the number of larvae present in host muscle tissue (albeit with $P > 0.05$). Antibodies do not play a significant role in eliminating adult parasites during a primary infection, but have been implicated in reducing the number of migrating larvae (reviewed by Luebke, 2010). Thus, the increase in antibody titre was not protective, as the overall effect appeared to be reduced resistance to infection. HCB exposure also resulted in a dose-dependent reduction in resistance to the intracellular bacterium *Listeria monocytogenes*. The LD_{50} values were 14×10^5 bacteria in the control group, 7.1×10^5 bacteria in the 2.5 mg/kg body weight per day group and 5.0×10^5 bacteria in the 7.5 mg/kg body weight per day group ($P < 0.05$). A separate prenatal/postnatal exposure study by this group found no significant effect on antibody responses to *T. spiralis* or on the body burden of parasite larvae at doses equivalent to 0.2 or 1 mg/kg body weight per day (Vos et al., 1983).

It can be concluded that HCB exposure did alter the outcome of the host resistance assay by stimulating the humoral immune response to *Trichinella spiralis* infection, but the increased response was not protective. In addition, resistance to *Listeria monocytogenes* infection, which depends on cell-mediated immunity, was reduced, illustrating the need to consider the entire data set when assessing evidence of immunostimulation. In the case of HCB, there is no evidence to suggest that exposure increased resistance to infection.

C2.3.1.3 Is there evidence that exposure to the chemical is associated with unintended stimulation of immune function (antibody production, DTH responses) or alters the balance of immunoregulatory cytokines?

Yes. In rats, dietary HCB exposure stimulates several functional immune parameters.

The primary IgM and early IgG responses to the thymus-dependent antigen tetanus toxoid were increased ($P < 0.1$ and $P < 0.05$, respectively) in adult Wistar rats exposed to a dietary HCB concentration equivalent to 50 mg/kg body weight per day for 3 weeks; the response was not assessed in rats exposed to the equivalent of 25 mg/kg body weight per day (Vos et al., 1979a). Increased humoral responses to tetanus toxoid were also observed in two separate studies in which Wistar rats were exposed to HCB via maternal dosing during gestation and lactation and direct exposure after weaning. Exposure to 2.5 or 7.5 mg/kg body weight per day increased the primary and recall IgG responses to tetanus toxoid (Vos et al., 1979b). In

another study (Vos et al., 1983), dams and weaned offspring were exposed to HCB at a dose of 0.2, 1 or 5 mg/kg body weight per day; however, high offspring mortality (67% by postnatal day 21) precluded testing all end-points at the highest (5 mg/kg body weight per day) dose. IgM and IgG titres to tetanus toxoid were increased in the 0.2 and 1 mg/kg body weight per day dose groups after one or two immunizations. In contrast, IgM and IgG titres to ovalbumin were not increased, but DTH responses were significantly increased in the 1 and 5 mg/kg body weight per day dose groups.

It appears that the developing immune system is particularly susceptible to HCB-induced immune effects. However, it is difficult to compare the susceptibility after perinatal and adult exposures, as the lowest concentration used to assess immune function in the adult studies was 50 mg/kg body weight per day.

In summary, HCB increased humoral responses to tetanus toxoid and DTH in the offspring of rats after perinatal exposure. This increase was dose dependent, and the lowest dose was 0.2 mg/kg body weight per day. In adults, increased humoral responses to tetanus toxoid were observed after exposure to 50 mg/kg body weight per day for 3 weeks.

C2.3.1.4 Is there evidence from general immune assays (phenotyping, cytokines, total immunoglobulins, etc.) that the chemical stimulates immune function?

Yes. In rats, dietary exposure to HCB stimulates responses in general immune assays.

Dietary exposure of adult Wistar rats to concentrations of HCB equivalent to 7.5 or 22.5 mg/kg body weight per day for 6 weeks increased concanavalin A–induced IL-2 and IFN-γ mRNA levels, but not IL-4 mRNA levels, suggesting that HCB may stimulate the expression of Th1 but not of Th2 cytokines (Vandebriel et al., 1998).

Several studies have shown that oral exposure to HCB increased total serum levels of antibodies. In adult Wistar rats, oral exposure to 22.5–100 mg/kg body weight per day for 3 weeks significantly increased total IgM, but not IgG, concentrations (Vos et al., 1979a; Schielen et al., 1993; Michielsen et al., 1997). Longer exposure (13 weeks) to 7.5 or 15 mg/kg body weight per day increased serum levels of IgM at both dose levels. Furthermore, in the high-dose group, IgA antibodies were increased as well (Schielen et al., 1995a). In another study in Wistar rats exposed for 4 weeks to HCB, it was shown that exposure to 30 or 100 mg/kg body weight per day increased IgM antibody levels, whereas 3 mg/kg body weight per day did not (Schulte et al., 2002). In Brown Norway rats, oral exposure to 22.5, but not 7.5, mg/kg body weight per day in the diet for 4 weeks increased IgM, IgG and IgE antibodies (Michielsen et al., 1997). Prenatal and postnatal exposure of Wistar rats to 7.5 mg/kg body weight per day increased total IgM, but not IgG, concentrations in the serum (Vos et al., 1979b). Another prenatal/postnatal study by this group on Wistar rats exposed to 0.2, 1 or 5 mg/kg body weight per day found that only total IgM concentrations were increased, and only at the highest dose (Vos et al., 1983).

It can be concluded that exposure to HCB increased total serum immunoglobulin levels and expression of mRNA coding for Th1 cytokines. The lowest dose of HCB associated with increased responses in these studies was 7.5 mg/kg body weight per day. These results appear to be in conflict, because Th1 cytokines are known to downregulate production of Th2 cytokines, and the latter are associated with antibody production, particularly IgG, IgA and IgE isotypes. Independent reports of increased total IgM by separate research groups suggest that increased total IgM is independent of Th1/Th2 cytokine gene transcript profiles.

However, the latter may be functionally associated with the increased DTH responses reported in section C2.3.1.3.

C2.3.1.5 Is there histopathological evidence or are there haematological changes that suggest that the chemical causes immunostimulation or modulates autoimmunity or allergy?

Yes. Oral HCB exposure induced histopathological and haematological changes in rats, monkeys and dogs suggestive of immunotoxicity.

Oral exposure of Rhesus monkeys to HCB at 8, 32, 64 or 128 mg/day (equivalent to about 1.6, 6.4, 12.8 and 25.6 mg/kg body weight per day) for 60 days induced dose-dependent morphological changes in the thymus, which were observed at all doses. The changes consisted of reduction or absence of individual lobules and hyperplasia of reticular cells, macrophages and plasma cells in the medulla (Iatropoulos et al., 1976). In Wistar rats, dietary HCB exposure for 3–13 weeks to doses equivalent to 25 and 50 mg/kg body weight per day increased extramedullary haematopoiesis in the red pulp of the spleen and induced hyperplasia of B lymphocytes in splenic marginal zones and follicles (Vos et al., 1979a; Schielen et al., 1993; Michielsen et al., 1997; Schulte et al., 2002). These stimulatory effects on B lymphocytes may be related to increased antibody synthesis. In Wistar, Lewis and Brown Norway rats, HCB exposure for 3–4 weeks to dietary concentrations equivalent to 7.5–50 mg/kg body weight per day increased the number of high endothelial venules in lymph nodes (Vos et al., 1979a; Michielsen et al., 1997; Schulte et al., 2002), indicative of lymphocyte migration activity.

Dietary exposure to HCB induced inflammatory skin and lung lesions in various rat strains (Vos et al., 1979a, 1983; Michielsen et al., 1997). The most susceptible strain was the Brown Norway rat, but skin lesions were also observed in Lewis and Wistar rats. It has been shown that the onset and severity of these inflammatory skin and lung lesions are dependent on T cells and macrophages (Ezendam et al., 2004a, 2005).

The lowest dose that induced skin lesions was 7.5 mg/kg body weight per day in Brown Norway and Lewis rats. The first lesions occurred after 22 and 27 days in Brown Norway and Lewis rats, respectively. In Wistar rats, only rats dosed at 22.5 mg/kg body weight per day developed skin lesions after 24 days of exposure. The lesions were morphologically characterized by dermal inflammatory infiltrates of mainly eosinophils in Brown Norway rats and mononuclear cells in Lewis and Wistar rats (Michielsen et al., 1997). Unlike the skin lesions observed in adults in Turkey, these skin lesions were not related to porphyria (Den Besten et al., 1993; Schielen et al., 1995a) and correlated with indicators of immunostimulation, including enlarged lymph nodes, increased serum levels of IgM, IgG and IgE and increased levels of autoantibodies, suggesting an important role of the immune system in lesion development (Michielsen et al., 1997). The lesions were similar to those observed in the breastfed children discussed in section C2.3.1.1 above, which were not related to porphyria but caused by an unknown process. HCB also induced inflammatory lung lesions in rats, which were characterized by accumulations of alveolar macrophages and proliferation of the lung vessels. In contrast to the skin lesions, the induction of inflammatory lung lesions by HCB was not strain dependent; exposure to 7.5 mg/kg body weight per day for 4 weeks induced the lung effects in all strains. Unlike the skin lesions, lung effects did not correlate with assessed immune parameters (Michielsen et al., 1997). The appearance of macrophages in the lungs after only 4 days of exposure to HCB suggests that these cells play an important role in the induction of the HCB-induced lung inflammation.

Effects of HCB were studied in Beagle dogs using daily concentrations of 1, 10, 100 or 1000 mg HCB in gelatine capsules (equivalent to 0.1, 1, 10 or 100 mg/kg body weight per day) for 1 year. In the highest-dose group, the number of neutrophils increased after 4 weeks of exposure and remained elevated during the course of the study. In the group receiving 10 mg/kg body weight per day, an elevation of total neutrophil counts was measured from 16 weeks onward. In the lowest-dose groups, the number of neutrophils was not affected by HCB treatment. Histopathology showed that after 1 year of HCB exposure, hyperplasia of the gastric lymphoid tissue, indicative of lymphocyte proliferation, was observed in 41% of the dogs in the 100 mg/kg body weight per day group, 83% of the dogs in the 10 mg/kg body weight per day group, 92% of the dogs in the 1 mg/kg body weight per day group and 41% of the dogs in the 0.1 mg/kg body weight per day group (Gralla et al., 1977).

In Wistar rats, dietary exposure to an HCB dose equivalent to 100 mg/kg body weight per day for 3 weeks increased the number of peripheral blood leukocytes and monocytes. Furthermore, at an exposure of 50 mg/kg body weight per day, the numbers of basophils and neutrophils increased (Vos et al., 1979a). In Wistar rats, HCB exposure to 30 and 100 mg/kg body weight per day for 4 weeks induced a dose-dependent increase in the numbers of neutrophils. In the high-dose group, absolute numbers of lymphocytes and monocytes were increased as well (Schulte et al., 2002). Increased numbers of eosinophils were reported in Wistar rats exposed to HCB at a dietary concentration equivalent to 7.5 mg/kg body weight per day during gestation and lactation and for the first 5 weeks after birth (Vos et al., 1979b). In contrast, this group reported that only basophil numbers were increased in Wistar offspring exposed prenatally and postnatally to 20 mg/kg body weight per day, a dose that was associated with 67% mortality by postnatal day 21.

It can be concluded that HCB induced histological and haematological changes indicative of immunostimulation in rats, monkeys and dogs. A dose of 0.1 mg/kg body weight per day administered to dogs in gelatine capsules for a period of 1 year was the lowest dose showing effects on the histopathology of the gastric lymphoid tissue. In Rhesus monkeys, histopathological changes were observed in the thymus at concentrations of 1.6 mg/kg body weight per day administered for 60 days. In adult rats, the lowest dose that induced histopathological changes in the lymph nodes was 7.5 mg/kg body weight per day administered for 3–4 weeks. The lowest dose inducing macroscopic and microscopic skin and lung lesions was 7.5 mg/kg body weight per day. The lowest dose that induced haematological changes was 30 mg/kg body weight per day administered for 4 weeks.

C2.3.1.6 Is there evidence that the chemical increases immune organ weight (thymus, spleen, lymph nodes, etc.)?

Yes. In rats, oral exposure to HCB dose-dependently increased the weight of the spleen and lymph nodes, but did not affect the weight of the thymus.

Dietary exposure of adult Wistar rats to 7.5–100 mg/kg body weight per day for 3 or 13 weeks increased the weights of spleen and popliteal and mesenteric lymph nodes (Vos et al., 1979a; Schielen et al., 1993, 1995a). In Wistar rats, exposure to HCB at 30 or 100 mg/kg body weight per day for 4 weeks increased the weights of spleen and popliteal lymph nodes, but not of mesenteric lymph nodes (Schulte et al., 2002). Lower doses (22.5 and 7.5 mg/kg body weight per day) were required to increase spleen and lymph node weights in Lewis and Brown Norway rats (Michielsen et al., 1997). Prenatal and postnatal exposure of Wistar rats to 1 or 5 mg/kg body weight per day during gestation and lactation and after weaning

increased popliteal lymph node weights at 5 weeks of age; only the high dose increased node weight at 7 months of age (Vos et al., 1983).

In summary, oral exposure of adult rats to HCB for 3 or 13 weeks dose-dependently increased the weights of the spleen and lymph nodes. The lowest dose showing effects was 7.5 mg/kg body weight per day. The lowest prenatal and postnatal dose to increase lymph node weights in 5-week-old animals was 1 mg/kg body weight per day.

C2.3.2 Hazard characterization

This case-study was performed to illustrate the application of the weight of evidence approach presented in chapter 5, rather than as a comprehensive risk assessment for health effects or immunotoxicity that takes into account all immunotoxic effects of HCB. However, risk assessors should be aware that stimulation of some types of immune function and suppression of others may be induced by a single compound and occur simultaneously. For this case-study, we will use only those parameters that are linked to immunostimulation in the hazard characterization of HCB.

Studies in rats exposed to HCB during pregnancy and lactation (Vos et al., 1979b) and in adult dogs chronically exposed to HCB orally for 1 year (Gralla et al., 1977) provide the lowest exposure levels suggestive of immunostimulation induced by HCB. Gralla et al. (1977) reported hyperplasia of gastric lymphoid tissue after 1 year in dogs, with a relatively high incidence at all dose levels without a clear dose–response relationship. Therefore, it can be questioned whether the effects are treatment related. No functional immune parameters were assessed in this study. In the rat studies (Vos et al., 1979b, 1983), the antibody response to immunization with tetanus toxoid and the DTH response to ovalbumin were increased in rats exposed to HCB at 0.2 mg/kg body weight per day during gestation and lactation and after weaning. These functional end-points are good predictors of immune system modulation because they reflect the dynamic response of the immune system to novel antigens.

Therefore, a LOAEL of 0.2 mg/kg body weight per day in rats following perinatal exposure was chosen as the POD for the derivation of an AEL. Uncertainty factors as described in section 3.3.10 of chapter 3 of the guidance were applied as follows:

- The default uncertainty factor for interspecies (extrapolation from laboratory animals to humans) differences is 10. Currently, there are no arguments to support consideration of a different uncertainty factor.
- The intraspecies uncertainty factor (to account for interindividual differences) is 10. Exposure occurred during immune system development and maturation (gestation, lactation, early juvenile phases) and thus spanned the most vulnerable life stages. Furthermore, the dose of HCB associated with an unintended stimulation of the antibody response in Wistar rats was approximately 250 times lower if exposure occurred during development than if it occurred in adulthood (HCB equivalents of 0.2 and 50 mg/kg body weight per day, respectively), and stimulation of the DTH response was observed following developmental exposure (Vos et al., 1979b, 1983), but not in exposed adults (Vos et al., 1979a). Although it could be argued that an intraspecies uncertainty factor for toxicodynamic differences is not necessary because a sensitive subpopulation was assessed, guidance provided in section 3.3.10.1 suggests that an uncertainty factor of 10 should be applied unless the POD is based on human developmental immunotoxicity data.

- The subchronic to chronic uncertainty factor would not be applied to this study because it is a developmental exposure study.
- The uncertainty factor for LOAEL to NOAEL extrapolation is 10.
- The database uncertainty factor is 1. A variety of immune system end-points were assessed in adults and in developing animals.

The overall uncertainty factor is therefore 1000 (10 for interspecies × 10 for intraspecies × 10 for LOAEL to NOAEL). The AEL based on immunostimulation can be calculated as follows: 0.2 mg/kg body weight per day/1000 = 0.2 μg/kg body weight per day.

C2.4 Conclusion

This case-study describes the use of a weight of evidence approach for immunostimulation induced by HCB. The immunotoxic effects of HCB are very complex, differ within species and encompass both immunostimulation and immunosuppression. The goal of this case-study was to test the applicability of the guidance for immunostimulation provided in chapter 5, and therefore we have focused only on parameters associated with immunostimulation. Furthermore, this exercise does not represent a comprehensive risk assessment of or regulatory position on HCB. Although the case-study was limited to unintended immune system stimulation, when performing an assessment of data that suggest immunostimulation, other consequences of exposure (immunosuppression, allergy, autoimmunity) must also be considered.

The weight of evidence approach determined that HCB can be considered as an immunostimulatory chemical that affects many end-points that are described in chapter 5. The AEL for these immune effects is much lower for developmental exposure than for adult exposure. This shows that the developing immune system is particularly vulnerable to HCB-induced immune effects.

It could be argued that immunostimulation should not be considered as an unwanted, adverse human health effect, because in some cases stimulation is a desirable, intended effect (e.g. inclusion of adjuvants in vaccines). However, uncontrolled and unintended immunostimulation caused by exposure of humans to chemicals should be considered as unwanted and thus adverse. Therefore, HCB should be considered as an immunostimulatory compound that at certain concentrations could lead to unwanted adverse effects in humans.

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CASE-STUDY 3: ASSESSMENT OF SENSITIZATION AND ALLERGIC RESPONSE TO HALOGENATED PLATINUM SALTS

C3.1 Introduction

Allergic sensitization to halogenated platinum (Pt) salts via inhalation is a well-established human health hazard associated with occupational exposure (IPCS, 1991; WHO, 2000). Platinum-specific sensitization is supported by numerous case reports and occupational studies of workers who develop allergic sensitization to halogenated platinum salts. The sensitizing potential of halogenated platinum salts is also supported by experimental animal data. Sensitization appears to be restricted to halogenated platinum salts, as the available evidence does not support allergic sensitization to insoluble forms of platinum (e.g. platinum oxide or platinum metal) or non-halogenated soluble platinum compounds. Therefore, the following analysis is restricted to halogenated platinum salts.

This case-study illustrates the use of the risk assessment guidance provided for the assessment of sensitization and allergic response as presented in chapter 6. Platinum was selected as a case-study because it is well established that halogenated platinum salts are potent sensitizers, and available data include both occupational exposure and health effects data for platinum. The case-study for halogenated platinum salts highlights a database with strong support for respiratory sensitization and provides limited information on elicitation.

The risk assessment of halogenated platinum salts begins with a brief summary of the available evidence for sensitization associated with halogenated platinum salts, followed by an application of the decision-trees for the assessment of sensitization and allergic response (Figures 6.2A, 6.2B and 6.2C in chapter 6). The analysis is not intended to be a full risk assessment of the health effects associated with exposure to halogenated platinum salts nor a comprehensive risk assessment of sensitization associated with exposure to halogenated platinum salts. Rather, the following assessment was developed to illustrate the process for conducting a risk assessment of sensitization and allergic response by considering the available human, laboratory animal and mechanistic data for sensitization associated with exposure to halogenated platinum salts.

C3.2 Background: sensitization data for halogenated platinum salts

There are numerous case reports and occupational studies of workers who develop allergic sensitization to halogenated platinum salts; however, most studies do not include adequate exposure assessment. Of the available studies with exposure estimates, several occupational studies report increased prevalences of workers with allergic sensitization in halogenated platinum salt–contaminated workplaces with estimated air concentrations below the threshold limit value of 2 µg of soluble platinum per cubic metre (Merget et al., 1988, 2000; Baker et al., 1990; Brooks et al., 1990; Bolm-Audorff et al., 1992; Linnett & Hughes, 1999). Although available data from experimental animal studies are inadequate to characterize the exposure–response relationship for induction of allergic sensitization to halogenated platinum salts in experimental animals exposed to platinum compounds via inhalation, the sensitizing potential of halogenated platinum salts in humans is supported by several inhalation exposure studies in primates (Biagini et al., 1983, 1985b, 1986) and a larger number of dermal and parenteral

exposure studies in rodents (Murdoch & Pepys, 1984a,b, 1985, 1986; Schuppe et al., 1992, 1997a,b; Dearman et al., 1998).

There are six epidemiological studies that inform the effect level for platinum-specific sensitization (Merget et al., 1988, 2000; Baker et al., 1990; Brooks et al., 1990; Bolm-Audorff et al., 1992; Linnett & Hughes, 1999). Effect levels based on these studies are presented in Table C3.1. Baker et al. (1990) and Brooks et al. (1990) report sensitization in 11% of workers down to the lowest soluble platinum level (LOAEL = 400 ng/m^3); however, they reported a higher concentration (600 ng/m^3 soluble platinum) in an area associated with no positive SPTs (0/15 in the offices) than in other work areas associated with positive SPT (e.g. 2/19 at 400 ng/m^3 soluble platinum in the analytical laboratories). Linnett & Hughes (1999) identified sensitization in 51% of workers, and exposure data from the study support an effect level for soluble platinum of less than 500 ng/m^3. Absolute platinum concentrations were not reported in Linnett & Hughes (1999), and a clear LOAEL is not identified, because the data are reported as relative frequency of exposure levels above and below 2 µg/m^3 of soluble platinum. Bolm-Audorff et al. (1992) reported sensitization in 19% of workers in a study that included exposure data down to 80 ng/m^3 in stationary samples and below the limit of detection (50 ng/m^3) in personal samples. Merget et al. (1988) reported sensitization in 20% of workers with exposure below 80 ng/m^3. The WHO (2000) report on platinum in air quality guidelines for Europe reported a LOAEL based on these studies of 50 ng/m^3 for platinum allergic sensitization.

Table C3.1: Effect levels for platinum-specific allergic sensitization in human studies.

Effect level (ng/m^3)	*Sensitization (% of workers)*	*Exposure data*	*Reference*
LOAEL			
52.9	11	28 samples; stationary sampler	Merget et al. (2000)
80	20	exposure measured by refinery "generally below 8 × 10^{-8} g/m^3"	Merget et al. (1988)
50–100	19	2 stationary samples <0.2 ng/m^3 2 stationary samples 80 and 100 ng/m^3 2 personal samples <50 ng/m^3 limit of detection	Bolm-Audorff et al. (1992)
50			WHO (2000) (review of available studies)
400	11	3 relevant samples at the analytical laboratory (out of 75 air measurements in company's environmental monitoring from 1977 to 1979)	Baker et al. (1990)
<500	51	88% of personal samples were <50 ng/m^3 6% of samples were 500–1000 ng/m^3	Linnett & Hughes (1999)
NOAEL			
3.37	—	Stationary samples	Merget et al. (2000) Only study with NOAEL Only prospective study

Among the available occupational studies of platinum-specific sensitization, Merget et al. (2000) provided the best exposure assessment data with sufficient documentation of health effects to establish a dose–response relationship. The Merget et al. (2000) prospective cohort study among German catalyst production workers tested baseline allergic sensitivity to halogenated platinum salts among 275 new and current workers with reassessment after 5 years of follow-up. Conversion to a positive SPT to the halogenated platinum salt hexachloroplatinic acid was used as an indicator of allergic sensitization to halogenated platinum salts. For the exposure assessment, workers were assigned to different exposure categories (high, low, no exposure) based on job title and location within the plant. The study analyses excluded atopic individuals and workers with a positive SPT at the start of the study. Of the 115 workers in the high-exposure category, 13 (11.3%) developed a positive SPT response by the end of the 5-year follow-up period. No positive SPT responses were reported in the other exposure categories.

Air monitoring samples were available to quantify platinum exposure in terms of soluble platinum[1] for each category, and limited personal monitoring samples were available for the high-exposure group. Concentrations for the stationary air samples are reported as the arithmetic mean in Table C3.2. The pooled arithmetic mean ± standard error (SE) concentrations from the high-, low- and no-exposure catalyst production areas were 52.9 ± 19.7, 3.37 ± 0.773 and 0.048 ± 0.005 ng of soluble platinum per cubic metre. As demonstrated in Table C3.1, the LOAEL of 52.9 ng/m^3 based on these data is supported by data from Bolm-Audorff et al. (1992), Merget et al. (1988) and the WHO (2000) report. Personal air monitoring data were limited to 1993 and collected only from the high-exposure group; however, the limited personal air sampling data suggest that stationary air sampling may have underestimated exposure in work areas by up to 10-fold.

C3.3 Assessment of sensitization and allergic response to halogenated platinum salts

The decision-trees presented in Figures 6.2A, 6.2B and 6.2C in chapter 6 are intended to aid in organizing and evaluating immunotoxicity data for a given chemical in the process of performing a risk assessment for sensitization and allergic response associated with exposure to that chemical. The weight of evidence conclusions developed by answering the questions in these decision-trees summarize the hazard identification for sensitization and allergic response and should describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps. When a chemical is characterized as a sensitizer as indicated by the weight of evidence, the data are brought forward for dose–response assessment beginning with selection of the most appropriate end-point(s) (critical effects) and developing PODs. The questions in those decision-trees are reproduced and answered below, followed by a discussion of the supporting data.

[1] Although exposure data in available occupational studies are characterized only to the extent that soluble platinum concentrations are reported, the specificity of the SPT used to identify platinum-specific allergic response demonstrates that occupational allergic sensitization from exposure to platinum compounds is to chlorinated platinum salts. Furthermore, the data from Cleare et al. (1976) demonstrated that the SPT among occupationally exposed workers may also result in a positive response to other halogenated platinum salts, such as brominated platinum salts.

Table C3.2: Air concentrations of soluble platinum and incidence of positive SPT for three exposure groups of German catalyst production workers in Merget et al. (2000).

Exposure group		*Arithmetic mean soluble platinum concentration[a] (ng/m³)*			*Incidence of workers with positive SPT*
		1992	***1993***	***Pooled***	
High	Mean	61.6	41.4	52.9	13/115
	SE	34.0	9.62	19.7	
	N	16	12	28	
Low	Mean	6.06	0.675	3.37	0/111
	SE	0.664	0.211	0.773	
	N	8	8	16	
No	Mean	0.047	0.050	0.048	0/48
	SE	0.007	0.000	0.005	
	N	8	4	12	

[a] Arithmetic means calculated from raw data in Merget et al. (2000) as provided by the lead author.

C3.3.1 Is there evidence that the substance is a skin sensitizer (e.g. data from LLNA, GPMT, HRIPT, human experience, QSAR, in vitro tests)? (see Figure 6.2A)

Yes. There is evidence that halogenated platinum salts are skin sensitizers. Urticaria and contact dermatitis are reported as symptoms of platinum-specific allergic sensitization in numerous case reports and occupational studies that identify health effects in workers exposed to halogenated platinum salts (Hunter et al., 1945; Marshall, 1952; Pepys et al., 1972; Pepys, 1984; Merget et al., 1988, 1999, 2000; Baker et al., 1990; IPCS, 1991; Bolm-Audorff et al., 1992; Calverley et al., 1995, 1999; Merget, 2000; WHO, 2000; Cristaudo et al., 2005). Experimental animal data also provide some support for skin sensitization. Schuppe et al. (1997a) reported significant positive reaction in BALB/c mice to 5% sodium hexachloroplatinate (Na_2PtCl_6) in an LLNA adapted to avoid radioactive labels as well as a modified mouse ear swelling test. Data from Dearman et al. (1998) support sensitization from topical application of several halogenated platinum salts (e.g. $(NH_4)_2PtCl_6$ or $(NH_4)_2PtCl_4$) to the ears of BALB/c mice based on induction of cytokines from auricular lymph nodes. Dearman et al. (1998) reported substantial production of interleukin (IL-10) for all of the halogenated platinum salts tested at exposure concentrations of 1%, 0.5% and 0.25%; however, they did not develop EC_3 values using radiolabel incorporation or using alternative methods. Although Dearman et al. (1998) concluded that the cytokines are characteristic of respiratory sensitizers (i.e. a type 2 cytokine response pattern with vigorous secretion of IL-4 and IL-10), the use of cytokine profiling to discriminate between dermal and respiratory sensitizers has not been fully supported, and recent studies suggest that the use of cytokine profiling for hazard identification may be premature (Selgrade et al., 2006).

C3.3.1.1 Is information on skin sensitizing potency available as LLNA EC_3 or human NOEL to derive a quantitative POD? (see Figure 6.2A)

No. Most of the case reports and occupational studies with health effects data on platinum-specific allergic sensitization do not include exposure data. In addition, the database of case reports and occupational studies does not provide adequate information on the role of dermal

versus inhalation exposure to halogenated platinum salts to enable quantification of the dermal exposure. The laboratory animal data were also insufficient to allow evaluation of a dose–response relationship, as a single dose was used in the Schuppe et al. (1997a) study.

C3.3.1.2 Is sufficient information on skin sensitizing potency available to group the substance into a skin sensitizing potency category? (see Figure 6.2A)

No. As discussed previously, dermal exposure data from the occupational studies are lacking, and the laboratory animal data are from studies that utilized a single dose. Conclusions from this single dose could underestimate the potency category for halogenated platinum salts. Therefore, a qualitative risk assessment of induction of skin sensitization is recommended, including the collection of use and exposure information, as well as a description of use and exposure scenarios that may pose a skin sensitization risk.

Although the SPT is the most common diagnostic test for platinum-specific allergic sensitization, data from patch tests on halogenated platinum salts are sometimes reported as part of the occupational platinum literature (e.g. Linnett & Hughes, 1999; Cristaudo et al., 2005). One example comes from a study of 153 workers in a catalyst manufacturing and recycling factory reported by Cristaudo et al. (2005). The report indicates that 2 of the 153 workers had a positive patch test to the halogenated platinum salt (H_2PtCl_6), and 22 workers had a positive SPT to the same compound. The patch test was performed with 15 µl of H_2PtCl_6 at a single concentration (10^{-2} mol/l). Although the patch test in these previously sensitized individuals may inform an evaluation of elicitation associated with halogenated platinum salts, no exposure data were reported in Cristaudo et al. (2005), and therefore these data provide no information with which to evaluate sensitization. Qualitative evaluation for the low exposure to halogenated platinum salts associated with sensitization (e.g. the association of the exposure measurements in Merget et al. [2000] and positive SPT described in section C3.2 above) would suggest that halogenated platinum salts are potent or strong sensitizers, but there are no dermal exposure measurements in any of the available occupational studies.

In addition to the lack of human dermal exposure data, there are data gaps that increase the uncertainty for developing a qualitative risk assessment of skin sensitization from the experimental animal data on halogenated platinum salts. Two experimental animal studies (Schuppe et al., 1997a; Dearman et al., 1998) provide evidence that several halogenated platinum salts are dermal sensitizers. Schuppe et al. (1997a) developed an auricular lymph node index of 22.8 for Na_2PtCl_6 administered at 5% in acetone using an LLNA adapted to avoid the use of radioactive labels. At that single test concentration, the SI exceeds 3. If that value were to be used as the EC_3, then the potency category for this halogenated platinum salt would be in the moderate range (see Figure 6.1 in chapter 6 for potency categories). However, given that no lower doses were tested, the potency category of strong or extreme cannot be excluded. Dearman et al. (1998) reported substantial production of IL-10 for both $(NH_4)_2PtCl_6$ and $(NH_4)_2PtCl_4$ at exposure concentrations down to 0.25%, an exposure that would represent a potency category of strong. However, Dearman et al. (1998) evaluated cytokine release only, and the experiment did not include LLNA or development of EC_3 values. The available laboratory animal data suggest that the potency category for some halogenated platinum salts would be moderate or strong. However, there are significant data gaps, and data are not available at lower exposure levels to evaluate the possibility that some halogenated platinum salts may be classified as strong or extreme sensitizers.

C3.3.1.3 Is information on elicitation potency (e.g. a BMD or NOEL from human patch tests or ROAT) available to derive a quantitative POD? (see Figure 6.2A)

No. Data suggest that non-occupationally exposed individuals are not already sensitized to halogenated platinum salts (e.g. none of the 800 consecutive subjects with contact dermatitis or urticaria tested by Santucci et al. [2000] had a positive patch test or SPT to halogenated platinum salts). Data are not available to evaluate the elicitation potency of halogenated platinum salts in humans or experimental animals by the dermal or inhalation route. Therefore, a qualitative risk assessment of elicitation of skin sensitization is recommended, including the collection of use and exposure information, as well as a description of use and exposure scenarios that may pose a risk of elicitation in the allergic subpopulation. The lack of dose–response data on both elicitation and the limited exposure data relating to sensitization from individuals sensitized to halogenated platinum salts present a challenge to developing an informative qualitative risk assessment of elicitation. There is information, however, with which to make some conclusions about elicitation potency. For example, there is considerable evidence from other chemicals of a relationship between the exposure doses associated with sensitization and the subsequent doses required for elicitation. In general, as the sensitization dose of an allergen increases, the dose needed to elicit a response on rechallenge decreases (Scott et al., 2002; Hostynek & Maibach, 2004). Multiple exposure factors are likely to affect this relationship, including frequency of exposure and single versus multiple exposures (Scott et al., 2002). The strength of sensitization also affects the elicitation dose such that there is a clear inverse correlation between the strength of the sensitization and the subsequent dose required to elicit a contact hypersensitivity response in humans (Friedmann, 2007).

In one of the few examples where the results of a patch test for a halogenated platinum salt were reported, two workers in a catalyst manufacturing and recycling factory were positive to H_2PtCl_6 by the patch test (Cristaudo et al., 2005). Although the Cristaudo et al. (2005) study did not report exposure information, the patch test was performed with 15 µl of H_2PtCl_6 at a single concentration of 10^{-2} mol/l, and this provides a single concentration that is associated with elicitation. The clinical symptoms reported (rhinitis, asthma, urticaria and eczema) for individuals positive by SPT or patch test suggest that the positive patch test may have been related to eczema. Among workers with a positive response, the only two workers with eczema had positive patch test results (Cristaudo et al., 2005). Although data from patch tests are limited, there are some occupational data on challenge doses associated with a positive SPT response to halogenated platinum salts. For example, Brooks et al. (1990) and Biagini et al. (1985a) reported that the lowest exposure concentration of halogenated platinum salts required to elicit a positive SPT ranged from 10^{-9} to 10^{-3} g/ml (6 orders of magnitude) among individuals previously sensitized to halogenated platinum salts. Conclusions for the elicitation dose for halogenated platinum salts based on the occupational exposure data and SPT data would suggest that halogenated platinum salts are at the higher end of sensitizers (see Figure 6.1 in chapter 6 for potency categories)—that is, potent or strong sensitizers with potent or strong associated elicitation. However, the SPT involves placing a drop of the test substance on the surface of the skin and then pricking the skin with a needle inserted to penetrate the skin and then drawn up through that test drop. The SPT is not equivalent to a patch test. There are insufficient data on dermal exposure to fully evaluate the elicitation dose for halogenated platinum salts and little information to inform a qualitative evaluation. The low doses and large variation (6 orders of magnitude) for elicitation in the SPT suggest that halogenated platinum salts may have a strong dermal elicitation potency.

C3.3.2 Is there evidence that the substance is a respiratory sensitizer (e.g. data from epidemiological studies, human experience or laboratory animal studies)? *(see Figure 6.2B)*

Yes. Halogenated platinum salts are known respiratory sensitizers (IPCS, 1991; WHO, 2000). Among the various symptoms of allergic sensitization, asthma, rhinitis and conjunctivitis are reported in numerous case reports and occupational studies that identify health effects in workers exposed to halogenated platinum salts (Hunter et al., 1945; Marshall, 1952; Pepys et al., 1972; Pepys, 1984; Merget et al., 1988, 1999, 2000; Baker et al., 1990; IPCS, 1991; Bolm-Audorff et al., 1992; Calverley et al., 1995, 1999; Merget, 2000; WHO, 2000; Cristaudo et al., 2005). Additional reports provide support for one or more of the above effects consistent with respiratory sensitizers: asthma (Brooks et al., 1990; Merget et al., 1991, 1994, 1995, 1996), respiratory difficulties (Karasek & Karasek, 1911), inflammatory changes in the respiratory tract (Roberts, 1951; Merget et al., 1996), bronchospasms (Calverley et al., 1999) and bronchial hyperactivity (Biagini et al., 1985a; Brooks et al., 1990; Merget et al., 1991).

C3.3.2.1 Is information on sensitization potency (e.g. BMD or NOEL from an epidemiological or laboratory animal study) available? (see Figure 6.2B)

Yes. Therefore, a quantitative risk assessment of induction of respiratory sensitization using SAFs to derive acceptable non-sensitizing air concentrations is recommended, as well as a quantitative exposure assessment including a risk characterization. A comprehensive risk assessment is beyond the scope of this case-study; however, the preliminary steps in the analysis will be presented below to illustrate the approach suggested by the guidance.

As described in section C3.2 above, several epidemiological studies have found an increased prevalence of workers with allergic sensitization in halogenated platinum salt–contaminated workplaces with estimated air concentrations below the threshold limit value of 2 µg of soluble platinum per cubic metre (Merget et al., 1988, 2000; Baker et al., 1990; Bolm-Audorff et al., 1992; Linnett & Hughes, 1999). Merget et al. (2000) provided the best exposure assessment data with sufficient documentation of health effects to establish a dose–response relationship and is the only study to provide a NOAEL for allergic sensitization to halogenated platinum salts. No workers in the low-exposure category converted to a positive SPT during the 5-year study; therefore, the exposure level in the low-exposure group (3.37 ± 0.773 ng of soluble platinum per cubic metre) represents a NOAEL. The exposure level in the high-exposure group (52.9 ± 19.7 ng of soluble platinum per cubic metre) represents a LOAEL, as 13 out of 115 workers in this group developed allergic sensitization as determined by a conversion to a positive SPT during the 5 years of the study. As demonstrated in Table C3.1, the LOAEL of 52.9 ng/m^3 based on these data is supported by data from Bolm-Audorff et al. (1992), Merget et al. (1988) and the WHO (2000) report. The NOAEL can be used as a POD for risk assessment, or the exposure data presented in Table C3.2 could be used to derive BMCs. However, it is useful to note that the data are of marginal adequacy for BMC modelling, because only three exposure groups (high, low and no exposure) are available, and only one of these groups has a non-zero response. A large degree of uncertainty exists when modelling data sets that do not contain at least two non-zero response levels, because a wide range of curves can be drawn through a single point and a control value (Barnes et al., 1995). It is also useful to point out that reliance on a NOAEL for risk assessment has its own set of limitations (e.g. the number and spacing of doses represented in a given study will influence the dose that is identified as the NOAEL) (Filipsson et al., 2003).

To continue the illustration in this case-study, the Merget et al. (2000) NOAEL has been selected as the POD because of the uncertainty associated with BMC modelling of these data, as described above. The next step in the risk assessment process would be the application of extrapolation, uncertainty or assessment factors, as described in section 6.5 of the guidance, for assessment of sensitization and allergic response and referring to the general guidance presented on uncertainty factors in section 3.3.10 of chapter 3. Following the guidance:

- The interspecies uncertainty factor would be 1, as human data from Merget et al. (2000) are used.
- The intraspecies uncertainty factor to account for interindividual variability would be 10 in the absence of more definitive data.
- The LOAEL to NOAEL uncertainty factor would be 1, as the human data from Merget et al. (2000) represent a NOAEL.
- The matrix factor would be 1; however, the matrix factor of 1 may be replaced by a factor of 3 or 10 for exposure situations that involve ozone (discussed below).
- The use and time factor would be 10 for a chronic exposure assessment, as the study length was 5 years; application of this uncertainty factor depends on the scope defined in the problem formulation stage of the risk assessment (e.g. lifetime/chronic, subchronic); a use and time factor of 1 would be applied for a subchronic risk assessment.
- The database uncertainty factor would be 1 for a risk assessment that was designed to evaluate sensitization, as there are numerous studies to demonstrate sensitization risk, and data are available with which to perform a dose–response assessment.

As a comparison, the LOAEL from Merget et al. (2000) of 52.9 ng/m^3 could also be used as a POD to illustrate the support for the NOAEL in this study, given the range of platinum exposure data available and the limited number of exposure groups. As demonstrated in Table C3.1, the LOAEL of 52.9 ng/m^3 based on these data is supported by data from Bolm-Audorff et al. (1992), Merget et al. (1988) and the WHO (2000) report, which present LOAELs of 50–80 ng/m^3. The derivation of health-based guidance values from LOAELs rather than NOAELs requires the application of an additional 10-fold uncertainty factor to extrapolate from a LOAEL to a NOAEL. Reference values based on the LOAELs would differ by less than a factor of 3 (1.48–2.37) from the reference value based on the NOAEL of 3.37 ng/m^3 from Merget et al. (2000).

The following calculation is presented to illustrate the derivation of a reference value for halogenated platinum salts using the NOAEL (3.37 ± 0.773 ng of soluble platinum per cubic metre; 3.37×10^{-6} mg of soluble platinum per cubic metre used in the calculation) from Merget et al. (2000) as the POD and the uncertainty factors described above. Adjustments to occupational exposure data are required to develop a reference value applicable to continuous lifetime exposure. The adjusted NOAEL (or $NOAEL_{ADJ}$) presented below is developed using standard practice (USEPA, 1994) to compensate for differences between occupational 8 hours/day, 5 days/week exposures and non-occupational 24 hours/day, 7 days/week predicted exposures using the following equation:

$$NOAEL_{ADJ} = NOAEL\ (mg/m^3) \times (VE_{ho}/VE_h) \times 5\ days / 7\ days$$

where the NOAEL is the TWA occupational exposure level at which no adverse effect was observed; VE_{ho} is the human occupational default minute volume (10 m^3/day); and VE_h is the human ambient default minute volume (20 m^3/day). Using this conversion, the $NOAEL_{ADJ}$ is calculated as follows:

$$\text{NOAEL}_{\text{ADJ}} = 3.37 \times 10^{-6}\ \text{mg/m}^3 \times (10\ \text{m}^3/\text{day} / 20\ \text{m}^3/\text{day}) \times 5\ \text{days} / 7\ \text{days}$$
$$= 1.20 \times 10^{-6}\ \text{mg of soluble platinum per cubic metre}$$

To complete the derivation of a health-based guidance value (e.g. RfC or AEL), the guidance recommends consideration of groups at risk (i.e. children, elderly, etc.) and then dividing the POD by the total sensitization uncertainty factor (the sensitization assessment factor, or SAF) described above. For this case-study, relevant considerations would include the possibility that asthmatics or individuals with changes in airway integrity would have an increased risk of developing respiratory sensitization to halogenated platinum salts. Co-exposure to adjuvants or irritants may also influence respiratory sensitization. For halogenated platinum salts, there is evidence that smoking is a risk factor in the development of platinum-specific allergic sensitization in several occupational studies of workers in platinum refineries and catalyst production plants (Venables et al., 1989; Baker et al., 1990; Calverley et al., 1995; Linnett & Hughes, 1999; Merget et al., 2000; Cristaudo et al., 2005). Although no data are available on co-exposure to other relevant irritants or adjuvants in the epidemiological studies, results of the laboratory animal study by Biagini et al. (1986) suggest that ozone promotes development of allergic sensitization to platinum. In monkeys, inhalation of high concentrations of $(NH_4)_2PtCl_6$ (200 µg/m^3, 4 hours/day, 5 days/week, for up to 12 weeks) produced minimal evidence of sensitization (e.g. one of eight monkeys was positive in hexachloroplatinate SPTs). In contrast, four out of eight monkeys exposed to ozone at 2.14 mg/m^3 with hexachloroplatinate at 200 µg/m^3 had positive SPTs to Na_2PtCl_6, compared with none of seven monkeys exposed to ozone alone at the same concentration. These results provide support for the hypothesis that airway damage from exposure to adjuvants or irritant materials in combination with exposure to halogenated platinum salts may promote the development of allergic sensitization. To complete the derivation of a health-based guidance value, the final step is to divide the POD by the total SAF described above.

Using the SAF mentioned above, for a risk assessment designed to evaluate sensitization, the total SAF applied would be 100 (1 for interspecies, 10 for intraspecies, 1 for matrix, 10 for use and time and 1 for database). A chronic reference value for halogenated platinum salts can be calculated by dividing the POD by the total SAF, as follows:

For the sensitization-specific risk assessment of soluble platinum:

$$\text{Reference value} = 1.20 \times 10^{-6}\ \text{mg/m}^3 \div 100$$
$$= 1.20 \times 10^{-8}\ \text{mg/m}^3$$

The exposure assessment for this case-study would comprise the qualitative and quantitative description of the contact of an individual with halogenated platinum salts for specific durations of time (IPCS, 2009). The exposure in the Merget et al. (2000) study was determined by stationary air samplers using sampling periods of 12–17 hours. Thus, the reported air concentrations from the stationary air samples represent 12- to 17-hour TWA concentrations. Although data from personal air samplers would be preferred (particularly in a heterogeneous exposure scenario), the Merget et al. (2000) study included personal air sampler data only in the high-exposure group and for a single year of the study. The personal air sampler data were approximately 10-fold higher than the stationary data, suggesting that the stationary samplers may underestimate exposure. However, the personal air sampler data cannot be used to derive a NOAEL because there are no data from workers in the low-exposure group.

Application of the data from Merget et al. (2000) to a continuous subchronic or lifetime exposure would require dosimetric adjustments. One of the additional challenges illustrated in this case-study is the lack of specificity of the exposure measurements. Although exposure data in available occupational studies are characterized only to the extent that soluble platinum concentrations are reported, the specificity of the SPT used to identify platinum-specific allergic response demonstrates that occupational allergic sensitization from exposure to platinum compounds is to chlorinated platinum salts. Furthermore, the wider application of platinum-specific allergic sensitization to halogenated platinum salts rather than chlorinated platinum salts is suggested by the data from Cleare et al. (1976) and Cristaudo et al. (2005) demonstrating that workers with platinum-specific allergic sensitization may have a positive SPT to chlorinated or brominated platinum salts. Therefore, exposure measurements that are limited to soluble platinum concentrations have direct implications for development of sensitization to halogenated platinum salts. The WHO (2000) report also describes sensitization associated with exposure to halogenated platinum complexes in terms of exposure to soluble platinum salts. Data are not available to further characterize exposure levels or speciate measurements of soluble platinum to concentrations of individual halogenated platinum salts.

The completion of the risk assessment for respiratory sensitization to halogenated platinum salts involves the risk characterization stage, in which the summary and integration of the information in the hazard characterization, qualitative and quantitative dose–response assessment and exposure assessment are combined, along with a critical appraisal of the toxicity information. A principal example of strength in the risk assessment that would be highlighted in this case-study is the relative strength of the database of case reports and occupational studies supporting allergic sensitization resulting from inhalation exposure. Conversely, the consideration of data gaps and uncertainties would include several aspects of the exposure data: lack of data on levels of exposure to specific halogenated platinum salts, lack of personal air sampler data and variation in the stationary air sampler data. Standard considerations include the uncertainty associated with extrapolating from occupational exposure to non-occupational exposure and the potential "healthy worker" effect that may be further exacerbated by self-selection against atopics in a work environment where allergic sensitization is of concern. Finally, the discussion above focuses on the use of a positive response in the SPT to hexachloroplatinic acid as a measure of allergic sensitization resulting from exposure to halogenated platinum salts. This represents another source of uncertainty, as the SPT detects IgE-mediated, type 1 allergic responses. The potential for a second, non-IgE-mediated mechanism to be responsible for allergic sensitization in some individuals is suggested by the existence of both IgE-mediated and non-IgE-mediated hypersensitivity responses to known sensitizers, such as diisocyanate (Kimber et al., 1998; Kimber & Dearman, 2002; Redlich & Karol, 2002). An IgE-mediated and a second, non-IgE-mediated mechanism may play a role in platinum sensitization. For example, close to 10% (10 out of 110) of workers identified as having allergic sensitization to halogenated platinum salts had a negative SPT in the retrospective study of 406 United Kingdom refinery workers reported by Linnett & Hughes (1999).

C3.3.2.2 Is information on elicitation potency (e.g. a NOEC from an epidemiological or human provocation study) available? (see Figure 6.2B)

No. There are no human provocation studies that determine a range of airborne concentrations of halogenated platinum salts associated with development of respiratory sensitization. There are, however, several studies that demonstrate the range of doses in the SPT required to produce a positive SPT in individuals previously sensitized to halogenated platinum salts.

Data from other refinery workers with allergic sensitization to platinum demonstrate that the elicitation doses required to produce a positive SPT to $(NH_4)_2PtCl_6$ range from 10^{-9} to 10^{-3} g/ml (Biagini et al., 1985a), whereas doses of either $(NH_4)_2PtCl_6$ or Na_2PtCl_6 range from 10^{-8} to 10^{-3} g/ml (Brooks et al., 1990). Therefore, a qualitative risk assessment of elicitation of respiratory sensitization is recommended, including the collection of use and exposure information, as well as a description of use and exposure scenarios that may pose a risk of elicitation in the allergic subpopulation. The lack of dose–response data on elicitation from individuals sensitized to halogenated platinum salts presents a challenge to developing an informative qualitative risk assessment of elicitation. As described for dermal elicitation, there is an accepted general inverse relationship between the sensitization dose and the elicitation dose (Scott et al., 2002; Hostynek & Maibach, 2004). The data for this relationship are primarily based on dermal sensitization rather than respiratory sensitization. Nevertheless, there are multiple exposure factors that are likely to affect this relationship for either route of sensitization, including frequency of exposure and single versus multiple exposures (Scott et al., 2002). The strength of sensitization is also expected to affect the elicitation dose, resulting in an additional inverse correlation between the strength of the sensitization and the subsequent elicitation dose (Friedmann, 2007).

Merget et al. (1996) reported the results of specific bronchial challenge to halogenated platinum salts in 57 workers in a platinum refinery and catalyst production. Although the Merget et al. (1996) study did not report exposure information, evaluation included bronchial provocation to methacholine, bronchial challenge to halogenated platinum salts, SPTs, serum IgE level, lung function and other measures. The concentration of halogenated platinum salts causing a 50% fall in specific airway conductance varied widely (from 2×10^{-7} to 10^{-2} mol/l) (Merget et al., 1991, 1996). Conclusions for the respiratory elicitation dose for halogenated platinum salts based on the occupational exposure data and SPT data would suggest that halogenated platinum salts are at the higher end of sensitizers (see Figure 6.1 in chapter 6 on potency categories for skin sensitizers for an analogous scale)—that is, potent or strong sensitizers with potent or strong associated elicitation. Although data from specific bronchial challenge to halogenated platinum salts are limited, there are some occupational data on challenge doses associated with a positive SPT response to halogenated platinum salts. The concentration of halogenated platinum salts required to elicit a positive SPT varies by 6 orders of magnitude (ranging from 10^{-9} to 10^{-3} g/ml) among individuals previously sensitized to halogenated platinum salts (Biagini et al., 1985a; Brooks et al., 1990). As described previously, the SPT is not equivalent to an elicitation test for respiratory sensitizers (i.e. the specific bronchial challenge or test), but SPT data may be indicative of the elicitation response for respiratory sensitization for halogenated platinum salts. Merget et al. (1996) reported a clear correlation between specific bronchial responsiveness to halogenated platinum salts and the SPT to halogenated platinum salts ($r = 0.6$; $P < 0.0001$).

In conclusion, there are limited data with which to evaluate the elicitation potency of halogenated platinum salts; however, based on the low doses associated with sensitization and the huge variation (5–6 orders of magnitude) for elicitation response, halogenated platinum salts are likely to have a strong elicitation potency.

C3.3.3 Is there evidence that the substance causes oral or parenteral sensitization (e.g. data from epidemiological studies, human experience or laboratory animal studies)? (see Figure 6.2C)

Yes. The halogenated platinum salts considered for skin and respiratory sensitization were evaluated separately from the platinum anticancer drugs, because the platinum anticancer drugs are not expected to represent a significant source of environmental exposure to platinum. The platinum anticancer drugs currently approved for clinical use are cisplatin, carboplatin and oxaliplatin, although a large number of carboplatin analogues have been developed and tested to various degrees in clinical trials (Sanderson et al., 1996). The extensive clinical experience with platinum anticancer drugs provides a large body of information regarding adverse effects of those platinum compounds in humans at high doses and generally after acute or short-term parenteral exposure. Although a comprehensive review of the systemic sensitization properties of platinum anticancer drugs is beyond the scope of this document, numerous reports indicate that hypersensitivity reactions to platinum anticancer drugs occur (e.g. Markman, 2003; Navo et al., 2006; Kim et al., 2009; Lee et al., 2009). Evidence suggests that although there is some cross-reactivity in the hypersensitivity reactions to platinum anticancer drugs, there is predominance of specificity of the response for the individual drugs (Leguy-Seguin et al., 2007). Treatment can often be resumed with a different platinum anticancer drug without further complication from hypersensitivity reactions (Leguy-Seguin et al., 2007). Therefore, a qualitative (or semiquantitative) risk assessment of systemic sensitization is recommended, including the collection of use and exposure information, as well as a description of use and exposure scenarios that may pose a systemic sensitization risk. It is important to note that the exposure to platinum anticancer therapeutics is dictated by the exposure necessary to treat the cancer, and a qualitative risk assessment may not be possible or appropriate, as treatment-related exposure to platinum anticancer therapeutics would be based on medical decisions. A limited qualitative evaluation of the risks of systemic sensitization may be possible for platinum anticancer drugs, by summarizing studies that examine particular risk factors or exposure/treatment conditions for platinum anticancer drugs that may be associated with a greater risk of hypersensitivity reactions. For example, in patients treated with carboplatin for recurrent ovarian cancer, the hypersensitivity reaction rate was 4 times higher in patients with retreatment intervals approaching 2 years compared with those with shorter intervals (Gadducci et al., 2008). Further discussion of a potential qualitative risk assessment for sensitization via oral or parenteral exposure to platinum is beyond the scope of this document.

C3.4 Conclusions

The halogenated platinum salts case-study is an illustration of the use of the risk assessment guidance for the assessment of sensitization and allergic response. Platinum was selected because of the strong database for halogenated platinum salts as a respiratory sensitizer. However, the example serves to illustrate that chemical sensitizers often have properties of skin, respiratory and systemic sensitization. The case-study also illustrates an example of the kind of variation often seen in occupational exposure measurements.

It should be noted that this case-study on platinum is provided with the purpose of illustrating how the risk assessment guidance can be used for sensitization, but it does not represent a comprehensive risk assessment, nor does it represent a final regulatory position.

C3.5 References

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CASE-STUDY 4: ASSESSMENT OF SKIN SENSITIZATION TO CITRAL

C4.1 Introduction

Citral is commonly used as a fragrance material in consumer products, and it belongs to the group of most frequently reported allergens in cosmetic and household consumer products. This case-study illustrates the use of the risk assessment guidance provided for the assessment of sensitization and allergic response as presented in chapter 6. Citral was selected as a case-study because it represents an example from a group of fragrance ingredients that are well established as skin sensitizers. The case-study for citral highlights a database with strong support for skin sensitization and provides limited information on an elicitation dose–response relationship.

The risk assessment of citral follows the application of the decision-trees for the assessment of sensitization and allergic response provided in chapter 6. This case-study is not intended to be a full risk assessment of the health effects associated with exposure to citral or a comprehensive risk assessment of sensitization associated with citral. Rather, the following assessment was developed to illustrate the process for conducting a risk assessment of sensitization and allergic response by considering the available human, experimental animal and mechanistic data for citral-associated sensitization.

C4.2 Background on sensitization against fragrance ingredients and citral

A number of allergenic substances have been identified in a wide range of food and consumer products. Substances in products that come in contact with skin play an important role as exogenous factors in the triggering of allergic contact eczemas at work, but also at home. Respiratory allergy can be induced by substances in consumer products, but information on this is very scarce.

For consumer products, most information is available on allergenic substances in cosmetic products, although data indicate the presence of such substances in other products, including detergents, toys, textiles and do-it-yourself products. The presence of a substance as such is not always a problem: a substance can exert its sensitizing action only as it is available for dermal contact, can be released from its matrix or can enter the lungs (Wijnhoven et al., 2008).

Fragrances represent one of the five main categories of allergenic substances that can be distinguished in consumer products (Wijnhoven et al., 2008). Today, more than 5000 fragrance substances are frequently used as mixtures, particularly in cosmetics (perfumes, shampoos, creams, shower gels, toothpastes), household products (room fresheners and carpet shampoos), textiles, shoes and toys. Fragrances have been identified as the most frequent cause of allergic contact dermatitis from cosmetic products (reviewed in SCCNFP, 1999).

In 1999, the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP, now re-established as the Scientific Committee on Consumer Safety) identified 24 fragrance chemicals potentially resulting in contact allergy and divided them

into two different lists. Table C4.1 is a list of fragrances most frequently reported and well recognized as contact allergens, and Table C4.2 is a list of fragrances less frequently documented as consumer allergens.

Table C4.1: Fragrances most frequently reported as consumer allergens.

Common name	*CAS No.*
Amyl cinnamal	122-40-7
Amylcinnamyl alcohol	101-85-9
Benzyl alcohol	100-51-6
Benzyl salicylate	118-58-1
Cinnamal	104-55-2
Cinnamyl alcohol	104-54-1
Citral	5392-40-5
Coumarin	91-64-5
Eugenol	97-53-0
Geraniol	106-24-1
Hydroxycitronellal	107-75-5
Hydroxymethylpentyl-cyclohexenecarboxaldehyde	31906-04-4
Isoeugenol	97-54-1

CAS, Chemical Abstracts Service
Source: SCCNFP (1999)

Table C4.2: Fragrances less frequently reported as consumer allergens.

Common name	*CAS No.*
Anisyl alcohol	105-13-5
Benzyl benzoate	120-51-4
Benzyl cinnamate	103-41-3
Citronellol	106-22-9
Farnesol	4602-84-0
Hexyl cinnamaldehyde	101-86-0
Lilial	80-54-6
d-Limonene	5989-27-5
Linalool	78-70-6
Methyl heptine carbonate	111-12-6
3-Methyl-4-(2,6,6-trimethyl-2-cyclohexe-1-yl)-3-buten-2-one (= γ-methylionone)	127-51-5

CAS, Chemical Abstracts Service
Source SCCNFP (1999)

It has to be emphasized that fragrance chemicals in these lists are not the only compounds that can elicit allergic reactions. Other fragrance chemicals have also been reported to cause cases of skin sensitization or may be allergenic but have not been identified as such owing to a lack of data. When allergic contact dermatitis is suspected in a patient, the dermatologist

will usually apply two different fragrance mixtures (fragrance mixture I and II) to the patient's skin in a patch test to screen for skin sensitization to fragrances. Components of fragrance mixture I are cinnamyl alcohol, cinnamaldehyde, eugenol, α-amyl-cinnamaldehyde, hydroxycitronellal, geraniol, isoeugenol and oak moss absolute (consisting of atranol and chloroatranol). Fragrance mixture II is composed of α-hexyl cinnamaldehyde, citral, citronellol, farnesol, coumarin and hydroxymethylpentyl-cyclohexenecarboxaldehyde (Wijnhoven et al., 2008).

Citral is a mixture of two acyclic monoterpenoids, neral and geranial, which can be regarded as branched-chain aliphatic unsaturated aldehydes (*cis*- and *trans*-3,7-dimethyl-2,6-octadien-1-al). Citral is therefore also known as geranial (or citral a) and neral (or citral b) (Figure C4.1). It is common in lemongrass, lemon and other citrus fruits. Next to limonene, citral is one of the characteristic odours related to the citrus family.

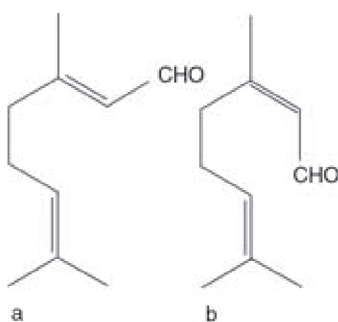

Figure C4.1: Structure of citral a (geranial) and citral b (neral) (from http://www.food-info.net).

Citral occurs widely in varying component isomer ratios in many natural products, including citrus oils, such as lemon oil and orange oil, lemongrass oil, *Litsea cubeba* oil, black pepper oil, verbena oil, melissa oil and ginger oil. Most people are exposed to citral in their daily lives when citrus fruits are peeled and cut by hand. Also, citral is regularly found in the diet as a natural or synthetic flavouring component of some spices and in fruit-based or fruit-flavoured soft drinks (Lalko & Api, 2008). Citral is generally recognized as a safe food additive and has been approved by the USFDA (2009) for use in foods.

Citral is commonly used as a fragrance material in consumer products, and it belongs to the group of most frequently reported allergens, according to the SCCNFP (see Table C4.1).

In 2003, Directive 2003/15/EC, the 7th amendment of the European Cosmetic Directive 76/768/EEC, was published based on the recommendations of the SCCNFP (EC, 2003). Within the EU, the directive requires that citral be listed on the ingredient label of consumer products when present in concentrations greater than or equal to 10 mg/kg (0.001%) in leave-on cosmetic products and greater than or equal to 100 mg/kg (0.01%) in rinse-off cosmetics.

C4.3 Assessment of sensitization and allergic response to citral

Decision-trees are presented in Figures 6.2A, 6.2B and 6.2C in chapter 6 that are intended as a guide through the process of assessing sensitization and allergy caused by chemical substances via the dermal, inhalation and systemic routes. The questions in those decision-trees are reproduced and answered below, followed by a discussion of the supporting data.

C4.3.1 Is there evidence that the substance is a skin sensitizer (e.g. data from LLNA, GPMT, HRIPT, human experience, QSAR, in vitro tests)? (see Figure 6.2A)

Yes. There is sufficient evidence that citral is a skin sensitizer from both human data and experimental animal studies.

Based on structural analysis, QSAR reveals structural alerts for potential toxic dermal effects such as sensitization (Ford et al., 2000). Application of the rule-based DEREK (Deductive Estimation of Risk from Existing Knowledge) system also identifies citral as a potential contact allergen, as it has a relative molecular weight of 154.24 and a calculated octanol/water partition coefficient (log K_{ow}) of 3.45. This suggests that it would fairly readily permeate human skin. As citral is a volatile organic chemical, it can be expected that (under non-occlusive conditions) evaporation of citral is competing with dermal absorption, which would result in an exposure lower than the applied dose. Quantitative information on dermal absorption of citral is limited; however, citral has been demonstrated to penetrate readily through both human and experimental animal skin (Meyer & Meyer, 1959; Barbier & Benezra, 1983; Mutalik & Udupa, 2003). Studies investigating peptide reactivity have identified citral as being protein reactive (Gerberick et al., 2004).

Citral is irritating to skin but not irritating to the eyes of rabbits. In humans, this chemical was marginally irritating to skin, at 8% (Lalko & Api, 2008).

In the past, citral has been tested extensively for skin sensitization in guinea-pigs, mice and humans. In all species, citral has tested positive for skin sensitization. Whereas the results in guinea-pigs and in humans are predominantly useful for identifying a skin sensitizer, the LLNA test can provide input for both hazard identification and dose–response relationship.

Citral was found to be sensitizing in the guinea-pig at 1% in petrolatum (the highest non-irritating concentration, test performed before publication of the OECD test guideline) (OECD, 2001). Lalko & Api (2008) reported extensively on guinea-pig test results using different test methods (e.g. GPMT, Buehler assay, Draize test, Maguire test). An overview of all reported results in guinea-pigs can be found in Table C4.9 in Appendix C4.1 at the end of this case-study.

A survey of sensitization data from tests on materials containing citral was conducted under the auspices of the Soap and Detergent Association (OECD, 2001). This survey was restricted to skin patch tests on human subjects conducted in the USA by member companies of the Soap and Detergent Association and by perfume suppliers. None of the personal care or household products containing citral induced hypersensitivity attributed to citral in 10 660 patch tests, and there were no confirmed reactions to citral in 2098 patch tests on fragrance blends containing the substance.

In various HRIPTs and HMTs, sensitization was induced in a total of 105 of 405 subjects when concentrations of 2–8% citral in ethanol or petrolatum were used, whereas no induction occurred at 1.2% or lower in 182 tested subjects. An overview of the results of these tests as reviewed by Lalko & Api (2008) is given in Table C4.10 in Appendix C4.1. The results of routine clinical diagnostic patch tests reported in the literature are summarized in Table C4.11 of Appendix C4.1 (adapted from Lalko & Api, 2008).

In mice, citral has been evaluated extensively in the LLNA following the method of Kimber and Basketter as formalized in OECD Test Guideline 429 and OPPTS Guideline 870.26 (OECD, 2002). Results of 11 different LLNA tests (as summarized by Lalko & Api, 2008) are shown in Table C4.12 of Appendix C4.1. Citral showed skin sensitizing properties in all LLNAs.

C4.3.1.1 Is information on skin sensitizing potency available as LLNA EC_3 or human NOEL to derive a quantitative POD? (see Figure 6.2A)

Yes. Both LLNA data as well as human HRIPT data are available for citral.

To derive a POD for the induction of skin sensitization, Lalko & Api (2008) used the LLNA data, because these results provide data on the dose–response relationship of induction of skin sensitization. From the 11 EC_3 values, ranging from 300 to 3250 µg/cm^2 (see Table C4.12 in Appendix C4.1), Lalko & Api (2008) calculated a weighted mean EC_3 of 1414 µg/cm^2; however, the weighting procedure was not explained in the paper. Therefore, for the purposes of this case-study, a new mean vehicle-based mean EC_3 was calculated. If more than one EC_3 was available for a particular vehicle, a mean value for that vehicle was calculated first, and then the mean over all vehicles was derived, as shown in Table C4.3. Use of a vehicle-weighted mean, rather than the lowest EC_3 value, is justified, because OECD Test Guideline 429 gives acetone:olive oil (4:1) vehicle priority when testing, provided the test substance is sufficiently soluble in it. This preferred standard vehicle resulted in the highest EC_3. Diethyl phthalate and ethanol are not among the standard vehicles listed in the OECD test guideline and may in fact act as penetration enhancers and thus result in lower EC_3 values. Use of a vehicle-based mean is also justified because LLNA EC_3 values, when tested repeatedly, tend to vary within a factor of 2–3 from the mean value, and the variability of the EC_3 value caused by different vehicles leads to uncertainty in the risk assessment that is taken into account in setting the matrix factor (see section 6.5.3 and below).

Table C4.3: Calculation of vehicle-based mean EC_3 value from LLNA results (as reported in Table C4.12 in Appendix C4.1).

Vehicle group	*Vehicle*	*EC_3 (µg/cm^2)*	*Vehicle mean EC_3 (µg/cm^2)*
A	EtOH:DEP (1:3)	300	937.5
A	EtOH:DEP (1:3)	1575	
B	EtOH:DEP (3:1)	1150	1237.5
B	EtOH:DEP (3:1)	1325	
C[a]	EtOH:DEP (3:1) + 0.1% Toc	375	1012.5
C	EtOH:DEP (3:1) + 0.1% Toc	1700	
C	EtOH:DEP (3:1) + AO Mix	525	
C	EtOH:DEP (3:1) + AO Mix	1150	
C	EtOH:DEP (3:1) + 0.1% TrlC	925	
C	EtOH:DEP (3:1) + 0.1% TrlC	1400	
D	Acetone:olive oil (4:1)	3250	3250
Overall mean			1609

AO Mix, antioxidant mix of 0.3% butylated hydroxytoluene/tocopherol/eugenol; DEP, diethyl phthalate; EtOH, ethanol; Toc, α-tocopherol; TrlC, Trolox C

[a] EtOH:DEP (3:1) plus antioxidant vehicles were lumped together.

Human data from sensitization tests also provide relevant data for derivation of the threshold level (summarized in Table C4.10 in Appendix C4.1). In the HRIPT, the NOEL was 1400 µg/cm^2 and the LOEL was 3876 µg/cm^2. Generally, the induction phase in the HRIPT comprises a total of nine 24-hour occluded patch applications, and the application skin site is changed if moderate or stronger skin reactions are observed upon removal of a patch. In contrast, the HMT induction phase typically consists of five alternate 48-hour patches made on sodium lauryl sulfate–irritated skin if the test substance is not irritating itself. These conditions are considered unsuitable when characterization of the skin sensitizing potency is the goal. Also, the HMT is no longer perfomed for ethical reasons. Therefore, the HRIPT data were given precedence over the HMT data (Api et al., 2008). The study from which the POD is derived was conducted relatively recently according to a standardized protocol and in a sufficient number of subjects (Politano & Api, 2008).

The derived human NOEL of 1400 µg/cm^2 from the HRIPT data is well supported by the vehicle-weighted mean LLNA EC_3 of 1609 µg/cm^2 and was therefore set as the POD for the assessment of induction of skin sensitization (also referred to as no expected sensitization induction level [NESIL] in the methodology developed by IFRA/RIFM) (Api & Vey, 2008).

Based on the LLNA EC_3 value of 5.6% (Api et al., 2008) or 5.7% (Loveless et al., 2010), citral can be classified in the weak to moderate potency range of skin sensitizers (ECETOC, 2003) (see chapter 6, Figure 6.1).

C4.3.1.2 Do quantitative risk assessment of elicitation of skin allergy using SAFs to derive an acceptable non-eliciting skin area dose; do quantitative exposure assessment, describe risk characterization for elicitation in allergic subpopulation. (see Figure 6.2A)

(a) Exposure

** Presence in consumer products*

Citral is found in several consumer products, as shown in the inventory report of Wijnhoven et al. (2008), which is the source of the information given in the following. Citral was identified by the Danish Environmental Protection Agency in 26.1% (23 out of 88) of the cosmetic products containing a fragrance substance, with concentrations ranging from 38.8 to 553.9 mg/l. In children's cosmetics, it was present in 8.2% (17 out of 208) of products in concentrations between 4.0 and 73 mg/l. In the Netherlands, the Food and Consumer Product Safety Authority determined the presence of citral in 8.7% (2 out of 23) of children's cosmetic products in a concentration range of 109–168 mg/l. Citral is also used in cleaning products and detergents. The Danish Environmental Protection Agency investigated 43 cleaning products, of which 7 products (16.3%) contained citral in concentrations up to 0.0501% by weight. Similarly, the Food and Consumer Product Safety Authority measured fragrances in 52 products and found citral in 1 product at a concentration of 8 mg/l. In air fresheners, the Danish Environmental Protection Agency determined citral to be present in 36.8% (7 out of 19) of the products tested in a concentration range of 200–26 000 mg/l. The European Consumers' Organisation measured emissions from 74 different air fresheners. Citral could be detected in 2 (2.6%) air fresheners, resulting in airborne concentrations of 2.0–48 µg/m^3. Furthermore, citral was found in a toy at a concentration of 27 mg/kg by the Danish Environmental Protection Agency.

This overview shows that citral is present in a number of consumer products at concentrations ranging from 4 to 26 000 mg/l. For the exposure estimation in the next section, upper bounds

of citral concentrations in cosmetic products and household cleaning products of 0.06% and 0.05%, respectively, will be assumed.

** Quantitative exposure assessment*

As mentioned above, citral has been demonstrated to be present in several different consumer products varying from cosmetics to detergents to air fresheners. Because of this wide variety of potential sources of exposure, it is conceivable that aggregate exposure (defined here as the total area dose of citral to one skin site from different consumer products over 1 day) should be used for exposure estimation. In this case, the relevant route of concern was restricted to the dermal route. Therefore, for this quantitative exposure assessment example, an exposure scenario has been chosen in which several consumer products are applied onto the same skin site within a relatively short time frame. Three different products that are likely to contain citral—two cosmetic products (shower gel and hand cream) in combination with a household cleaner—were selected. The aggregate exposure estimate did not take into account the fact that only a fraction of the products on the market contain citral (worst-case scenario).

Furthermore, for reasons of simplicity, it was decided not to estimate population exposure or to apply probabilistic techniques to scale the variability and uncertainty in the assessment. Also, exposure from other sources, such as citral in food, was not taken into account here, although it is known that in highly allergic individuals, oral uptake of a sensitizer may lead to manifestation of allergic symptoms in the skin. It is acknowledged that these aspects could be considered in a full-blown risk assessment, but they are beyond the scope of this case-study testing the applicability of the decision-trees.

The following exposure scenario has been chosen: an individual uses a hand cream, cleans the kitchen, takes a shower and uses the hand cream again. It is anticipated that these exposures would lead to an aggregate exposure in which the exposure estimates for the individual products are added.

** Exposure parameters*

The WHO guidance/decision-trees give no clear preference for the exposure assessment method. Therefore, the proposed approach by IFRA/RIFM (Api et al., 2008; IFRA, 2008) and exposure modelling using the ConsExpo computer model were used in this case-study.

A) Using the ConsExpo 4.1 computer model (developed by the Dutch National Institute for Public Health and the Environment [RIVM] and freely available at http://www.rivm.nl/en/healthanddisease/productsafety/ConsExpo.jsp), exposure parameters were derived from the ConsExpo fact sheets on cosmetics (i.e. hand cream and shower gel) and cleaning products (i.e. cleaning agent) (developed by RIVM; Bremmer et al., 2006; Prud'homme de Lodder et al., 2006). Weight fractions of citral in cosmetic and cleaning products were obtained from Wijnhoven et al. (2008), and upper limits of 0.06% and 0.05%, respectively, were chosen for a worst-case estimation (see above). Corrections were made for dilution and retention (rinse-off) of the product. The data in Table C4.4 show the exposure parameters for the individual products as well as the aggregate exposure, derived by simple addition, for the chosen scenario.

Table C4.4: Exposure parameters and estimates for the hands calculated with ConsExpo and based on the accompanying fact sheets on cosmetics and cleaning products (see Appendix C4.2).

Exposure parameters	*Values*
Skin cream	
Weight fraction citral product (%)	0.06
Amount on skin (g)	1.7 × 2 uses/day
Surface area of contacted skin (cm^2)	860
Exposure estimate (mg/cm^2)	*0.0024*
Shower gel	
Weight fraction citral product (%)	0.06 / 3 (dilution factor)
Amount on skin (g)	26.1 (use amount 8.7 g × dilution factor 3)
Surface area of contacted skin (cm^2)	17 500
Exposure estimate (mg/cm^2)	*0.000 30*
Cleaning agent	
Weight fraction citral product (%) for mixing / application	0.05 / 0.05 with 80 dilution factor during application
Amount on skin (g) mixing / application	0.010 / 19
Surface area of contacted skin (cm^2) mixing / application	215 / 1900
Exposure estimate (mg/cm^2)	*0.000 086*
Aggregate exposure (mg/cm^2)	***0.0028 (rounded)***

B) Another exposure estimation was done using the IFRA/RIFM method as described by Api & Vey (2008) and IFRA (2008). In this method, all consumer products containing fragrances are categorized into 1 of 11 different product categories (Api et al., 2008). Categorical exposure estimates are used to determine the "surrogate" exposure for a product within that category. Generally, a combination of a relatively high use amount and small exposed skin area was selected, rendering a high area dose exposure estimate for that specific category. (Comment: The consumer exposure levels [CELs] posed as defaults are mainly based on unpublished data gathered by industry.)

Quantitative exposure assessment using the IFRA/RIFM method is relatively straightforward. The estimate of exposure for a specific product is based on the exposure estimate for the category to which that product belongs. The products hand cream, shower gel and household cleaner belong to categories 5, 9 and 10, respectively (for details, see Api & Vey, 2008, and Appendix C4.2), with corresponding product exposure estimates of 4.2, 0.2 and 0.1 mg/cm^2 per day. The corresponding exposure estimates for these products are calculated and described in Table C4.5.

C) In addition to the upper bounds of measured citral concentrations in products, the IFRA standards (available at http://www.IFRAorg.org) for citral were also used for exposure estimation (right-hand column of Table C4.5). The IFRA standards define safe use levels of individual fragrance ingredients. They are subject to regular amendments, based on new data and scientific developments. They are part of the IFRA Code of Practice.

Table C4.5: Exposure parameters and estimates for the hands calculated according to the IFRA/RIFM method using the category approach.

Exposure parameters	*Values*	
	Using upper limit of citral concentrations measured in marketed products	*Using IFRA standard concentrations for citral*
Hand cream		
Weight fraction citral product (%)	0.06	0.3
Product category 5 exposure estimate (mg/cm^2 per day)	4.2	4.2
Exposure estimate (mg/cm^2)	*0.002 5*	*0.013*
Shower gel		
Weight fraction citral product (%)	0.06	5.0
Product category 9 exposure estimate (mg/cm^2 per day)	0.2	0.2
Exposure estimate (mg/cm^2)	*0.000 12*	*0.010*
Cleaning agent		
Weight fraction citral product (%)	0.05	2.5
Product category 10 exposure estimate (mg/cm^2 per day)	0.1	0.1
Exposure estimate (mg/cm^2)	*0.000 05*	*0.002 5*
Aggregate exposure (mg/cm^2)	***0.002 7***	***0.025***

(b) Risk characterization

** Risk of sensitization following exposure to individual products*

The main focus of the IFRA/RIFM method is safe product use. For this, the risk per product is determined by comparing the AEL (= NESIL/SAF) with the CEL. The CEL is determined per product category (Api et al., 2008). If the ratio AEL/CEL is greater than 1, the product is not considered to pose a risk for skin sensitization.

To extrapolate from the experimental (defined and controlled exposure conditions) to real-life consumer exposure (variable exposure controlled by the consumer), SAFs are applied in dermal sensitization risk assessment. These SAFs take account of three parameters: inter-individual variability (same as in general toxicology, with a default of 10), vehicle/product matrix effects and use considerations (specific for dermal sensitization) (Api et al., 2008). The interspecies parameter in this IFRA/RIFM method is determined to be 1 and is incorporated in the NESIL, because of the use of human data as the POD (i.e. no interspecies extrapolation).

The total SAF, according to the IFRA/RIFM method, for the different products of this example—including the rationale for the matrix and use factors—is given in Table C4.6.

Using the methodology for deriving a skin sensitization assessment factor proposed by Griem et al. (2003) would also lead to a total factor of 100, composed of 1 for interspecies extrapolation (not applicable because human data as starting point), 10 for intraspecies (interindividual) extrapolation and 10 for more frequent exposure.

Table C4.6: Derivation of SAFs for fragrance ingredients in different products of this example.

Product type	*Inter-individual SAF*	*Matrix SAF*	*Rationale*	*Use SAF*	*Rationale*	*Total SAF*
Hand cream	10	3.2	Matrix for the product not the same as the experimental conditions and may be designed to enhance penetration	3.2	The area is mainly the hands, which may include dry skin; there may be compromised skin due to dermatitis, but occlusion does not occur	100
Shower gel	10	3.2	Matrix is different from experimental conditions and may be designed to enhance penetration; may contain irritating ingredients	3.2	Area is entire body, may include dry skin, abraded skin and mucous membranes	100
Household cleaner	10	3.2	Matrix is different from experimental conditions and may contain solvents and other irritating ingredients	3.2	Hands and lower arms, may involve skin sites with dermatitis	100

Source: Adapted from Api et al. (2008)

After determination of the total SAF, the risk of sensitization after using a product can be determined as demonstrated in Table C4.7 for the separate products selected here.

The derived AELs for citral are compared with the CELs derived with the ConsExpo software tool (A) or with the IFRA/RIFM methodology (B) using the upper bound of measured citral concentrations and with the IFRA/RIFM methodology using the IFRA standards (upper use concentration limits) for citral (C). As can be seen from Table C4.7, all AEL/CEL ratios are greater than 1, and therefore each of the individual product exposure scenarios can be considered safe with regard to induction of skin sensitization.

Table C4.7: Determination of the sensitization risk for citral in the separate products.

Methodology	*Parameter*	*Hand cream*	*Shower gel*	*Household cleaner*
	NESIL (µg/cm^2)	1400	1400	1400
	SAF	100	100	100
	AEL (mg/cm^2 per day)	0.014	0.014	0.014
A) ConsExpo	CEL (mg/cm^2 per day)	0.0024	0.000 30	0.000 086
	Risk ratio AEL/CEL	5.8 (**safe**)	47 (**safe**)	163 (**safe**)
B) IFRA/RIFM	CEL (mg/cm^2 per day)	0.0025	0.000 12	0.000 05
	Risk ratio AEL/CEL	5.6 (**safe**)	117 (**safe**)	280 (**safe**)
C) IFRA/RIFM standard	CEL (mg/cm^2 per day)	0.013	0.010	0.0025
	Risk ratio AEL/CEL	1.1 (**safe**)	1.4 (**safe**)	5.6 (**safe**)

** Risk of sensitization following aggregate exposure*

As already described above, it is conceivable that in real life a person is exposed to more than one product containing the same substance within a certain time frame (aggregate exposure). Therefore, in this example, the sensitization risk was also determined for the aggregate exposure estimation for an exposure scenario including three products containing citral: two cosmetic products as well as a household cleaner. Again, exposure estimations A, B and C were evaluated (Table C4.8).

Table C4.8: Determination of the risk with aggregate exposure to citral from the selected products: hand cream, shower gel and household cleaner.

Methodology/parameter	*Aggregate hand exposure*
NESIL (µg/cm^2)	1400
SAF	100
AEL (mg/cm^2 per day)	0.014
A) ConsExpo	
Aggregate CEL (mg/cm^2 per day)	0.0028
Risk ratio AEL/CEL	5.0 (**safe**)
B) IFRA/RIFM	
Aggregate CEL (mg/cm^2 per day)	0.0027
Risk ratio AEL/CEL	5.2 (**safe**)
C) IFRA standard	
Aggregate CEL (mg/cm^2 per day)	0.025
Risk ratio AEL/CEL	0.56 (**not safe**)

Using the upper bound of citral concentrations in marketed products, aggregate exposure estimates can be considered safe regarding induction of skin sensitization.

Using the maximum concentration limits for citral according to the IFRA standards, the AEL/CEL ratio for the aggregate exposure in this example is less than 1 and thus cannot be considered safe.

Exposure estimates resulting from the category approach following the IFRA/RIFM method might give an overestimation when used in an aggregate assessment. The exposure level within each category is a worst-case estimate based on one sentinel product. Relevant aggregation (taking into account time and location of exposure) possibly uses exposure parameters from a product for which aggregation would not have been relevant.

Although it cannot be excluded that some marketed products contain citral at the maximum use concentration limit according to the IFRA standards, it is obviously an overly conservative approach to assume that citral is present at the maximum concentration limit in all products of all product categories when doing aggregate exposure estimation. Here, an adaptation—for example, by using a probabilistic population-based approach (requiring more habits and use data input)—would be necessary in order to derive realistic worst-case estimations. This is, however, beyond the scope of this case-study.

The risk of dermal sensitization for citral in this case-study has been determined following exposure to different consumer products. As shown in the case-study, it is possible to derive a safe concentration limit of citral for individual consumer products. However, when taking into account the aggregate exposure of certain skin sites, as done here for the hands, from several different cosmetic products and other consumer products, such as air fresheners or household detergents, a further development of the methodology for both concerted establishment of concentration limits for a sensitizer in all relevant consumer products and estimation of aggregate exposures is deemed necessary.

C4.3.1.3 Is information on elicitation potency (e.g. a BMD or NOEL from human patch tests or ROAT) available to derive a quantitative POD? (see Figure 6.2A)

No. There are no quantitative data on the elicitation potency of citral.

There are some human patch test studies (diagnostic patch tests in patients) with citral available, in which test concentrations varied between 0.5% and 5% (Frosch et al., 2005; Lalko & Api, 2008). Patch test data are described in Table C4.12 of Appendix C4.1. However, these studies give only an impression of the number of individuals who scored positive in the test after a second exposure to a certain concentration of citral. No elicitation threshold can be derived from these data. In summary, the available data are insufficient to derive a POD (elicitation NOEL in individuals with acute contact dermatitis) for quantitative risk assessment.

C4.3.1.4 Do qualitative risk assessment of elicitation of skin allergy, collect use and exposure information—if available, compare with dermal elicitation threshold—and describe use and exposure scenarios that may pose a risk of elicitation in allergic subpopulation. (see Figure 6.2A)

A qualitative risk assessment of elicitation is difficult to explore. The only information available is that thresholds for elicitation are lower when compared with sensitization induction for the same chemical. In other words, higher levels are needed for sensitization of naive individuals than for elicitation of sensitized subjects. Griem et al. (2003) reported that in humans, no correlation could be shown between sensitization and elicitation thresholds; hence, thresholds for induction of sensitization cannot currently be used to predict elicitation thresholds.

Furthermore, specific exposure information for citral is not available. Sensitized patients who are allergic to a specific allergen should be advised to avoid exposure to that allergen. In the EU, this is achieved with the help of legislation (i.e. by setting concentration limits above which declaration on the labels of preparations and cosmetics is required). In this way, consumers can make an informed choice of their products and can avoid products in which a specific allergen is declared. However, it should be noted that the current EU declaration limits are not based on a quantitative risk assessment (neither for induction nor for elicitation) and that the 26 fragrance ingredients that have to be labelled were considered the most relevant at that time, taking into account sensitization test data and prevalence of clinical allergy. As the relevant dose metric for both induction and elicitation is the skin area dose (i.e. amount of sensitizer applied per square centimetre of skin), it may be expected that—assuming the same citral concentration in the products—cosmetic product categories resulting in the highest area dose confer a higher relative risk of eliciting allergic reactions in sensitized patients. The skin area doses for the IFRA product categories (Api & Vey, 2008) decrease in the following order: Category 1 (11.7 mg/cm^2 per day, lip products) > Category 2

(9.1 mg/cm^2 per day, deodorants/antiperspirants) > Category 5 (4.2 mg/cm^2 per day, hand and facial cream/masks/makeup) > Categories 3 and 4 (2.2 mg/cm^2 per day, hydroalcoholics, eye products, hair sprays, body creams) > Category 6 (1.4 mg/cm^2 per day, mouthwash, toothpaste) > Category 8 (1.0 mg/cm^2 per day, makeup removers, non-spray hair styling aids) > Category 9 (0.2 mg/cm^2 per day, rinse-off hair products, liquid soap, shaving creams) > Category 10 (0.1 mg/cm^2 per day, laundry detergents, household cleaners) > Category 11 (0.000 33 mg/cm^2 per day, air fresheners, candles, machine wash/dishwashing detergents).

C4.3.2 Is there evidence that the substance is a respiratory sensitizer (e.g. data from epidemiological studies, human experience or laboratory animal studies)? *(see Figure 6.2B)*

No. There is no evidence of citral being a respiratory sensitizer based on the very limited data available.

Usually, dose–response data on this end-point are derived from clinical studies (prospective or retrospective). However, for fragrances in general, there are only limited human data available on the effects of inhalation exposure. There are two case reports that have shown that occupational exposure to fragrances could lead to asthma and rhinitis (Baur et al., 1999; Quirce et al., 2008). These and other recent studies of Schnuch et al. (2010) on isoeugenol or Lyral indicate that inhalation of fragrances could lead to respiratory allergies. Nevertheless, no public human data on respiratory sensitization following exposure to citral are available. No case reports of respiratory sensitization to citral (or its constituents neral and geranial) have been reported in publicly available data banks (PubMed, Toxline). There are some experimental animal data available for citral. Ezendam et al. (2009a) described results on citral in a respiratory LLNA. Although a relatively high concentration of citral has been used and citral is known as a weak to moderate skin sensitizer, no significant induction of cell proliferation could be demonstrated in this short-term assay (Ezendam et al., 2009a).

C4.3.2.1 Is the substance a (likely) skin sensitizer or a high molecular weight compound containing or being a protein? (see Figure 6.2B)

Yes. There is evidence that citral is a skin sensitizer (see section C4.3.1).

Human data on respiratory exposure to citral (occupational or consumer) are very limited.

C4.3.2.2 Collect use and exposure information and describe use and exposure scenarios if relevant inhalation exposure occurs. (see Figure 6.2B)

It has been described in a recent RIVM letter report (Ezendam et al., 2009b) that citral is present in various scented products that can be inhaled by the consumer. The database of the Dutch National Poison Control Centre shows that citral is present in 32.7% of all scented products with a potential respiratory exposure (n = 113, with 48 air fresheners and 65 products intended for steam baths and saunas) (Ezendam et al., 2009b). The same trend is found on the publicly available web site of Sara Lee (http://www.saralee-int.info/), where citral has been demonstrated to be present in 36.7% of 49 scented products (mostly electrical room perfumes and scented car products) (Ezendam et al., 2009b). The European Consumers' Organisation performed a study in which emission levels of 11 fragrances in 74 air fresheners were reported. Citral was present in 2.6% of all tested air fresheners, resulting in airborne emission levels of 2–48 μg/m^3. An additional study of the Danish Environmental Protection Agency showed not only that citral was present in 36.8% of the scented products

investigated, but also that it was one of the five fragrances used in the highest concentrations (up to 2.6% of the product by weight).

For the workplace, monitoring data in a citral manufacturing plant indicated workplace air concentrations between 0.31 and 0.56 mg/m^3 (OECD, 2001).

In conclusion, these data indicate that there is potential respiratory exposure of consumers to citral. Owing to its skin sensitizing properties and volatility, a potential for respiratory sensitization of citral cannot be ruled out. Definitive data in the form of case reports or epidemiological evidence in humans or experimental animal studies evaluating respiratory sensitization are lacking. From the lack of any case reports, it may be concluded that the current inhalation exposures at the workplace and from consumer products are unlikely to pose a significant respiratory sensitization hazard.

C4.3.3 Is there evidence that the substance causes oral or parenteral sensitization (e.g. data from epidemiological studies, human experience or laboratory animal studies)? *(see Figure 6.2C)*

No. There is no evidence that citral is an oral or parenteral sensitizer.

No case reports of oral or parenteral sensitization to citral (or its constituents neral and geranial), for example, in the form of food allergy, have been reported in publicly available data banks (PubMed, Toxline). Likewise, no experimental animal studies evaluating the potential of citral to cause oral or parenteral sensitization could be located.

C4.3.3.1 Does the substance have uses with significant intentional or foreseeable exposure via the oral or parenteral route (e.g. in food, parenteral medicinal uses)? (see Figure 6.2C)

Yes. There is evidence that citral is used as a food additive and in some foods is a naturally occurring substance.

The Joint FAO/WHO Expert Committee on Food Additives has evaluated citral as a food additive and has derived a group ADI of 0–0.5 mg/kg body weight (expressed as citral) for a group of terpenoid flavouring agents, including citral, citronellol, geranyl acetate, linalool and linalyl acetate (FAO/WHO, 2004).

Parenteral applications of citral are not known.

C4.3.3.2 Collect use and exposure information, evaluate available information indicating interaction with the immune system (e.g. from repeated-dose toxicity studies, other sensitization studies, QSAR, in vitro tests, human experience) and decide whether further data (e.g. from hazard identification tests) have to be collected or generated. Fill information gaps, and restart if necessary. (see Figure 6.2C)

Citral occurs naturally in several fruits and spices and their essential oils (e.g. *Melissa officinalis*, lemongrass, myrtle trees, African basil, lemons, limes, oranges and tomatoes) (Ress et al., 2003). Synthetic citral is used primarily as lemon flavouring in foods, beverages and candies because of its strong lemon flavour and odour. Citral is a generally recognized as safe (GRAS) list chemical (USFDA, 2009). Citral concentrations have been reported for several foods: for example, chewing gum (~170 mg/kg), baked goods (~43 mg/kg), candy (~41 mg/kg), ice cream (~23 mg/kg) and beverages (~9 mg/kg) (NTP, 1990). The estimated

daily per capita intakes of citral in Europe and the USA are 6.85 mg and 6.99 mg, respectively (FAO/WHO, 2004).

From the lack of any case reports of food allergy, it may be concluded that the current exposure to citral via food is unlikely to pose a significant oral sensitization hazard.

There are no data to suggest that oral exposure to citral from natural or flavoured food induces immunological tolerance in the gastrointestinal tract–associated immune system. Oral tolerance could modify the responsiveness to citral as a skin sensitizer. However, no evidence for this has been reported to date.

C4.4 Conclusion

The citral case-study illustrates the use of the risk assessment guidance for the assessment of sensitization and allergic response (chapter 6). Citral was selected because it represents an example of the group of fragrance ingredients that are well established as skin sensitizers. The example was intended to illustrate the point that exposure to chemical sensitizers often results from several consumer products, which can make exposure assessment quite complex.

Risk assessment does not include decisions on whether and how risks can or should be controlled. Consequently, risk management measures were not discussed in this case-study. It would be the task of risk managers to decide, on the basis of the risk assessment outcome, whether and which measures of protection and communication are necessary to adequately control risk. Possible measures could include, for example, labels and use instructions on consumer products, bans or concentration limits for certain uses, and personal protection measures at the workplace.

It should be noted that this case-study on citral is provided with the purpose of illustrating how the risk assessment guidance can be used for assessing the risk of sensitization, but it does not represent a comprehensive risk assessment, nor does it represent a final regulatory position.

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Appendix C4.1: Supplementary data tables

Table C4.9: Overview of guinea-pig sensitization tests in which citral showed sensitizing effects.

Method	*Induction concentration*	*Challenge concentration*
Maximization	0.4% (intradermal; VNR), 1% (topical; VNR)	0.25% (VNR)
Maximization	0.2% in 4:1 acetone:olive oil (intradermal), 5% in acetone:olive oil (topical)	0.5% in 4:1 acetone:olive oil
Maximization	0.2% in 0.9% saline solution (intra-dermal), 5% in 70:30 acetone/PEG 400 (topical)	0.5% in 70:30 acetone/PEG 400
Maximization	10% for both intradermal and dermal (VNR)	10% (VNR)
Maximization	5% (intradermal; VNR), 25% in petrolatum (topical)	Dose reported as a sub-irritant concentration (VNR)
Buehler	20% in petrolatum	20% in petrolatum
Draize	0.1% (intradermal injection, VNR), 20% (topical, VNR)	0.25% (VNR)
Draize	0.4% (intradermal injection, VNR), 20% (topical, VNR)	1% (VNR)
Draize	0.1% in saline	0.1% in saline
Maguire	8% in petrolatum	8% in petrolatum
Maguire	8% in petrolatum	8% in petrolatum
FCAT	50% citral in FCA	Dose reported as a sub-irritant concentration in petrolatum
OET	10% citral (VNR)	1% (VNR)
CET	3% citral (VNR)	1% citral (VNR)
SIAT	0.4% in FCA	0.5% (VNR)
SIAT	0.4% in saline with FCA	0.5% in acetone/PEG

Buehler, Buehler DTH test; CET, closed epicutaneous test; Draize, modified Draize test; FCA, Freund’s complete adjuvant; FCAT, Freund’s complete adjuvant test; maximization, Magnusson and Kligman GPMT; Maguire, modified Maguire DTH test; OET, open epicutaneous test; PEG, polyethylene glycol; SIAT, single injection adjuvant test; VNR, vehicle not reported

Source: Modified from Lalko & Api (2008); see publication for references

Table C4.10: Overview of human sensitization tests with citral.

Test method	*Induction dose (μg/cm²)*	*Test material concentration and vehicle*	*Incidence of positive responses*
HRIPT	3876	5% in SDA39C	5/8
HRIPT	1400	1.2% in 3:1 DEP:EtOH	0/101
HRIPT	1240	4% in petrolatum	0/50
HRIPT	775	1.0% in SDA39C	0/40
HRIPT	388	0.5% in SDA39C	0/41
HMT	5517	8% in petrolatum	8/24
HMT	3448	5% in petrolatum	16/25
HMT	3448	5% in petrolatum	14/25
HMT	3448	5% in petrolatum	12/25
HMT	3448	5% in petrolatum	8/25
HMT	3448	5% in petrolatum	11/24
HMT	3448	5% in butylene glycol	0/25
HMT	2759	4% in petrolatum	3/25
HMT	2759	4% in petrolatum	3/25
HMT	2759	4% in petrolatum	9/25
HMT	2759	4% in petrolatum	5/25
HMT	2759	4% in petrolatum	4/25
HMT	2759	4% in petrolatum	5/25
HMT	1379	2% in petrolatum	2/24

DEP, diethyl phthalate; EtOH, ethanol; SDA39C, alcohol SDA39C

Source: Modified from Lalko & Api (2008); see publication for references

Table C4.11: Overview of diagnostic patch test studies with citral.

Concentration/vehicle	*Number of positive findings in total number of patients*
5% in petrolatum	4/155 cosmetic dermatitis patients
	5/159 eczema/dermatitis patients
	0/48 control subjects
5% VNR	8/310 cosmetic dermatitis patients
	9/408 non-cosmetic patients
	1/122 control subjects
2% VNR	21/1825 patients
2% in petrolatum	19/1825 patients
2% VNR	12/1701 patients
2% VNR	28/658 patients
2% VNR	1/240 cosmetic dermatitis patients
	2/584 non-cosmetic patients
	0/105 control subjects
1% VNR	6/1701 patients
1% VNR	4/228 patients
1% in petrolatum	8/192 patients
0.1% VNR	1/192 patients (reaction was questionable)

VNR, vehicle not reported

Source: Modified from Lalko & Api (2008); see publication for references

Table C4.12: Overview of LLNA results for citral.[a]

EC_3 value (%)	EC_3 value (µg/cm^2)	Vehicle
1.2	300	EtOH:DEP (1:3)
1.5	375	EtOH:DEP (3:1) + 0.1% Toc
2.1	525	EtOH:DEP (3:1) + AO Mix
3.7	925	EtOH:DEP (3:1) + 0.1% TrlC
4.6	1150	EtOH:DEP (3:1)
4.6	1150	EtOH:DEP (3:1) + AO Mix
5.3	1325	EtOH:DEP (3:1)
5.8	1400	EtOH:DEP (3:1) + 0.1% TrlC
6.3	1575	EtOH:DEP (1:3)
6.8	1700	EtOH:DEP (3:1) + 0.1% Toc
13.0	3250	Acetone:olive oil (4:1)
Weighted mean = 5.7	Weighted mean = 1414	

AO Mix, antioxidant mix of 0.3% butylated hydroxytoluene/tocopherol/eugenol; EtOH, ethanol; DEP, diethyl phthalate; Toc, α-tocopherol; TrlC, Trolox C

[a] Per cent EC_3 values were converted to their dose per unit area (µg/cm^2) equivalents assuming an application area of 1 cm^2 and a dose volume of 25 µl. Weighted mean EC_3 value based on the vehicle utilized.

Source: Modified from Lalko & Api (2008); see publication for references

Table C4.13: Product type consumer exposure levels that drive the IFRA quantitative risk assessment category.

IFRA QRA category	Category consumer exposure (mg/cm^2 per day)[a]	Product type that drives the category consumer exposure level
Category 1	11.7	Lip products
Category 2	9.1	Deodorants/antiperspirants
Category 3	2.2	Hydroalcoholics for shaved skin
Category 4	2.2	Hydroalcoholics for unshaved skin
Category 5	4.2	Hand cream
Category 6	1.4	Mouthwash
Category 7	4.4	Intimate wipes
Category 8	1.0	Hair styling aids
Category 9	0.2	Rinse-off hair conditioners
Category 10	0.1	Hard surface cleaners
Category 11	0.000 33	Candles

QRA, quantitative risk assessment

[a] The category consumer exposure level (mg/cm^2 per day) is driven by the product type in that category with the combined highest consumer exposure level and highest SAF. A higher SAF for the use of products on shaved skin explains the difference between categories 3 and 4.

Source: Modified from IFRA/RIFM quantitative risk assessment information booklet (IFRA, 2008)

Appendix C4.2: Exposure estimation with ConsExpo 4

ConsExpo 4.1 report [additions given in square brackets]

Report date: 02.03.2010

Compound
Compound name: Citral
CAS number:
Molecular weight: 152 g/mol
Vapour pressure: 0.2 mmHg [267 Pa]
K_{ow}: 2.9 linear

Product: Hand cream

General exposure data
Exposure frequency: 1/day
Body weight: 65 kg

Dermal model: Direct dermal contact with product: instant application
Weight fraction compound: 0.06%
Exposed area: 860 cm^2
Applied amount: 3.4 g

Uptake model: diffusion
Skin permeability: 0.002 82 cm/h [Fiserova-Bergerova diffusion QSAR]
Compound concentration: 0.6 mg/cm^3
Exposure time: 1.44E3 min [1440 min/day = leave-on use]

Output

Dermal: point estimates

Dermal load: ***0.002 37 mg/cm^2***
Dermal external dose: 0.0314 mg/kg
Dermal acute (internal) dose: 0.0314 mg/kg

Product: Showering with liquid soap

General exposure data
Exposure frequency: 1/day
Body weight: 65 kg

Dermal model: Direct dermal contact with product: instant application
Weight fraction compound: 0.02%
Exposed area: 1.75E4 cm^2
Applied amount: 26.1 g

Uptake model: diffusion
Skin permeability: 0.002 82 cm/h [Fiserova-Bergerova diffusion QSAR]
Compound concentration: 0.3 mg/cm^3
Exposure time: 4 min

Output

Dermal: point estimates

Dermal load: ***0.000 298 mg/cm^2***
Dermal external dose: 0.0803 mg/kg
Dermal acute (internal) dose: 0.0138 mg/kg

Product: Household all-purpose liquid cleaner [inhalation estimation not shown]

Use step: loading and mixing

Dermal model: Direct dermal contact with product: instant application
Weight fraction compound: 0.05%
Exposed area: 215 cm^2
Applied amount: 0.01 g

Uptake model: diffusion
Skin permeability: 0.712 cm/h [Fiserova-Bergerova diffusion QSAR]
Compound concentration 0.5 mg/cm^3
Exposure time: 0.75 min

Output

Dermal: point estimates

Dermal load: ***2.33E-5 mg/cm^2***
Dermal external dose: 7.69E-5 mg/kg
Dermal acute (internal) dose: 2.19E-5 mg/kg

Product: Household all-purpose liquid cleaner [inhalation estimation not shown]

Use step: application

Dermal model: Direct dermal contact with product: instant application
Weight fraction compound: 0.000 625% [80-fold dilution]
Exposed area: 1.9E3 cm^2
Applied amount: 19 g

Uptake model: diffusion
Skin permeability : 0.002 82 cm/h [Fiserova-Bergerova diffusion QSAR]
Compound concentration: 0.006 25 mg/cm^3
Exposure time: 20 min

Output

Dermal: point estimates

Dermal load: ***6.25E-5 mg/cm^2***
Dermal external dose: 0.001 83 mg/kg
Dermal acute (internal) dose: 0.000 164 mg/kg

Total dermal load for household cleaner: 8.58E-5 mg/cm^2

CASE-STUDY 5: ASSESSMENT OF MERCURY-RELATED AUTOIMMUNITY AND AUTOIMMUNE DISEASE

C5.1 Introduction

There is an extensive peer-reviewed literature that addresses the impact of exposure to mercury in its different forms on various aspects of the immune response in experimental animals and humans. Depending on the chemical form, the dose, the route of administration and the immunological status of the host, mercury can modulate immune mechanisms, resulting in either immunosuppression or autoimmune dysfunction (Moszczyński, 1997; Silbergeld et al., 2005). The potential for mercury to induce or exacerbate autoimmune disease, in particular, has lent itself to experimental inquiry through human population-based studies, animal models of disease, as well as cellular molecular mechanistic research. Indeed, mercury is viewed to be an example of an important ubiquitous environmental contaminant that has been linked to human autoimmune disease and for which well-established animal model systems have been developed. In this regard, mercury serves as a constructive candidate toxicant to illustrate the application of the weight of evidence schematic depicted in Figure 7.1 in chapter 7 to perform a risk assessment of autoimmunity induced by chemicals.

In this case-study, the evidence that mercury induces autoimmunity or autoimmune disease is evaluated by following the guidance presented in chapter 7 for the assessment of autoimmunity. The case-study is not meant to be a full risk assessment of the health effects associated with exposure to mercury or a detailed risk assessment of autoimmune disease–inducing potential associated with human exposure to mercury compounds. Rather, the following assessment is provided to illustrate the process for conducting a risk analysis of mercury-induced autoimmunity by considering the available human, experimental animal and mechanistic data required to do so.

C5.2 Background: data on the potential for mercury to induce autoimmunity

Mercury is a natural component of Earth's crust and atmosphere. It is found in three main chemical forms: elemental mercury (Hg^0), organic mercury (e.g. methyl, ethyl) and inorganic mercury (e.g. Hg^{2+}). Humans are mainly exposed to three sources of mercury: two organic compounds—namely, methylmercury through consumption of fish and thimerosal as a component of some vaccines—and the elemental form of mercury inhaled as mercury vapour from amalgam dental fillings (Clarkson, 2002). Each of these chemical forms of mercury has different toxicokinetic properties, although they are all converted to inorganic Hg^{2+} within the body. The toxicity of mercury compounds to the nervous and renal systems following acute high-level exposure has been well documented. However, the current public health concern has largely centred on the effects of low-level mercury exposure, which are relevant to large segments of the population as a result of ubiquitous environmental contamination. Much of this interest and scrutiny for adverse health effects associated with low-level mercury exposure has focused on the immune system.

A growing body of literature is providing evidence that exposure to mercury, particularly in the context of specific genetic variants, may increase the risk for disrupting immune system homeostasis and promote or induce autoimmunity. Epidemiological studies and case reports have suggested that exposure to occupational and environmental mercury levels may be a

factor that contributes to idiosyncratic autoimmune disease in humans. Generally speaking, however, these studies are underpowered and of limited value in predicting the risk of human autoimmune disease from mercury exposure. Rodent studies have provided the best direct evidence that exposure to mercury in its various forms gives rise to or exacerbates autoimmune disease. There are essentially four categories of rodent studies dealing with mercury exposure and its effects on the autoimmune disease process. The first category is known as mercury-induced autoimmune disease (HgIA). In this model, a de novo lupus-like systemic autoimmune disease characterized by lymphoproliferation, hyperglobulinaemia, antinuclear antibody production and systemic immune complex deposition leading to glomerulonephritis is produced following the administration of mercury to mice with a certain MHC haplotype. Induction of disease in HgIA depends on defined genetic elements within the murine H-2 locus and their interaction with mercury or the aftermath of mercury-mediated cellular toxicity. The second category involves de novo induction of autoimmune disease in rats following administration of mercury. As with murine HgIA, the rat model of disease involves interactions between mercury and genetic elements of the rat MHC. Although there are general similarities between the mouse and rat models of HgIA, the two are mechanistically distinct, and the clinical features of disease presentation differ between the two species. The third category involves spontaneously or genetically autoimmune disease–prone strains of mice in which exposure to mercury appears to exacerbate the onset or severity of features of the disease. The distinction between this third category and the previous two may simply be that interaction between mercury and the genetic elements of disease is more defined in the first two categories (i.e. MHC). Operationally, another important distinction is that mercury is clearly required for disease induction in the first two categories but not the third. The fourth and final category involves studies where again mercury exacerbates rather than induces disease. In these studies, the mouse strains are not spontaneously genetically autoimmune disease prone, but the disease is induced upon exposure to a defined environmental trigger other than mercury. Collectively, the relatively large database of rodent studies indicates that, depending on the circumstances, mercury can act as an inducer of de novo autoimmune disease, as a modifier of an existing genetic predisposition to autoimmune disease or as a cofactor with other non-genetic inducers that trigger autoimmune diseases.

C5.3 Assessment of the potential for mercury to induce or exacerbate autoimmunity

C5.3.1 Application of the weight of evidence approach

A series of questions is presented in chapter 7, section 7.7.1, “Weight of evidence approach to assessment of risk for autoimmunity”, that is intended to aid in organizing and characterizing immunotoxicity data for a given chemical from the strongest and most predictive data through the least predictive evidence supporting human risk for autoimmunity and autoimmune disease. The weight of evidence conclusions developed by answering these questions summarize the hazard identification for autoimmunity and should describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps. When autoimmunity is indicated by the weight of evidence, the data are brought forward for dose–response assessment beginning with selection of the most appropriate end-point(s) (critical effects) and developing PODs. The questions are reproduced and answered below, followed by a discussion of the supporting immunotoxicity data.

C5.3.1.1 Are epidemiological studies, clinical studies or case-studies available that provide human data on end-points relevant to chemical-induced autoimmunity (i.e. increased incidence of all or specific autoimmune diseases, changes in immune parameters indicative of autoimmunity, increased levels of autoantibodies, decreased regulatory T cell function, evidence of nonspecific stimulation of the immune system, increased levels of markers of inflammation)?

Yes. Human exposure to mercury from various sources has been implicated as a trigger for autoimmune disease; however, no large-scale, properly powered epidemiological studies have been conducted in which mercury exposure has been evaluated and associated with elements of autoimmune disease. As such, the availability of human data to apply to the risk assessment of mercury and autoimmune disease is limited.

A few small-scale population studies provide some support for an association between mercury exposure and manifestation of autoimmune disease in humans. For example, in a small case–control study in patients with systemic sclerosis, the authors reported an association between elevated urinary mercury levels and the severity of the connective tissue autoimmune disease, scleroderma. In this report, urinary mercury levels correlated with heightened serum antibody levels to fibrillarin, an autoantibody specificity associated with scleroderma, and more severe features of the disease (Arnett et al., 2000). In another example, a community comparison study examining people living near an oil field waste site showed significant associations between presumed toxicant exposure (petroleum products and mercury) and prevalence of rheumatic diseases (OR = 10.78) and lupus (OR = 19.33) (Dahlgren et al., 2007). However, as is the case with many epidemiological studies, residents near the oil field waste site were exposed to a mixture of compounds in addition to mercury, including elevated levels of compounds with the potential to induce autoimmunity (pristane and phytane). Therefore, the Dahlgren et al. (2007) study cannot attribute the observed increase in autoimmune diseases to the effects of mercury alone. In a separate study, statistically significant correlations (OR = 3.6) were seen in self-reported occupational exposures to mercury and the prevalence of systemic lupus erythematosus among dental workers (Cooper et al., 2004). Although these human studies are conceptually informative with respect to linking mercury exposure to human autoimmune disease, the lack of adequate assessment and documentation of the mercury exposures limits their utility for risk assessment and analysis.

Another approach has been to test for elements of autoimmune disease (e.g. presence of autoantibodies above normal levels and increased T cell subpopulations) in mercury-exposed populations. Elevated levels of circulating antilaminin antibodies were found in a subset of chloralkali workers who were occupationally exposed to mercury vapour (Lauwerys et al., 1983). However, the increased levels of antilaminin antibodies in that cohort were not predictive of glomerulonephritis. An additional cross-sectional study of chloralkali workers showed no statistically significant association between elevated blood mercury levels and any indices of autoimmune disease (Barregård et al., 1997). Moszczyński et al. (1995) reported that quantitative changes in T cell numbers (ranging from 40% to 96%, depending on T cell subset analysed and mercury vapour exposure group) were an immunological index of occupational exposure to mercury. Male workers stratified by mercury vapour exposure group (median TWA concentration = 0.036 mg/m^3) with duration of exposure either up to 10 years or above 10 years showed increased numbers of CD3+ T cells by 45% and 55%, of CD4+ T cells by 42% and 60%, and of CD8+ T cells by 80% and 96%, respectively. A follow-up study in a second cohort by the same investigators more extensively documented the mercury exposure assessment, where mercury vapour exposure (TWA concentration = 0.028 mg/m^3) resulting in urine (range = 10–240 µg/l) and blood (range = 4–30 µg/l) mercury

burdens correlated with increased numbers of total T cells by 35% and 38%, of CD4+ T cells by 42% and 60%, and of CD8+ T cells by 85% and 96%, with the magnitude of the increase in each T cell subpopulation correlating with the duration of exposure—either up to 10 years or more than 10 years, respectively (Moszczyński et al., 1996). Although diagnoses of autoimmune disease and more direct markers of autoimmunity (e.g. autoantibody titres) were not studied in these cohorts, the increased number of T cells associated with occupational exposure to mercury vapour was proposed as a factor contributing to autoimmune disease. This, generally, is in keeping with rodent studies in which mercury promotes T cell expansion and T cell–dependent autoimmune disease. In contrast, Herrström et al. (1994) found no significant change relative to controls in total T cells or T cell subsets in a population of Swedish teenagers with amalgam fillings who had average plasma mercury burdens of 3.2 μg/l. Moreover, Park et al. (2000) reported decreases in T lymphocyte subpopulations and NK cells in 20 fluorescent lamp makers who had urinary inorganic mercury concentrations ranging from 1.8 to 163 μg/l. These studies demonstrate alterations in immune measures associated with autoimmunity as well as data from general immune assays that provide some support for mercury-induced autoimmunity in humans. However, the above examples also demonstrate the inconsistencies characteristic of the literature investigating the incidence of human autoimmune disease associated with mercury exposure.

Additional evidence of mercury-associated changes in general immune end-points is provided by changes in serum immunoglobulin levels reported in various worker cohorts exposed to mercury. Here, again, the effects of mercury exposure on markers of humoral immunity are variable, but there is evidence of an effect at higher doses. For example, Herrström et al. (1994) found no correlation between mercury burden and levels of IgG, IgM, IgA or IgE. Similarly, no significant changes in plasma levels of IgG, IgM or IgA were found in workers exposed to inorganic mercury resulting in a mean urinary mercury concentration of approximately 25 μg/g creatinine (Langworth et al., 1992). However, increases in serum IgG, IgA and IgM levels were found to be elevated in a cohort of 44 male workers exposed in a mercury-producing plant (Queiroz et al., 1994). These workers had urinary mercury levels ranging from 3.5 to 68 μg/g creatinine (mean = 24.7 μg/g). Another study reported increased serum IgA and IgM levels in mercury-exposed workers with urinary mercury concentrations of 29–545 μg/l (Bencko et al., 1990).

There are also a number of particularly controversial topics that surround the issue of mercury and human autoimmune disease. For example, the potential contribution of dental amalgam as a continuous source of mercury exposure to human diseases, including autoimmunity, remains a controversial topic. The fact that the vast majority of individuals with mercury-containing amalgam do not display an association with any autoimmune disease argues strongly against amalgam as a source of mercury contributing to disease incidence. Indeed, a large-scale retrospective cohort study showed no associations between the number of mercury-containing fillings and adverse health effects, including autoimmunity (Bates et al., 2004), and no evidence of anti–glomerular basement membrane IgG was found in individuals with mercury dental amalgam fillings relative to controls (Guzzi et al., 2008). However, another report provided a meta-analysis of dental amalgam exposure and development of multiple sclerosis, an organ-specific autoimmune disease (Aminzadeh & Etminan, 2007). In keeping with this, clinical benefit has been reported following replacement of amalgam fillings in patients with systemic lupus erythematosus, thyroiditis or multiple sclerosis (Prochazkova et al., 2004; Sterzl et al., 2006). A second controversial topic involves the potential for adverse health effects following exposure to mercury as thimerosal in vaccines.

However, there are no human data that demonstrate frank autoimmunity or elements of autoimmune disease following exposure to thimerosal.

In summary, as described above, the epidemiological data provide some evidence that mercury exposure induces or exacerbates autoimmune disease in humans. In particular, an association between mercury exposure and autoimmune disease is supported by some case–control studies, aspects of autoimmune disease observed in mercury-exposed populations and reduced symptoms of systemic lupus erythematosus, thyroiditis or multiple sclerosis following replacement of amalgam fillings. However, the database is notably lacking a definitive, large-scale epidemiological study evaluating mercury exposure and elements of autoimmune disease. Human studies in which exposure cannot be definitively linked to mercury provide limited support because of the considerable uncertainty in the relationship between mercury and the observed effect when there is co-exposure to other chemicals linked to autoimmunity, as is the case with co-exposure to pristane in the Dahlgren et al. (2007) study. The risk assessor has to determine whether the exposure questions for individual studies result in equivocal data. The available epidemiological data for mercury provide little information on a potential dose–response relationship between mercury exposure and reported symptoms of autoimmune disease in humans and do not support a quantitative risk assessment. These human data add to the weight of evidence presented by substantial data supporting mercury-induced autoimmunity in animal models, which will be discussed below in section C5.3.1.2.

C5.3.1.2 Is there evidence that the chemical causes changes in disease incidence or progression in animal models of autoimmune disease?

Yes. The case for a connection between mercury exposure and autoimmune disease has been solidified through extensive research using a variety of rodent models. There is a large body of literature that supports a connection between mercury exposure and the induction of autoimmune disease as well as the pathogenesis and progression of autoimmune disease in a variety of rodent models. Mercury induces de novo autoimmune disease in certain mouse and rat strains (Druet, 1995; Vas & Monestier, 2008; Schiraldi & Monestier, 2009; Pollard et al., 2010). The features of HgIA differ between rats and mice, but both models share genetic susceptibility factors and an autoimmune etiology. The rat and mouse models of HgIA show that, in the context of susceptible MHC backgrounds (H-2 locus in mouse, RT-1 locus in rat), exposure to low doses of mercury in its various forms is sufficient to induce autoimmune disease. In addition, mercury exposure in the context of non-mercury-susceptible MHC backgrounds, which may more closely approximate most human exposures, as a mercury-susceptible MHC haplotype in humans has not been discovered, still appears to contribute to autoimmune disease by acting with other intrinsic and extrinsic cofactors, thereby increasing the risk of clinical disease progression.

Numerous studies support the concept that mercury exposure synergizes with other intrinsic (e.g. genetic) and extrinsic (e.g. immunogen) factors to influence disease pathogenesis and progression in susceptible mouse models. For example, lupus-prone strains such as NZBWF1 or MRL+/+ injected subcutaneously with mercury(II) chloride (1.6 mg/kg body weight 3 times a week for 10 weeks) show accelerated age of onset of disease, including increased systemic autoantibody titres and renal immune complex deposition (Al-Balaghi et al., 1996; Pollard et al., 1999). An additional study (Pollard et al., 2001) examined the influence of mercury exposure and dosage on the expression of autoimmunity in lupus-prone BXSB mice. As with the findings with other lupus-prone mice, subcutaneous injection of mercury(II) chloride at doses of 0.04–40 μg/0.1 ml twice per week for 4 weeks increased the polyclonal

immunoglobulin levels and autoantibody titres to antinuclear and antichromatin antigens and accelerated pathological changes in the kidney attributable to the disease in BXSB mice. Exposure assessment of the kidney mercury burden (range = ~76–3600 ng/g wet weight) showed that the mercury levels in the kidneys of these animals fell within the normal range found in non-occupationally exposed humans. Collectively, these studies are useful in demonstrating mercury exacerbation of disease progression in animal models of autoimmune disease, but the risk assessor should note that the route of exposure (injection) and form of mercury (inorganic mercury(II) chloride) utilized in these studies generally differ from the most prevalent human exposure scenario (oral exposure to organic mercury).

Low levels of mercury have also been found to exacerbate disease in several models of acquired autoimmunity. For example, the parent into F_1 model of chronic graft-versus-host disease is a murine model of acquired lupus-like autoimmune disease. The transfer of parent donor CD4+ T cells into the F_1 hosts results in the responsiveness of the donor T cells to the host allo-antigens, thereby driving the autoreactive immune response, resulting in polyclonal host B cell activation, autoantibody production, immune complex formation and renal deposition followed by glomerulonephritis. The parent (DBA/2) into $B6D2F_1$ pairing produces a severe and chronic disease, where morbidity and mortality are related to the extent of glomerulonephritis, which correlates directly with the number of activated donor T cells transferred to the host. Donor T cells derived from mice administered inorganic mercury in low doses (defined as non-nephrotoxic doses of 20 or 200 µg/kg body weight injected every other day for 15 days) worsened the graft-versus-host disease course, as characterized by histological evidence of more severe glomerulonephritis, accelerated appearance of proteinuria, elevated anti–single-stranded DNA autoantibodies and accelerated mortality (Via et al., 2003). The implication of these findings is that exposure to inorganic mercury might interact with other genetic and environmental risk factors, thereby lowering the threshold for activated T cell autoreactivity and autoimmune disease in susceptible individuals (Via et al., 2003).

In the collagen-induced arthritis disease model, another murine model of acquired autoimmunity, arthritis is induced by sensitization followed by challenge (typically 3 weeks later) with type II collagen injection. The arthritic disease is characterized by joint swelling, synovial inflammation and infiltration of mononuclear cells, and these features are accompanied by elevated production of proinflammatory cytokines and collagen-specific pathogenic antibodies of the IgG1 and IgG2a subclasses. The later stages of the disease involve overt and severe attributes of morbidity, such as cartilage and bone erosion. The influence of mercury on the development and progression of collagen-induced arthritis was assessed through an experimental design that involved injecting mercury(II) chloride subcutaneously into DBA/1 mice at 1.6 mg/kg body weight every third day for a period of 4 weeks at various time points before, during and after the induction of collagen-induced arthritis. When administered during and after collagen but not before, mercury increased the severity of collagen-induced arthritis as scored observationally, histologically and serologically (Hansson et al., 2005). Analogous to the collagen-induced arthritis model, mercury(II) chloride injection has been shown to increase the severity and prevalence of autoimmune myocarditis in an animal model where injection with cardiac myosin peptide in adjuvant induces an inflammatory autoimmune disease (Nyland et al., 2004; Silbergeld et al., 2005). Taken together, these studies suggest that mercury exposure functions as an environmental cofactor in autoimmune disease that promotes disease progression by acting during the induction and effector phases of the disease, thereby increasing the risks and severity of clinical disease in the presence of other genetic and immunological triggering events.

As mentioned above, mercury-induced autoimmune disease represents specialized and experimentally contrived models where exposure to mercury alone induces de novo systemic autoimmune disease in susceptible strains of mice and rats. Susceptibility to HgIA is mainly determined by the MHC genotype (i.e. the H-2 locus in the mouse and the RT-1 locus in the rat). Not surprisingly, the influence of genotype on HgIA has been most studied in the mouse model, as the tools necessary for thorough immunogenetic analysis exist for this species. In susceptible strains of mice, HgIA is characterized by elevated levels of serum IgG1 and IgE as well as the generation and persistence of serum antinucleolar autoantibodies with high specificity to fibrillarin, a 34 kDa ribonucleoprotein (Hultman et al., 1989, 1992, 1993). The antifibrillarin autoantibody response is under the control of the H-2 I-A locus; mice with H-2 haplotypes -*s* and -*q* develop strong antifibrillarin autoantibody titres and immune complexes accompanied by heavy granular IgG deposits in the renal mesangium and glomerulonephritis (Enestrom & Hultman, 1984; Hultman et al., 1989). Although susceptibility to the development of antinucleolar autoantibodies clearly lies within the H-2 I-A locus, the magnitude, persistence and specificity of the autoantibody response appear to be under the control of non-H-2 loci (Hultman et al., 1996). These non-H-2 genes contribute to the disease by controlling immunological factors (Johansson et al., 1997; Häggqvist & Hultman, 2003), as well as mercury toxicokinetics (Hultman & Nielsen, 1998; Ekstrand et al., 2010) and splenic mercury burden (Griem et al., 1997). The autoimmune kidney damage associated with murine HgIA reportedly occurs at mercury body burdens similar to those reported in some occupationally exposed humans (Hultman & Enestrom, 1992).

Although having a susceptible H-2 haplotype is a prerequisite for induction of antifibrillarin autoantibodies, there are properties of the mercury dose–response relationship and thresholds that influence the development and progression of disease in HgIA. Certain attributes of the disease appear to show differential sensitivity to mercury dose. For example, the threshold for elevated IgE levels appears to be higher than that required for induction of antifibrillarin autoantibodies (Nielsen & Hultman, 2002). Thus, whereas IgE and antifibrillarin autoantibodies are characteristic markers of development of autoimmune reactions following mercury exposure in susceptible strains, the mechanisms underlying elevated IgE levels and production of antifibrillarin autoantibodies are likely to be distinct. Elevated IgE level appears to be induced by relatively short exposures to mercury (e.g. 1-week exposure of 1 mg/l via the drinking-water), whereas prolonged exposure to mercury (i.e. 10-week exposure to 0.5 mg/l via the drinking-water resulting in renal mercury burden as low as 1.1 µg/g wet weight in susceptible strains) favours induction of antifibrillarin autoantibodies. The interplay between genetics and exposure is also revealed in experiments comparing renal mercury burdens following various dosing regimens in mice with H-2 susceptible and non-susceptible strains of mice. These experiments demonstrate that the toxicokinetics of inorganic mercury varies among mouse strains. Increasing the body and target organ burden of mercury does not supersede the prerequisite for having a susceptible haplotype for induction of HgIA. Furthermore, despite the presence of a susceptible haplotype, there are thresholds below which no autoimmune reactions develop. Intraspecies variability in this threshold value is seen among different mouse strains with susceptible H-2 haplotypes, which highlights the additional complexity of mercury burden interacting with genetic determinants outside the H-2 locus as well as other intrinsic or extrinsic factors.

One complication for risk assessment is that exposure for the bulk of the research performed with these animal models has been via subcutaneous injection of mercury(II) chloride; however, administration of mercury in forms and routes that are more relevant to human exposures has also been shown to induce HgIA. For example, mice with the MHC haplotype $H\text{-}2^s$

develop antinucleolar autoantibodies targeting fibrillarin following injection with methylmercury (Hultman & Hansson-Georgiadis, 1999) or exposure to methylmercury via the drinking-water (Havarinasab et al., 2007). In comparison with HgIA disease features following injection with inorganic mercury, methylmercury induces a weaker polyclonal B cell response, a lower antinucleolar autoantibody titre and no systemic immune complex deposits. The weaker autoimmune response elicited by methylmercury appears to be due to its immunosuppressive activities; however, the immunosuppressive phase post–initial exposure does not preclude subsequent immunostimulation and development of autoimmune disease in susceptible strains of mice (Häggqvist et al., 2005). The demethylation of methylmercury by macrophage enzymatic activities is believed to lead to the accumulation of inorganic mercury in lymphoid tissues (Havarinasab & Hultman, 2005; Havarinasab et al., 2007). The role of transformation from an organic mercurial to the autoimmune disease–inducing inorganic form is also seen with the ethylmercurithiosalicylate, thimerosol, which is rapidly metabolized to ethylmercury. As an organomercurial compound, thimerosal shares some of the immunosuppressive activities of methylmercury; however, perhaps because of the more rapid conversion of ethylmercury to inorganic mercury, exposure of susceptible mice to thimerosal produces HgIA that more closely resembles that induced by inorganic mercury (Havarinasab et al., 2004, 2005). Thimerosal treatment also accelerates the development of antinuclear antibodies and renal glomerular and systemic vessel wall immune complex deposits in autoimmune disease–prone NZBWF1 mice at lower doses of thimerosal than are required for HgIA in H-2^{s} mice and within a theoretical dose range encountered through vaccination (Havarinasab & Hultman, 2006). HgIA, complete with high-titre antifibrillarin autoantibodies, immune complex deposition and glomerulonephritis, has been reported in H-2^{s} mice exposed to mercury vapour under conditions where the exposure reportedly produced kidney mercury burdens relevant to occupationally exposed humans (Warfvinge et al., 1995). Finally, as another example where exposure produces HgIA, intraperitoneal implantation of dental amalgam into H-2^{s} mice resulted in a time- and dose-dependent development of hypergammaglobulinaemia, high serum titre antifibrillarin autoantibodies and systemic immune complex deposits (Hultman et al., 1994).

A human counterpart to murine HgIA has not been described. That is, no human HLA loci appear to impart susceptibility to mercury-induced frank autoimmune disease, as MHC loci do in susceptible rodents. However, features of HgIA in mice may be relevant to low-level mercury exposure in humans. As in HgIA in rodents, autoantibodies to fibrillarin occur in some patients with systemic sclerosis and other autoimmune connective tissue diseases. As discussed above, a human epidemiological study has reported higher urinary mercury excretion values in antifibrillarin autoantibody–positive subjects than in antifibrillarin autoantibody–negative subjects with systemic sclerosis and healthy controls. All subjects and controls in this report were within the “normal” range for urinary mercury excretion; however, the findings suggest that exposure to mercury might be a contributing factor in systemic sclerosis (Arnett et al., 2000).

The first demonstration of HgIA in an animal model was reported in 1978 with the observation that injection with mercury(II) chloride induced immune-type glomerulonephritis in Brown Norway rats (Druet et al., 1978). As with the murine model of HgIA, the induction of autoimmune disease in rats is strain specific, with susceptibility mapping to the rat RT-1 locus of the MHC class II complex. Brown Norway rats (RT-1^{n}) are highly susceptible, whereas Lewis strain rats (RT-1^{l}) are resistant (Aten et al., 1991). Similar to the mouse model, mercury(II) chloride leads to polyclonal T and B cell activation, increased serum immunoglobulin levels, including IgE, autoantibody production and immune complex

deposition, accompanied by a biphasic glomerulonephritis and proteinuria (Druet et al., 1977; Hirsch et al., 1982; Sapin et al., 1984). Despite these similarities, the rat model is distinct in that the autoantibody specificities differ and rats spontaneously recover and develop resistance to further mercury-induced disease (Dubey et al., 1993; Castedo et al., 1994). In the rat, autoantibody reactivities to phospholipids, DNA, glomerular basement membrane and laminin are found. The early phase of the disease manifests with linear deposition of anti–glomerular basement membrane autoantibodies. Later, immune complex granular IgG deposits form, leading to the development of nephritis (Druet et al., 1982). HgIA can be induced in Brown Norway rats by exposure to mercury vapour as well as by injection with mercury(II) chloride (Hua et al., 1993). Both vapour and injection produce similar disease features and demonstrate autoimmune disease manifestation that is mercury dose dependent; however, the kidney content of mercury was similar in all exposed groups. Rabbits injected with mercury(II) chloride reportedly develop autoimmune disease with features similar to those observed in Brown Norway rats (Roman-Franco et al., 1978).

From a risk assessment perspective, the data set described above constitutes a large body of evidence supporting a connection between mercury exposure and the induction of autoimmune disease as well as the pathogenesis and progression of autoimmune disease in a variety of rodent models. Mercury induces de novo autoimmune disease in certain susceptible rodent strains, exacerbates the onset and/or severity of disease in several strains of genetically autoimmune disease–prone mice and exacerbates disease in several mouse models of acquired autoimmunity. These animal data present clear evidence of an effect of mercury on autoimmunity and support a dose–response relationship. In rodent models, an increasing dose of mercury results in a greater autoimmune response as well as a greater accumulation of whole-body mercury levels and mercury deposition in target organs such as the kidney. Data support a threshold below which mercury exposure will not induce autoimmunity, even in susceptible rodent strains, and indicate that prolonged exposures to low doses of mercury are associated with accumulation of mercury in target tissues prior to developing autoimmune responses (Nielsen & Hultman, 2002). Along with the limited human data described above in section C5.3.1.1, the animal data present a strong weight of evidence for a mercury-associated increase in autoimmune disease incidence and progression.

As described above in section C5.3.1.1, the human data contain considerable uncertainty owing to the general lack of exposure data and the lack of a definitive, large-scale epidemiological study evaluating mercury exposure and elements of autoimmune disease. Therefore, human data are not available to evaluate a potential dose–response relationship between mercury exposure and reported symptoms of autoimmune disease in humans, and the available animal data should be used to develop a quantitative risk evaluation. The database of animal studies for mercury autoimmunity has two characteristics that need to be discussed in the context of developing a quantitative risk evaluation for human risk. First, as with other risk assessments, the use of data from the most relevant exposure route in humans is preferred, and therefore experimental animal data from studies involving oral exposure to mercury should be selected for the risk assessment over data from subcutaneous exposure studies. This is a particular issue for mercury, because so many of the animal studies of mercury-induced autoimmunity involve parenteral exposure. Second, the use of animal data from autoimmune disease–prone rodent models needs to be considered explicitly in the uncertainty factors applied to the animal data in estimating human risk of mercury-induced autoimmunity. The application of these uncertainty factors will be discussed in detail below, but as described in chapter 7 of the guidance document, these animal models are considered good models of susceptible humans rather than the general population, and therefore the

intraspecies uncertainty factor should generally be reduced from 10 to 1 when human risk is estimated from data obtained in autoimmune disease–prone rodents.

The oral drinking-water study by Hultman and Nielsen (study results reported in two publications: Hultman & Nielsen, 2001; Nielsen & Hultman, 2002) provides the lowest effect level from animal data by a relevant route of exposure with a clear dose–response relationship (see Table C5.1 for a summary of exposure and effects data) and therefore was selected for the quantitative risk assessment. The LOAEL from the studies was 0.5 mg/l for mercury(II) chloride in drinking-water for 10 weeks in male and female A.SW mice, and the effect was increased titre of antifibrillarin autoantibodies. The 0.25 mg/l dose of mercury(II) chloride represented a NOAEL in female A.SW mice, and a no-effect level was not determined in males, because the lowest dose tested in males was 0.5 mg/l (Hultman & Nielsen, 2001). It is important to note that the end-point selected (increased antifibrillarin autoantibodies) is an end-point that represents a relatively early effect in the etiology of mercury-induced or mercury-exacerbated autoimmunity in these mice. Antifibrillarin autoantibodies are considered an adverse effect, although there are other effects, such as immune complex deposition, that are more closely related to a clearly adverse outcome (i.e. renal damage).

Table C5.1: Mercury accumulation in male and female A.SW mice after drinking-water exposure to mercury(II) chloride for 10 weeks.

Sex	*Dose (mg/l)*	*AFA positive/ total*	*AFA reciprocal titre*	*Renal mercury accumulation (μg/g wet weight)*	*Splenic mercury accumulation (μg/g wet weight)*	*Whole-body retention (μg)*
Female	0	0/8	—	NR	NR	0
	0.25[a]	0/8	—	0.23	0.009	0.29
	0.5[b]	2/8	340 ± 424	0.71	0.0232	0.85
	1	8/8	1890 ± 1667	1.63	0.0472	1.76
	2	8/8	4880 ± 2735	3.76	0.120	4.08
Male	0	0/8	—	NR	NR	0
	0.5[b]	2/8	5200 ± 7127[c]	1.56	0.0294	1.19
	1	5/8	1688 ± 2176	2.68	0.0664	2.14
	2	8/8	2600 ± 1875	6.97	0.114	5.24
	4	8/8	5440 ± 2136	27.3	0.335	16.1

AFA, antifibrillarin autoantibody; NR, not relevant

[a] 0.25 mg/l was the NOAEL for autoantibodies to nuclear protein fibrillarin in females.

[b] 0.5 mg/l was the LOAEL for autoantibodies to nuclear protein fibrillarin in males and females.

[c] The authors reported that one male mouse had an extremely high AFA titre (10 240) that was not observed in any other mouse, regardless of dose level.

Sources: Hultman & Nielsen (2001); Nielsen & Hultman (2002)

The duration of dosing had an effect on the observation of an autoimmune response, with the 0.5 mg/l dose of mercury producing antifibrillarin autoantibodies only after 10 weeks of exposure and having no effect in males or females following 2.5 weeks of mercury exposure (Hultman & Nielsen, 2001; Nielsen & Hultman, 2002). The incidence of autoantibodies in each exposure group increased with increasing dose from two of eight individuals of each sex at 0.5 mg/l to eight out of eight of each sex at higher doses (see Table C5.1). The increase in

antifibrillarin autoantibody titre also displayed a dose–response relationship in the females; however, the data for antifibrillarin autoantibody titre did not display a dose–response relationship in male mice. The authors explained the lack of a dose–response relationship in the male mice as an exception caused by one male mouse at the 0.5 mg/l dose exhibiting an extremely high antifibrillarin autoantibody titre that was not observed in any other mouse, regardless of dose level (Nielsen & Hultman, 2002). The study utilized mercury(II) chloride labelled with a gamma-emitting isotope and therefore provided direct data on the administered dose (mg/l drinking-water) as well as internal dose reflecting the steady-state[1] whole-body retention of mercury, splenic mercury accumulation and renal mercury accumulation (see Table C5.1).

Although various authors (e.g. Nielsen & Hultman, 1999; Pollard et al., 2001) have used the kidney dose as a potential dose metric for mercury-induced autoimmunity because kidney is a principal organ of mercury accumulation as well as a principal site for toxic effects, the most appropriate dose metric for induction of autoimmunity or specific effects such as antifibrillarin autoantibody is unknown. Griem et al. (1997) hypothesized that splenic mercury may be the best dose metric for induction of mercury-induced autoimmunity because they found a higher correlation between splenic mercury accumulation and susceptibility to mercury(II) chloride–induced autoimmunity across mouse strains compared with other measures, including blood, liver and kidney levels of mercury. Use of a PBTK model would help estimate human risk from an internal dose metric such as blood or tissue levels of mercury from the animal data if the model had validated toxicokinetic and toxicodynamic parameters. Although there are toxicokinetic models for mercury distribution and elimination in humans and rodents (Nordberg & Skerfving, 1972; Bernard & Purdue, 1984; Carrier et al., 2001a,b; Berlin et al., 2007), there is no accepted model at present (Berlin et al., 2007; Ekstrand et al., 2010). A more comprehensive risk assessment should carefully reconsider the utility of the available toxicokinetic models to inform a human health risk assessment and extrapolate relevant human internal and external doses associated with increased risk of autoimmunity from the available animal data. However, that is beyond the scope of this case-study; therefore, this example is based on oral exposure and provides a brief discussion of the internal dose data on the spleen, kidney and whole-body retention that should be considered by the risk assessor. The data on internal dose are only useful for quantification with a validated PBTK model.

The 0.5 mg/l LOAEL needs to be converted to units of mg/kg body weight for standard calculations of a POD and the estimation of the reference value. The mg/kg body weight dose can be calculated from the drinking-water dose by multiplying the mercury concentration in drinking-water by the average water intake and dividing by the average body weight, as follows:

Female LOAEL = 0.5 mg/l × (0.0029[2] l/day) / (0.018 35 kg body weight)
= 0.079 mg/kg body weight per day

[1] Steady-state levels of mercury in mice are reached in 3–5 weeks of exposure (Nielsen & Hultman, 2002).

[2] The following dose information for the A.SW mouse is from personal correspondence with J.B. Nielsen: Average water intake = 2.9 ml (female) and 3.3 ml (male). Mean body weight of 17.1–19.6 g (female) at start of experiment and after last dose; mean body weight of 19.8–24.4 g (male) at start of experiment and after last dose (10–20 weeks). Therefore, the average weight of females = 18.35 g ((17.1 + 19.6)/2) and of males = 22.1 ((19.8 + 24.4)/2).

Male LOAEL = 0.5 mg/l × (0.0033 l/day) / (0.0221 kg body weight)
= 0.075 mg/kg body weight per day

LOAEL = 0.08 mg/kg body weight per day in both sexes, rounded to one significant digit

Female NOAEL = 0.25 mg/l × (0.0029 l/day) / (0.018 35 kg body weight)
= 0.0395 mg/kg body weight per day

NOAEL = 0.04 mg/kg body weight per day, rounded to one significant digit

To continue the illustration of this case-study, the adjusted NOAEL of 0.04 mg/kg body weight per day for mercury(II) chloride from the Hultman and Nielsen study (Hultman & Nielsen, 2001; Nielsen & Hultman, 2002) will be used to derive the health-based guidance value or reference value. As described in chapter 3 (section 3.3.7.3), the risk assessor should apply BMD modelling to the data to derive a POD near the low end of the available data. For the purposes of this case-study, the NOAEL will be used as the POD rather than selecting a model and performing the calculations, but the BMD is generally the preferred method.

The next step in the risk assessment process is the application of uncertainty factors, as described in section 3.3.10 in general and section 7.10 with reference to autoimmunity:

- The intraspecies uncertainty factor would be 1, because the data used to determine human risk were from an autoimmune disease–prone rodent model. These animal models are considered good models of susceptible humans rather than the general population. Therefore, the intraspecies uncertainty factor should generally be reduced from 10 to 1 when human risk is estimated from data obtained in autoimmune disease–prone rodents (see chapter 7 for further discussion). Most animal data on mercury induction or exacerbation of autoimmunity are from susceptible strains, because the interplay between genetic susceptibility, exposure and other environmental factors appears to be particularly important for mercury-induced autoimmunity (Fournié et al., 2001). As described above, researchers have begun to identify the genetic and mechanistic bases for some of the susceptible rodent strains, which include toxicokinetic differences that result in higher organ burdens of mercury in these strains. Furthermore, because animal data suggest that accumulation of mercury (whole body or levels in target organs such as the kidney) is directly related to autoimmune response, toxicokinetic variation in humans is likely to be associated with susceptibility. Although similar gene–environment interactions are expected for human susceptibility to mercury, there are no data on potential variations in human susceptibility to mercury immunotoxicity (Silbergeld et al., 2005).
- The interspecies uncertainty factor would be 10 to extrapolate from experimental animal data to human risk.
- The use and time factor would be 10 for a chronic exposure assessment, as the study length was 10 weeks, below the 2-year study generally considered chronic. Application of this uncertainty factor depends on the scope defined in the problem formulation stage of the risk assessment (e.g. chronic, subchronic, acute); an uncertainty factor of 1 would be applied for a subchronic risk assessment. The animal data suggest that mercury reaches a steady state internally with continuous exposure in 3–5 weeks (Nielsen & Hultman, 2002), and dropping or reducing the chronic uncertainty factor should be considered. However, the data utilized for the risk assessment are

from a 10-week exposure period, and there are no chronic exposure data on animals of this strain and examining these end-points (antifibrillarin autoantibody) to confirm that the subchronic uncertainty factor could be reduced.

- The database uncertainty factor would be 1, because the database for mercury induction or exacerbation of autoimmunity is extensive. There is some weakness to the database in regards to relevant routes of exposure, as the majority of the data are from parenteral studies, and therefore the effects of oral exposure to mercury are less well studied. However, sufficient studies are available for oral exposure to mercury that include low doses and establish no-effect levels for mercury-induced autoimmunity.

To complete the derivation of a health-based guidance value or reference value, the guidance recommends consideration of groups at risk (i.e. children, elderly and genetically susceptible individuals) and then dividing the POD by the total uncertainty factor described above. For this autoimmunity and autoimmune disease case-study, the consideration of susceptible populations is particularly important. As described above and in chapter 7, the autoimmune disease–prone rodents are considered a good model of humans susceptible to mercury-induced autoimmunity. Therefore, the use of additional adjustments for susceptible life stages or human populations is generally not warranted unless chemical-specific data indicate an increased risk for a particular population. An additional consideration may be warranted for susceptible life stages because there may be a general increased risk for autoimmunity in older individuals linked to a modulation in adaptive immunity (see Hakim & Gress, 2007, and discussion in section 7.8 of chapter 7), but there are no human data to inform this potential susceptibility for mercury-induced autoimmunity. The use of data from the autoimmune disease–prone mice is assumed to account for the full range of susceptible human life stages and populations.

In light of the observation that most autoimmune diseases are sex-biased towards females, sex is another important intrinsic factor to consider in association with environmental exposures, including to mercury. In the murine HgIA model, although males and females are both susceptible (given appropriate H-2 haplotype), females exhibit higher sensitivity, which manifests as a lower threshold for induction of antifibrillarin autoantibodies as well as higher responsivity. Therefore, the possibility of a greater sensitivity to mercury-induced autoimmunity in women is supported by data from animal models in the absence of human data to inform this potential sex bias in susceptibility. However, in this case, the data are from female mice and the LOAEL was identical for male and female A.SW mice, so the consideration of sex differences is not warranted.

The data above apply to mercury(II) chloride, because the animal data used in the quantification are from experiments in which the animals were exposed to mercury(II) chloride. These data can be used to develop reference values for exposure to mercury(II) chloride, or they can be adjusted to derive a reference value for inorganic mercury based on certain assumptions. For example, the USEPA (1995) and California Environmental Protection Agency (2000) derive oral reference values for inorganic mercury based on animal data from mercury(II) chloride exposure studies. The dose conversion applied for the USEPA and California Environmental Protection Agency assessments is to multiply by 0.739 to convert from the mercury in mercury(II) chloride to Hg^{2+} by weight.

Using the above values, the total uncertainty factor applied for a risk assessment for derivation of a chronic reference value for mercury would be 100 (1 for intraspecies, 10 for interspecies, 10 for subchronic to chronic and 1 for database).

For the chronic risk assessment of autoimmunity associated with mercury(II) chloride:

Reference value = 0.04 mg of mercury(II) chloride per kilogram body weight per day ÷ 100
= 0.0004 mg of mercury(II) chloride per kilogram body weight per day

For the chronic risk assessment of autoimmunity associated with inorganic mercury:

Reference value = 0.04 mg of mercury(II) chloride per kilogram body weight per day × 0.739 ÷ 100
= 0.0003 mg of inorganic mercury per kilogram body weight per day

The reference values derived from the LOAEL of 0.5 mg of mercury(II) chloride per litre (0.08 mg/kg body weight per day) in male and female A.SW mice and the NOAEL of 0.25 mg of mercury(II) chloride per litre (0.04 mg/kg body weight per day) in female A.SW mice for antifibrillarin autoantibodies are supported by a number of studies in the literature presented previously. In particular, the LOAEL is slightly lower than, but consistent with, a LOAEL of 1.25 mg of mercury(II) chloride per litre and a NOAEL of 0.625 mg of mercury(II) chloride per litre in drinking-water for 10 weeks in female SJL mice for induction of antinucleolar antibodies reported by Hultman & Enestrom (1992). The Hultman and Nielsen study (Hultman & Nielsen, 2001; Nielsen & Hultman, 2002) also reported antinucleolar antibody data with a LOAEL of 1.0 mg of mercury(II) chloride per litre and a NOAEL of 0.5 mg of mercury(II) chloride per litre in drinking-water for 10 weeks in male and female A.SW mice as well as female B10.S mice. Therefore, the mercury-induced antifibrillarin autoantibodies in A.SW mice represent the most sensitive end-point for autoimmunity in the most sensitive strain and are supported by other animal data.

The inductions of antifibrillarin and antinucleolar antibodies by mercury are end-points that represent relatively early effects in the etiology of mercury-induced or mercury-exacerbated autoimmunity in these mice. Data are also available on end-points more closely related to clearly adverse outcomes. In general, these effects are associated with a slightly higher level of mercury exposure. For example, the LOAEL for increased renal deposits of IgG in female SJL mice given mercury(II) chloride in drinking-water for 10 weeks was 5 mg/l, with a NOAEL of 2.5 mg/l (Hultman & Enestrom, 1992). The 5 mg/l dose of mercury(II) chloride was also associated with glomerular endocapillary cell hyperplasia and tubular atrophy. Data from Hultman & Nielsen (2001) and Nielsen & Hultman (2002) also support the same LOAEL for renal IgG deposits in female A.SW mice under the same 10-week drinking-water exposure regimen.

As described previously, many of the animal data on mercury-induced autoimmunity are from experiments in which mercury was administered subcutaneously. Data from subcutaneous exposure studies in rodents can be used to support the risk assessment qualitatively, but they are generally not used for quantitative risk evaluations because of the failure of the parenteral exposure route to pass through the digestive tract or lung, as with the relevant

human oral or inhalation exposure. The potential utility of parenteral studies to support the risk assessment is illustrated by the following consideration of the autoantibody data in BXSB mice. Pollard et al. (2001) demonstrated in BXSB mice that subcutaneous exposure at doses down to 0.4 µg of mercury(II) chloride given twice per week for 40 weeks increased autoantibody titres to antichromatin antigens (from 3.74 ± 2.02 in controls to 10.70 ± 4.10 in treated mice) and increased proteinuria by more than 2-fold. Higher doses resulted in a more rapid increase in autoantibody titres over the 40 weeks of exposure, but resulted in similar titres. Obviously, these subcutaneous data cannot readily be converted to an estimated daily oral exposure, because compounds administered via the parenteral route do not enter the digestive tract or lung, as with the relevant human oral or inhalation exposure, and therefore parenteral exposure has unknown effects on the toxicokinetics, including absorption, excretion and biotransformation. Noting these differences and the absence of an appropriate PBTK model, comparison of the internal dose at the LOAEL between oral and parenteral exposures may provide some insight for a relevant internal dose metric. The subcutaneous exposure in Pollard et al. (2001) resulted in a mean mercury level in the kidney of 0.0762 ± 0.006 µg/g wet weight at the NOAEL of 0.04 µg of mercury(II) chloride and 0.6627 ± 0.0847 µg/g wet weight at the LOAEL of 0.4 µg of mercury(II) chloride in female BXSB mice. The kidney level of mercury in the female BXSB mice at the LOAEL (0.66 µg/g wet weight) is comparable to the concentration reported in female A.SW mice at the LOAEL (0.71 µg/g wet weight) and suggests the potential utility of this dose metric.

As described above, there is some support for the use of the kidney concentration as a dose metric, but the most appropriate dose metric for induction of autoimmunity or specific effects such as antifibrillarin autoantibodies is unknown. Therefore, the risk assessor is left to compare the animal data on oral dose and internal metrics from the above quantitative risk evaluation with known human exposure levels to develop a margin of exposure and evaluate human risk. The internal dose metrics such as kidney levels of mercury can also be used for comparisons with human exposure. Most notable in these comparisons is that the kidney levels of mercury in A.SW mice associated with induction of antifibrillarin autoantibodies of 0.71–27.3 µg/g wet kidney tissue are within the range of those in non-occupationally exposed humans (i.e. non-detectable to 2.1 µg/g wet kidney tissue) (Nylander et al., 1987; Barregård et al., 1999).

C5.3.1.3 Is there evidence that the chemical alters immune measures associated with autoimmunity (i.e. autoantibody levels, inflammatory markers, regulatory T cells, lymph node proliferation, etc.) in animal models of autoimmune disease?

Yes. There are many examples of mercury modulation of immune end-points associated with autoimmunity. Numerous studies present data on autoantibody production associated with mercury exposure. Although the majority of autoantibody data are from rodent strains that are susceptible to HgIA, a few studies also reported induction of antinucleolar autoantibodies following mercury exposure in outbred strains (e.g. ICR, NMRI and Black Swiss mice; Abedi-Valugerdi, 2009). The PLNA provides additional evidence of an autoimmune-associated end-point. Mercury produces a well-characterized positive response in the PLNA in a number of mouse strains (Stiller-Winkler et al., 1988), such that mercury has been used as a control in evaluating the potential for other metals to induce autoimmunity and hypersensitivity (Carey et al., 2006). There is also limited evidence that mercury exposure is associated with proinflammatory cytokine release; however, there is no clear pattern of cytokine production across susceptible or outbred rodent strains (reviewed in Vas & Monestier, 2008). In the mercury model of autoimmunity in rodents, the polyclonal B cell

activation ultimately responsible for the immunopathology is widely believed to be due to a selective stimulation of Th2 cells (Badou et al., 1997). In this model, upregulation of IL-4 expression has been shown in response to mercury treatment both in vivo and in vitro in rodents (Gillespie et al., 1995; Badou et al., 1997; Häggqvist & Hultman, 2001) and following in vitro treatment of human peripheral blood mononuclear cells (De Vos et al., 2007; Hemdan et al., 2007). However, the importance of an imbalance of Th1 and Th2 in the susceptibility to mercury-mediated autoimmunity has been called into question (Kono et al., 1998), and mercury-induced autoimmune disease and IL-4 production have been dissociated from each other (Bagenstose et al., 1998). Hence, despite a well-established literature supporting the view that mercury-induced systemic autoimmunity is a prototypic Th2-mediated disease, the cellular immune mechanisms underlying the disease process are not understood as clearly as they previously were thought to be. Furthermore, although some progress has been made in understanding the biochemical signalling mechanisms mediating the effects of mercury on Th2 cells (Badou et al., 1997), many of the details concerning the molecular components directly or indirectly targeted by mercury are essentially unknown. Irrespective of the data showing that IL-4 is not required for the antifibrillarin autoantibody response, IL-4 is required for the elevated IgE and IgG1 response characteristic of mercury-susceptible mouse strains (Ochel et al., 1991).

As described above, there are many examples of mercury-related changes in immune measures associated with autoimmunity, including increased autoantibody levels in non-autoimmune disease–prone mice and a positive response in the PLNA. These data alone present some evidence of an effect of mercury on autoimmunity. For the purposes of illustration, a risk assessor faced with evaluating a data set restricted to these immune measures associated with autoimmunity could conclude that there was a potential for mercury to induce or exacerbate autoimmunity. However, even as an exercise, it is difficult to separate these data from the larger database of mercury effects and mercury-induced changes in immune measures reported in animal models of autoimmune disease, because so many of the data on mercury immunotoxicity are from susceptible rodent strains. The indication that mercury alters immune measures associated with autoimmunity contributes to the weight of evidence that mercury induces and exacerbates autoimmunity, outlined in sections C5.3.1.1 and C5.3.1.2 above. Considered together, these data support the human epidemiological data and the large database from rodent models of autoimmune disease, resulting in a solid weight of evidence for a mercury-associated increase in autoimmune disease incidence and progression.

C5.3.1.4 Is there evidence from general or observational immune assays (lymphocyte phenotyping, cytokines, complement, lymphocyte proliferation, etc.) that the chemical has the potential to modulate autoimmune disease?

Yes. There are examples of mercury-induced changes in general immune assays; however, the data provide limited direct support to the hypothesis that mercury has the potential to modulate autoimmunity. Numerous rodent studies demonstrate that exposure to mercury induces changes in immune cell populations, cytokine secretion, selective T cell proliferation (generally CD4+ and, to a lesser extent, CD8+ T cells), polyclonal B cell activation, hypergammaglobulinaemia and other end-points (reviewed in Vas & Monestier, 2008). These effects are consistent with a role in autoimmunity, because they are supported by mechanistic studies in autoimmune disease–prone rodents and illustrate how mercury exposure affects self-tolerance, thereby promoting autoimmunity. For example, Laiosa et al. (2007) showed that in BALB/cJ mice exposed to mercury(II) chloride at 10 mg/l ad libitum via drinking-water for 2 weeks, attenuation of pro-apoptotic signalling due to mercury exposure may be a

factor contributing to autoreactive T cell activation. However, the predictive value of changes in lymphocyte cell subpopulations, cytokines or signalling pathways for chemically induced autoimmunity is unclear, and these data would provide equivocal support for autoimmunity without the larger database of mercury-induced changes in immune measures reported in animal models of autoimmune disease. The data set of mercury-associated changes from general immune assays does support and inform other aspects of the risk assessment, such as susceptible populations or life stages (i.e. greater sensitivity of females and the developing animal). Pilones et al. (2009) showed that in utero exposure to mercury(II) chloride (drinking-water at 10 mg/l ad libitum for the duration of gestation) induced phenotypic changes in the immune cells of F_1 progeny. Thymic and splenic tissues harvested at 10 weeks of age to assess T cell phenotypes and function showed a significant reduction in splenic CD4+CD25+ cells in mercury-exposed female, but not male, mice. Concanavalin A–stimulated splenocytes from mercury-exposed mice showed significant increases in proliferative responses relative to cells from control mice, regardless of sex. Cytokine secretion was also modulated, with concanavalin A–stimulated IL-4 and IFN being increased in splenocytes from mercury-exposed mice. The potential for life stage–related and sex-associated sensitivity to mercury-induced autoimmunity is discussed in greater detail above in the quantitative risk assessment presented in section C5.3.1.2.

Although the database of mercury-associated immune changes includes a number of general immune assays, these data alone provide equivocal evidence for mercury-induced autoimmunity. In the absence of the considerable database of mercury-induced changes in endpoints associated with autoimmunity from animal models of autoimmune disease, this type of immune data would suggest immunomodulation and would not be definitive for autoimmunity. Lymphocyte proliferation or cytokine data alone would identify a data gap suggesting the need for more conclusive autoimmunity and functional immune assays. As such, the data could be used to support the need for additional studies in animal models of autoimmune disease to determine autoimmune potential as well as functional immune assays to test for immunosuppression or immunostimulation. Of course, the equivocal nature of these data is largely due to the fact that we are evaluating the lymphocyte proliferation data out of context and for purposes of illustration. In fact, some of the data on these general immune assays were collected as part of mechanistic studies to characterize the MOA for autoimmune effects induced by mercury exposure. The reality is that the database supporting mercury-associated autoimmunity includes a large database of autoimmune-related effects reported in animal models of autoimmune disease (see section C5.3.1.2), numerous examples of immune measures associated with autoimmune disease (see section C5.3.1.3) and limited evidence from human epidemiological studies (see section C5.3.1.1). The data from general immune assays add to the considerable weight of evidence for mercury-induced autoimmunity.

C5.3.1.5 Is there histopathological evidence (thymus, etc.) or are there changes in immune organ weights or haematological changes that suggest that the chemical causes an immune response against self (i.e. immune complex deposition, inflammatory cell infiltrates)?

Yes. The main histopathological evidence from the mercury immunotoxicity database that supports autoimmunity comes from studies that investigated and reported immune complex deposition. Direct immunostaining for same-species IgG (i.e. staining for rat IgG along renal capillary walls and basement membranes in mercury-exposed rats) has been used to detect autoimmune response to mercury in autoimmune disease–prone rats such as the Brown Norway and MAXX strains (Henry et al., 1988). Mercury exposure is associated with

development of autoantibodies that result in immunoglobulin deposits in the renal basement membranes of rabbits, mice and rats. Similar deposits of IgG have been observed in the basement membranes of the spleen, liver, adrenal glands, heart and intestine in rats (reviewed in Bigazzi, 1999). Granular deposits of IgM, IgG1, IgG2a and Ig3 antibodies have been identified in the renal mesangium of mercury-injected NZBWF1 mice (Abedi-Valugerdi et al., 1997). Data that demonstrate the accumulation of immune complexes in various tissues rise to the level of providing some evidence of autoimmunity if the immune complexes are further characterized to identify autoantibodies. As discussed in section C5.3.1.3 above and section 7.7.1 of chapter 7, the presence of autoantibodies provides some evidence of chemical-induced autoimmunity. Among the many histopathological studies reporting mercury-induced immune deposits, there are numerous examples that identify autoantibodies. Therefore, in this case, the histopathology data set includes specific support for autoimmunity. This level of specificity is unlikely to be obtained from routine haematoxylin and eosin staining of paraffin-fixed tissue, and therefore it is unlikely that the risk assessor would be provided with this level of support from routine histopathological observations. The identification and characterization of antibodies in immune deposits would require more targeted techniques, including immunohistochemistry on appropriately fixed and prepared tissues.

As discussed above, the data set of histopathological evidence in immune tissues following mercury exposure includes specific demonstration of autoantibodies; therefore, these data alone provide some evidence for mercury-induced autoimmunity. The data set also provides mechanistic data suggesting an MOA. The database supporting mercury-associated autoimmunity includes a large number of studies reporting autoimmune-related effects observed in animal models of autoimmune disease (see section C5.3.1.2), numerous examples of immune measures associated with autoimmune disease (see section C5.3.1.3) and limited evidence from human epidemiological studies (see section C5.3.1.1). The specific, autoimmune-related histopathological evidence described above adds to the considerable weight of evidence for mercury-induced autoimmunity.

C5.3.2 Weight of evidence conclusions for hazard characterization

The individual discussions of different types of autoimmune data for mercury in sections C5.3.1.1–C5.3.1.5 (including the quantitative assessment presented above in section C5.3.1.2) do not represent a comprehensive risk assessment for health effects or immunotoxicity, but rather provide an illustrative example to outline the process for conducting an assessment of autoimmunity following the guidance from chapter 7. As described in chapters 3–7, the risk assessor should develop the weight of evidence conclusions based on the answers to all of the weight of evidence questions in chapter 7 and summarized in Figure 7.1. The weight of evidence conclusions for autoimmunity should also describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps.

Studies in autoimmune disease–prone A.SW mice exposed to mercury(II) chloride in drinking-water for 10 weeks (Hultman & Nielsen, 2001; Nielsen & Hultman, 2002) provide the lowest mercury exposure levels associated with autoimmunity. Therefore, because they represent the lowest effect levels, these data would be used to derive a POD in the quantitative risk assessment of mercury-induced autoimmunity. The procedures and calculations necessary to develop a reference value from the Hultman & Nielsen (2001) and Nielsen & Hultman (2002) data are presented above in section C5.3.1.2, including the application of

uncertainty factors. Calculations from the NOAEL are used to illustrate the process, although BMD modelling would probably be used for a full risk assessment, as it is the preferred approach. Section C5.3.1.2 also includes other considerations that would be part of a full risk assessment, such as a discussion of groups at risk, MOA and the selection of the appropriate dose metric. Each section on individual data types (sections C5.3.1.1–C5.3.1.5) includes a brief discussion of the consistency and strengths of the database for mercury-induced autoimmunity. A full risk assessment would generally include an expanded discussion of these points, data gaps and the associated uncertainties. For example, the human data do support the relevance of the experimental animal data, because the epidemiological data provide evidence that mercury exposure induces or exacerbates autoimmune disease in humans. The full risk assessment would detail these effects and the potential relationship between the laboratory animal data and effects in humans, such as symptoms of systemic lupus erythematosus. Data gaps would be highlighted; in particular, the lack of a definitive, large-scale epidemiological study evaluating mercury exposure and elements of autoimmune disease represents a data gap and contributes to the uncertainty in the risk assessment.

The weight of evidence discussion would reflect the relatively strong confidence in the data set described above and highlight the support from autoimmune-related effects observed in animal models of autoimmune disease (see section C5.3.1.2), the numerous examples of immune measures associated with autoimmune disease (see section C5.3.1.3), the evidence from general immune assays (see section C5.3.1.4), the autoimmune-related histopathological evidence (see section C5.3.1.5) and limited evidence from human epidemiological studies (see section C5.3.1.1). These studies add to the considerable weight of evidence for mercury-induced autoimmunity and support the drinking-water studies in autoimmune disease–prone A.SW mice (Hultman & Nielsen, 2001; Nielsen & Hultman, 2002) as principal studies appropriate for derivation of PODs and dose–response assessment of autoimmunity associated with mercury.

C5.4 Conclusions

This case-study of mercury-related autoimmunity and autoimmune disease is a demonstration of the application of the risk assessment guidance for the assessment of autoimmunity and autoimmune disease presented in chapter 7 of this document. Mercury was selected because of the strong database of the effects of mercury exposure in animal models of autoimmune disease as well as the epidemiological data relating to potential effects in humans. The case-study illustrates the limitations often encountered in evaluating epidemiological data (e.g. small sample sizes or co-exposure issues) and important issues in relating experimental animal data to human risk. The use of animal data from an autoimmune disease–prone rodent model required particular consideration in the quantitative risk assessment. These animal models of autoimmunity are considered good models of sensitive subpopulations of humans rather than the general population, and therefore the intraspecies uncertainty factor was reduced in the quantitative risk assessment of mercury-related autoimmunity and autoimmune disease.

It should be noted that this case-study on mercury is provided with the purpose of illustrating how the risk assessment guidance can be used for assessing the risk of autoimmunity, but it does not represent a comprehensive risk assessment, nor does it represent a final regulatory position.

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CASE-STUDY 6: ASSESSMENT OF AUTOIMMUNITY-STIMULATING EFFECT OF TRICHLOROETHYLENE

C6.1 Introduction

Exposure to trichloroethylene (TCE) has been associated with multiple forms of immuno-toxicity, including suppression, hypersensitivity and autoimmunity. The overwhelming majority of immune studies on TCE have examined autoimmune-related end-points. A large number of epidemiological studies have suggested that TCE, or at least organic solvent exposure in workers, is associated with systemic sclerosis and several other autoimmune diseases. This is balanced by an equally large number of experimental studies reporting that TCE exposure is associated with stimulation or exacerbation of end-points linked to auto-immunity in animal models of autoimmune disease.

This case-study illustrates the use of the risk assessment guidance provided for the assess-ment of autoimmunity and autoimmune disease as presented in chapter 7. TCE was selected as a case-study because there is a relatively large database of studies in both humans and animals that have explored the relationship between TCE and autoimmunity. The case-study for TCE illustrates a risk assessment that relies on animal data for quantification because the epidemiological data supporting the relationship between TCE and autoimmune disease lack good exposure data (as is often the case for human studies).

The risk assessment of TCE begins with a brief summary of the available evidence for auto-immunity associated with TCE. The weight of evidence conclusions that TCE exposure is associated with autoimmunity or autoimmune disease are then developed by following the guidance presented in chapter 7 for the assessment of autoimmunity. The case-study is not meant to be a full risk assessment of the health effects associated with exposure to TCE. Rather, the following assessment is provided to illustrate the process for conducting a risk analysis of TCE-associated autoimmunity by considering the available human, experimental animal and mechanistic data required to do so.

C6.2 Background: data on the potential for trichloroethylene to induce or exacerbate autoimmunity

TCE has been used extensively as an industrial degreasing agent and is commonly detected as a pollutant in ambient air, water supplies and soil (USEPA, 2011). Studies dating back to the late 1970s have reported an association between human exposure to organic solvents, including TCE, and development of autoimmune diseases, such as systemic sclerosis, con-nective tissue disease, multiple sclerosis, vasculitis (with increased antineutrophil cyto-plasmic antibodies) and rheumatoid arthritis. The human data demonstrating the strongest association between TCE exposure (or general solvent exposure) and autoimmune disease are for systemic sclerosis. For animal models, the majority of data are in the MRL+/+ mouse, an autoimmune disease–prone strain. Khan et al. (1995) published the first studies of TCE-associated autoimmunity in an animal model (female MRL+/+ mice) to clarify the role of TCE in autoimmune responses. High doses (10 mmol/kg body weight) of TCE via intra-peritoneal injections were associated with autoantibody formation in the Khan et al. (1995) study. A number of subsequent studies have examined mechanisms of action and, to a lesser extent, dose levels for TCE-associated autoimmune effects in the MRL+/+ mouse.

The underlying mechanism of TCE-stimulated autoimmune phenomena remains elusive, but specific T cell stimulation or inhibition of induction of apoptosis in these cells may be involved (Gilbert et al., 2006). This is underscored by findings in autoimmune disease–prone MLR+/+ mice, in which TCE exposure stimulates Th1 cells and exacerbates the lupus-like symptoms that these mice express (Khan et al., 1995; Griffin et al., 2000b; Wang et al., 2009). In addition, experimental animal studies have demonstrated that TCE is metabolized into reactive intermediates that form protein adducts and can also induce inducible nitric oxide synthase–dependent oxidation of proteins. Each event may give rise to neoantigens, culminating in autoimmunity as well as in allergic phenomena (Buben & O'Flaherty, 1985; Griffin et al., 2000a; Wang et al., 2009).

C6.3 Assessment of the potential for trichloroethylene to induce autoimmunity

C6.3.1 Application of the weight of evidence approach

A series of questions is presented in chapter 7, section 7.7.1, "Weight of evidence approach to assessment of risk for autoimmunity", that is intended to aid in organizing and evaluating immunotoxicity data from strong to weak evidence of significant risk for autoimmunity and autoimmune disease. The weight of evidence conclusions developed by answering these questions summarize the hazard identification for autoimmunity and should describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps. When autoimmunity is indicated by the weight of evidence, the data are brought forward for dose–response assessment beginning with selection of the most appropriate end-point(s) (critical effects) and developing PODs. The questions from chapter 7 are reproduced and answered below, followed by a discussion of the supporting immunotoxicity data.

C6.3.1.1 Are epidemiological studies, clinical studies or case-studies available that provide human data on end-points relevant to chemical-induced autoimmunity (i.e. increased incidence of all or specific autoimmune diseases, changes in immune parameters indicative of autoimmunity, increased levels of autoantibodies, decreased regulatory T cell function, evidence of nonspecific stimulation of the immune system, increased levels of markers of inflammation)?

Yes. Cross-sectional case–control studies are available suggesting that TCE induces clinical disorders similar to idiosyncratic drug hypersensitivity reactions, as well as clinical disorders that may be linked to autoimmunity, with the strongest data on autoimmunity in humans supporting an association between TCE and systemic sclerosis (scleroderma) (NRC, 2006; Cooper et al., 2009).

Adverse effects reminiscent of idiosyncratic drug reactions that have been described in TCE-exposed individuals range from rash, itching and fever to hepatic dysfunction (e.g. hepatitis) and severe generalized hypersensitivity dermatitis (Kamijima et al., 2008). As is the case with other idiosyncratic hypersensitivity reactions, the incidence of TCE-related adverse effects is usually low (1 case per 100) and displays a strong link to specific HLA loci, HLA-B*1301 and HLA-B*44 (OR = 36.8; 95% CI = 17.8–76.1) (Li et al., 2007). In addition, the dose–response relationship for TCE-related hypersensitivity disorders is not clear, reactive metabolic intermediates are involved and reactivation of human herpes virus 6 (HHV-6) has been found in TCE-affected individuals (Nakajima et al., 2003; Huang et al., 2006; Kamijima

et al., 2007). HHV and other infections are also linked to certain drug hypersensitivity reactions and may be susceptibility factors in TCE-induced adverse reactions, autoimmune or allergic, as well.

Cytokine levels found in serum of workers exposed to environmental TCE levels of 35 ± 14 mg/m^3 suggest a proinflammatory status (increased levels of IFN-γ and IL-2 and decreased levels of IL-4) (Iavicoli et al., 2005). A similar pattern was reported for newborns in a study in which increasing TCE concentrations in indoor air samples from the child's bedroom were associated with increased levels of IFN-γ and decreased levels of IL-4; IL-2 was unchanged (Lehmann et al., 2002). Although these changes in cytokine levels as such are not indicative of autoimmunity, they may indicate alterations in immune homeostasis that favour auto-immune disease development. In a recent study, Kamijima et al. (2008) showed that the skin disorders (DTH reactions) observed among workers exposed to TCE were not due to impurities or stabilizers and that they appeared to be associated with the extent of metabolism of TCE. The researchers compared affected workers in six factories with healthy exposed workers in two other (control) factories. Analysis of the urine from all workers detected levels of trichloroacetic acid (TCA), one of the major metabolites of TCE, at concentrations ranging from 318 to 1617 mg/l. The maximum TWA personal exposure of the healthy workers to TCE was between 164 and 2330 mg/m^3. Concentration ranges of TCE (from personal exposure measurements or determined by urinary TCA) overlap between healthy workers and patients with hypersensitivity. Available exposure data are not sufficient to explore a dose–response relationship for TCE-induced hypersensitivity disorders.

There are a number of epidemiological studies that support an association between solvent exposure, including TCE, and autoimmune diseases, such as systemic sclerosis, connective tissue disease, multiple sclerosis, vasculitis (with increased antineutrophil cytoplasmic antibodies) and rheumatoid arthritis. There are enough studies of solvent exposure and auto-immunity that meta-analyses have been performed for some autoimmune diseases. These meta-analyses found an association between solvent exposure and relative risk (RR) of systemic sclerosis (RR = 3.14; 95% CI = 1.56–6.33) and multiple sclerosis (RR = 2.6; 95% CI = 2.0–3.3) (Landtblom et al., 1996; Aryal et al., 2001). However, the data supporting an association between TCE exposure and autoimmunity are not as strong as the data on general solvent exposure. For example, Lacey et al. (1999) reported an association between self-reported solvent exposure (OR = 2.1; 95% CI = 1.5–3.0) and undifferentiated connective tissue disease in a case–control study involving 205 female patients; TCE alone was not associated with increased risk of connective tissue disease in these women.

Systemic sclerosis is the autoimmune disease that is most consistently associated with either TCE or general solvent exposure. Case–control studies in France and South Carolina, USA, both found an association between occupational exposure to TCE, or general solvents, and a 2- to 4-fold increased risk of systemic sclerosis (Nietert et al., 1998; Diot et al., 2002). Nietert et al. (1998) reported that the presence of anti-Scl-70 (DNA topoisomerase I) was an effect modifier that may be related to HLA genotypes. Workers with anti-Scl-70 (DNA topoisomer-ase I) autoantibodies and higher maximum or cumulative TCE intensity judged by a job exposure matrix had a 4-fold greater risk for systemic sclerosis. In a larger case–control study of women only, an association with solvent exposure was found (OR = 2.1; 95% CI = 1.7–2.6), but the OR for systemic sclerosis was not significant for self-reported TCE exposure (Garabrant et al., 2003).

Kilburn & Warshaw (1992) reported that symptoms of systemic lupus erythematosus or connective tissue disease and antinuclear autoantibodies were elevated in a population exposed to solvents including TCE and heavy metals through groundwater contamination. Cooper et al. (2009) suggested that the use of rheumatic disease as part of the selection criteria for the exposed population makes it difficult to attribute symptoms to exposure in the Kilburn & Warshaw (1992) study. More recent case–control studies addressing systemic lupus erythematosus have failed to show an association with solvents or TCE (Cooper & Parks, 2004; Parks & Cooper, 2006).

In summary, the human data provide some evidence that TCE exposure induces or exacerbates autoimmune disease in humans. The association between TCE and autoimmune disease is supported by a number of case–control studies linking TCE exposure and autoimmune disease (primarily systemic sclerosis), occupational studies demonstrating that TCE causes severe generalized hypersensitivity dermatitis and several epidemiological studies relating TCE and proinflammatory cytokine status. The strongest evidence to support this conclusion comes from the consistency of TCE-related effects observed across multiple case–control studies of TCE and systemic sclerosis. Although numerous case–control studies support a relationship between autoimmune diseases and exposure to solvents, including TCE, these studies provide limited support for TCE-induced autoimmunity because there is considerable uncertainty that the observed autoimmune-related effect is related to TCE exposure and not to the other solvents. The majority of the exposure data from the available studies are from job exposure matrices and involve multiple chemicals. The risk assessor has to determine whether the exposure questions for individual studies result in supporting or equivocal data for TCE-induced autoimmunity. Although Iavicoli et al. (2005) and Lehmann et al. (2002) provided some exposure data relating TCE to inflammatory cytokines, dose–response data on a potential association between TCE and autoimmune disease are not available. The database would benefit substantially from a well-conducted cross-sectional or prospective cohort study of autoimmune disease incidence with exposure data. The available epidemiological data for TCE provide little information on a potential dose–response relationship between TCE exposure and reported symptoms of autoimmune disease in humans and therefore do not support a quantitative risk assessment. The human data also do not provide much information on potential susceptible populations by age or sex. There is some evidence that TCE exposure is associated with a greater relative risk for systemic sclerosis for men than for women; however, this may reflect a greater ability to detect the disease due to the 10-fold lower background incidence of systemic sclerosis in men compared with women. Thus, human data add to the weight of evidence that TCE is associated with autoimmune disease, but, as a result of limitations in exposure data, cannot be used to perform a quantitative risk assessment.

C6.3.1.2 Is there evidence that the chemical causes changes in disease incidence or progression in animal models of autoimmune disease?

Yes. Most (Khan et al., 1995; Griffin et al., 2000a,b,c; Blossom et al., 2006, 2007, 2008; Gilbert et al., 2006, 2009; Blossom & Doss, 2007), but not all (Peden-Adams et al., 2008; Keil et al., 2009), studies using autoimmune disease–prone strains of mice suggest that TCE promotes pathogenesis and progression of autoimmune disease in several mouse models of autoimmune disease and induces biomarkers of autoimmune disease in wild-type mice (Keil et al., 2009). However, studies to date have not demonstrated that TCE induces autoimmune disease. Hence, the studies performed so far suggest that the appearance of autoimmune phenomena is accelerated, but not induced, by exposure to TCE.

A study using the autoimmune disease–prone MRL+/+ mouse demonstrated that TCE (10 mmol/kg body weight intraperitoneally) itself moderately stimulated the level of antinuclear antibodies (doubled optical density value in ELISA) and anti–single-stranded DNA antibodies (50% increase of optical density in ELISA), but not of anticardiolipin, anti-Sm, antihistone, anti–double-stranded DNA antibodies or circulating immune complexes (Khan et al., 1995). In addition, increases in relative spleen weight (36%) and total serum IgG level (45%) were detected. In the same study (Khan et al., 1995), one of the metabolites of TCE, dichloroacetyl chloride (DCAC) (at 0.2 mmol/kg body weight intraperitoneally), was tested and appeared to induce a higher (i.e. more than 300%) increase of IgG antibodies that were also affected by TCE. Notably, DCAC also induced DCAC-specific antibodies, giving 20–30 times higher optical density values. The disease- and autoantibody-promoting effect of DCAC (also using 0.2 mmol/kg body weight intraperitoneally) was confirmed by a more recent study by the same group (Cai et al., 2006). This study suggested that covalent adduct formation of proteins by the acylating agent DCAC may be the initial step in generating DCAC-specific immunity and possibly also in promoting autoantigen-specific antibody responses. Covalent binding of TCE, possibly as DCAC, to an array of macromolecules has been found in a number of tissues, including liver, lungs, kidneys and stomach. Interestingly, TCE also covalently binds to CYP2E1, the enzyme primarily responsible for TCE metabolism. Other metabolites of TCE have also been demonstrated to become immunogenic if adducted to albumin (Cai et al., 2007). In concordance with these studies, another oxidative metabolite of TCE, trichloroacetaldehyde hydrate (TCAH), has been shown to promote various autoimmune parameters in autoimmune disease–prone MRL+/+ mice (Gilbert et al., 2006; Blossom et al., 2007).

Similar studies provide evidence for the autoimmunogenic potential of TCE based on modulation of a range of immune parameters. Griffin et al. (2000a,b) exposed MRL+/+ mice to concentrations of TCE in the drinking-water resulting in doses of 21, 100 or 400 mg/kg body weight per day for 4 or 32 weeks. Only doses of 100 or 400 mg/kg body weight per day resulted in mononuclear infiltration (of both CD3+ and CD3− lymphocytes), adduct formation in the liver, activation of CD4+ T cells (increased activation markers and IFN-γ production) and increases in serum levels of antinuclear antibodies. Notably, compared with untreated mice, antinuclear antibodies were increased only at 4 weeks (at doses of 21 and 100 mg/kg body weight per day) but equalized to control at 32 weeks, whereas histological changes observed in the liver at 32 weeks (at doses of 100 and 400 mg/kg body weight per day) were not found after only 4 weeks of exposure. In a follow-up study (Griffin et al., 2000c), higher daily TCE doses of 455 and 734 mg/kg body weight per day resulted in increased levels of antinuclear antibodies after 6–8 weeks, which again normalized after longer exposure (22 weeks). These findings suggest that TCE may accelerate the onset of autoimmune responses in autoimmune disease–prone mice and that at a later time point, when spontaneous development of autoimmune disease has occurred in controls, the differences are no longer obvious. As liver pathology occurs only in TCE-treated MRL+/+ mice, this phenomenon is not likely due to autoreactivity, but rather is the result of a compound-specific, hypersensitivity-like process. The finding of increased levels of IgE in DCAC-treated MLR+/+ mice (Cai et al., 2007) may support this idea, although increased IgE levels point to a Th2-mediated hypersensitivity response. More recently, Cai et al. (2008) also showed that TCE (60 mg/kg body weight per day for up to 48 weeks) induced a slightly, but not significantly, enhanced level of serum antinuclear antibodies. In line with the above-mentioned study by Griffin et al. (2000b), pathological changes (CD3+ cell influx in liver, lungs and kidney and glomerular immunoglobulin deposits) were demonstrated only in TCE-exposed mice.

Other autoimmune disease–prone mouse strains—i.e. NZBWF1 (Keil et al., 2009) and C3H/HeJ mice (Blossom et al., 2006)—as well as non-autoimmune disease–prone mice—i.e. the B6C3F1 strain (Keil et al., 2009)—have been used to study the autoimmune-promoting effects of TCE. TCE did not induce autoimmune phenomena in C3H/HeJ (susceptible to autoimmune alopecia) and produced only minor changes in NZBWF1 mice (prone to systemic lupus erythematosus), as evidenced by autoantibodies. Keil et al. (2009) concluded that the transient increase in autoantibodies to glomerular antigen and double-stranded DNA at the low dose did not support a role for chronic exposure to TCE in the progression of autoimmune disease in NZBWF1 mice at 1.4 and 14 mg/l. In contrast to the findings in the NZBWF1 strain, non-autoimmune disease–prone B6C3F1 mice evaluated in the same experiment had consistently elevated serum levels of anti–single-stranded DNA after about 30 weeks of exposure to TCE (1.4 and 14 mg/l in drinking-water). A dose–response relationship for increased number of activated T cells (CD4+/CD44+) in the spleen was observed in the B6C3F1 mice that was significant at the 14 mg/l dose of TCE. Kidneys were analysed for pathological changes, and an increase of kidney score (based on inflammation, proliferation, etc.) was observed only in the B6C3F1 mice. It should be noted that because of their genetic predisposition to kidney disease, the basal kidney score was 3–9 times higher in NZBWF1 mice. Nonetheless, while the evidence is limited and has not been reproduced, this study suggests that TCE may have the ability to induce autoimmune disease spontaneously.

In three recent studies, the effects of TCE in MRL+/+ mice were examined following lifetime (developmental and early life) drinking-water exposure starting from the premating period (Peden-Adams et al., 2006, 2008; Blossom & Doss, 2007). The study by Blossom & Doss (2007), resulting in doses of up to 684 mg/kg body weight per day in dams, showed slight increases in IgG2a and antihistone levels (both parameters only at middle dose of 122 mg/kg body weight per day). Peden-Adams et al. (2006, 2008) exposed MRL+/+ and B6C3F1 mice to TCE at concentrations of 1.4 and 14 mg/l in the drinking-water (from mating until 12 months of age in MRL+/+ mice and until 8 weeks of age in B6C3F1 mice) and found no alterations in levels of anti–double-stranded DNA or antiglomerular antibodies.

Although other assays of immune function were not performed in MRL+/+ mice, a significant decrease in thymus cellularity was observed in male MRL+/+ mice at TCE concentrations of 1.4 and 14 mg/l; this decrease was also reflected in a decrease in all thymic T cell subpopulations at 14 mg/l (Peden-Adams et al., 2008). In contrast, a TCE concentration of 14 mg/l was associated with an increase in thymus cellularity in B6C3F1 mice of both sexes (Peden-Adams et al., 2006). Additional functional immune assays were performed in B6C3F1 mice. The primary antibody response to SRBCs (by PFC) was decreased in B6C3F1 of both sexes and both TCE dose groups, but an increase in SRBC-elicited DTH responses was observed in female B6C3F1 mice at a TCE concentration of 1.4 mg/l and both male and female B6C3F1 mice at 14 mg/l.

In summary, for purposes of a risk assessment, there is a relatively large body of animal data supporting the connection between TCE exposure and increased progression or pathogenesis of autoimmune disease in several rodent models. The majority of animal data indicating that TCE promotes autoimmune disease are from studies demonstrating that high doses of TCE (e.g. 100–2500 mg/l) exacerbate the onset and/or severity of symptoms of autoimmune disease in MRL+/+ mice, a genetically autoimmune disease–prone strain. There are also several studies demonstrating immunomodulation (immunosuppression, immunostimulation and promotion of markers of autoimmune disease) in non-genetically autoimmune disease–prone B6C3F1 mice. Data from the MRL+/+ mice support a dose–response relationship

without clear evidence of a threshold. Many of the studies use one or two doses because they are designed to determine mechanisms rather than for use in establishing a dose–response relationship or effect levels. The rodent data, together with the strong human data described above in section C6.3.1.1, present a strong weight of evidence for a TCE-associated increase in autoimmune disease severity and progression.

As described above in section C6.3.1.1, the human data contain considerable uncertainty owing to the general lack of exposure data and the lack of a well-conducted cross-sectional or prospective cohort study of autoimmune disease incidence with TCE exposure data. Therefore, human data are not available to evaluate a potential dose–response relationship between TCE exposure and systemic sclerosis or other reported symptoms of autoimmune disease in humans, and the available animal data should be used to develop a quantitative risk evaluation. Consideration of the database of animal studies should begin by evaluating studies that identify the lowest effect level with support for biological plausibility of the observed effects. First, as with other risk assessments, the use of data from the most relevant exposure route in humans is preferred, and therefore experimental animal data from studies involving oral exposure to TCE should be selected for the risk assessment over data from intraperitoneal exposure studies. Second, the use of animal data from autoimmune disease–prone rodent models needs to be considered explicitly in the uncertainty factors applied to the animal data in estimating human risk of TCE-induced autoimmunity. The application of these uncertainty factors will be discussed in detail below, but, as described in chapter 7 of this guidance document, these animal models are considered good models of susceptible humans rather than the general population, and therefore the intraspecies uncertainty factor should generally be reduced from 10 to 1 when human risk is estimated from data obtained in autoimmune disease–prone rodents.

When considering the database of available studies, it is readily apparent that many of the earlier studies use high doses of TCE (e.g. 100–2500 mg/l intraperitoneally or in drinking-water, resulting in an effective dose of 60–100 mg/kg body weight per day). More recent studies, such as those conducted by Keil et al. (2009) and Peden-Adams et al. (2006, 2008), have extended the dose range down to 14 and 1.4 mg/l in drinking-water for a 0.14–0.35 mg/kg body weight per day dose based on body weights from individual studies. Table C6.1 presents a summary of immune effects data from select studies at the lower dose range with a focus on autoimmune-related end-points. Most of these studies were designed to elucidate mechanisms of action of TCE at doses known to be toxic, and therefore the dose range does not extend below TCE doses of 1.4 mg/l in drinking-water. Even the lowest doses tested are higher than mean groundwater levels in the USA, but they are similar to levels found at sites on the United States National Priority List (Peden-Adams et al., 2008). It should also be noted that many of these studies point towards a complicated (at least two-step) mechanism of action, including phenomena related to both hypersensitivity (protein conjugation, metabolite-specific immune responses) and autoimmunity (increases of auto-antibodies). Hazard identification studies have demonstrated the potential of TCE to induce inflammation in various organs, but none of the studies established an autoimmune nature for the inflammation.

Table C6.1: Overview of animal immunotoxicity data for oral trichloroethylene exposures.

Reference	*Mouse strain*	*Body weight (g)*	*Doses (mg/l)*	*Exposure duration (days)*	*Effects*	*LOAEL (mg/kg body weight per day)*[a]
Griffin et al. (2000b)	MRL+/+	40	100, 500 and 2500	28	Adduct formation, increase of T cell activity, hepatitis, mononuclear infiltration of liver, antinuclear antibody increase	21
Keil et al. (2009)	NZBWF1	40	1.4 and 14	189	Increase in autoanti-bodies to double-stranded DNA at 19 weeks, and then 1.4 mg/l only at 32 and 34 weeks (not at 23, 24, 30 or 36 weeks); transient increase in autoantibodies to glomerular antigen (at 11 and 19 weeks only)	0.16[b]
Keil et al. (2009)	B6C3F1	30	1.4 and 14	210	Increase in autoanti-bodies to double- and single-stranded DNA at 32–39 weeks, increase renal pathology score, decrease thymus weight, increase activated T cells at 14 mg/l	0.19
Cai et al. (2008)	MRL+/+	26	500	336	Antinuclear antibody increase, hepatic T cell influx, signs of lung inflammation, renal effects	60
Peden-Adams et al. (2006)	B6C3F1	25	1.4 and 14	GD 0–56	Increase DTH (to SRBCs), decrease PFC (to SRBCs)	0.22
Peden-Adams et al. (2008)	MRL+/+	40	1.4 and 14	GD 0–386	Decrease thymus cellularity	0.14

GD, gestational day

[a] Daily dose depends on body weight, which may change over the course of long-term studies. Body weight readily available from the study report (initial or final) was used for these calculations and may not be the best calculation of dose.

[b] Keil et al. (2009) concluded that TCE did not contribute to the progression of autoimmune disease in MRL+/+ mice in their study.

Source: Table adapted from Peden-Adams et al. (2006, 2008)

The Keil et al. (2009) data on B6C3F1 mice provide the lowest dose from animal data for an autoimmune-related effect by a relevant route of exposure with evidence of a dose–response relationship and therefore were selected for the quantitative risk assessment. There are no data to identify a NOAEL for autoimmune effects associated with TCE. The autoimmune-related effects at the lowest dose (1.4 mg/l) represent a LOAEL for the study and include increased levels of autoantibodies to double- and single-stranded DNA and an increase in the

graded score indicating renal pathology. At the higher dose (14 mg/l), there was also an increase in activated (CD4+/CD44+) T cells. There is some evidence of a dose–response relationship for these end-points, but it is limited. For example, the increase in activated T cells supports a dose–response relationship, as the increase is not significant at the low dose (1.4 mg/l), but is significantly increased at 14 mg/l. The increase in autoantibodies to double-stranded DNA also supports a dose–response relationship, as the effect is observed sooner at the high dose (i.e. at 26 weeks at 14 mg/l TCE, whereas it is not significant until 32 weeks at 1.4 mg/l). There is no such difference in the autoantibodies to single-stranded DNA, and there are time points (e.g. 34 weeks) when the low dose (1.4 mg/l) is associated with increased autoantibody levels and the high dose is not.

The Keil et al. (2009) study is part of a series of studies on TCE-related autoimmunity in several mouse strains involving the 1.4 and 14 mg/l dose level in drinking-water. Using data from multiple studies, the importance of longer TCE exposure periods can be observed in the development of autoimmune-related effects at these dose levels. The developmental study reported in Peden-Adams et al. (2006) exposed B6C3F1 mice from gestation to 8 weeks of age and did not observe the elevation in autoantibodies reported by Keil et al. (2009) at 30+ weeks of age. This suggests that short-term or subchronic exposure to TCE is not sufficient to promote progression of autoimmune disease, and longer TCE exposure may be required to increase the expression of markers associated with autoimmune disease in the non-genetically prone B6C3F1 mice. The high-dose TCE studies in MRL+/+ mice described previously provide additional evidence that the duration of exposure influences the observed effects. Autoantibodies and T cell activation are generally observed with high doses of TCE, and short, 4-week exposures (e.g. Griffin et al., 2000b,c) and histopathological changes such as inflammatory and lymphocytic infiltrations in the liver are associated with exposures of 32–48 weeks (e.g. Griffin et al., 2000c; Cai et al., 2008). Data from these high-dose TCE studies also provide support for a dose–response relationship between TCE exposure and exacerbation of autoimmunity. Although the autoimmune effects at lower dose levels (i.e. 1.4 and 14 mg/l) do support a dose–response relationship, the lack of clear evidence for a dose–response relationship represents a source of uncertainty in the evaluation.

The LOAEL of 1.4 mg/l for TCE data in B63CF1 mice from Keil et al. (2009) is for several autoimmune-related end-points in a non-autoimmune disease–prone mouse strain. There are two additional oral drinking-water studies (Peden-Adams et al., 2006, 2008) that support a 1.4 mg/l TCE dose level as a LOAEL and two mouse strains (B6C3F1 and NZBWF1) that support autoimmune effects at the LOAEL. The same laboratory found limited evidence for TCE-related autoimmunity in autoimmune disease–prone NZBWF1 mice in the identical protocol and concluded that the evidence did not support a role for chronic exposure to TCE in the progression of autoimmune disease in NZBWF1 mice at 1.4 and 14 mg/l (Keil et al., 2009). Therefore, the NZBWF1 data from Keil et al. (2009) provide some support for the B6C3F1 data, but were not used to derive the POD. The Peden-Adams et al. (2008) data were also excluded from this analysis because the thymic cellular changes in MRL+/+ mice suggest a histopathological change that is not clearly related to autoimmunity. Keil et al. (2009) and Peden-Adams et al. (2006) also reported evidence of immunosuppression and immunostimulation (increased DTH and decreased PFC) at the 1.4 mg/l TCE dose in B6C3F1 mice in the developmental exposure study with exposure through 8 weeks of age. In a full risk assessment, these end-points (decreased PFC supporting suppression and increased DTH supporting stimulation) would be considered excellent candidate effects and support the same dose level for the LOAEL. Although the increase in DTH suggests immunostimulation and may be related to promotion of autoimmunity, the derivation of PODs or effect levels

from the DTH or PFC data is not included here, because this case-study is restricted to auto-immune-related effects.

Therefore, the lowest dose (1.4 mg/l) at which the autoimmune-related end-points of increased levels of autoantibodies to double- and single-stranded DNA and an increase in the graded score indicating renal pathology in B6C3F1 mice were observed represents a LOAEL for TCE-related exacerbation of autoimmunity from Keil et al. (2009). A NOAEL cannot be determined from the available data. Some of the end-points at the LOAEL, such as increased autoantibodies to single- and double-stranded DNA, represent a relatively early effect in the etiology of TCE-exacerbated autoimmunity in these mice. As discussed in chapter 7, DNA autoantibodies alone are not necessarily considered an adverse effect, as many individuals demonstrate these without clinical symptoms and they also may occur during normal ageing. However, there are other effects at 1.4 mg/l, such as renal score, which are more closely related to a clearly adverse outcome (i.e. renal pathology). The fact that there are multiple effects at 1.4 mg/l and higher dose levels increases the weight of evidence for TCE-related modulation of autoimmunity. The risk assessor can consider the reduction of uncertainty factors to account for LOAEL to NOAEL extrapolation when the end-point data being considered are for effects that are early in the etiology of disease and therefore the LOAEL is presumably approaching a NOAEL. Internal dose metrics associated with autoimmune effects, such as liver or kidney levels of TCE or metabolites, are not available for these studies. A more comprehensive risk assessment should carefully consider the utility of the available toxicokinetic models to inform a human health risk assessment and extrapolate relevant human internal and external doses of TCE associated with increased risk of autoimmunity from the available animal data. However, that is beyond the scope of this case-study; therefore, this example is based on the LOAEL from oral exposure data in B6C3F1 mice from Keil et al. (2009).

The 1.4 mg/l LOAEL needs to be converted to units of mg/kg body weight for standard calculations of a POD and the estimation of the reference value. The mg/kg body weight dose can be calculated from the drinking-water dose by multiplying the TCE concentration in drinking-water by the average water intake and dividing by the average body weight. Using an average body weight of 30 g from Table 1 in Keil et al. (2009) and assumptions of 4 ml drinking-water consumed per day from Table 5 of Peden-Adams et al. (2008), the calculations are as follows:

$$\begin{aligned} \text{LOAEL} &= 1.4\ \text{mg/l} \times (0.004\ \text{l/day}) / (0.030\ \text{kg body weight}) \\ &= 0.187\ \text{mg/kg body weight per day} \\ &= 0.19\ \text{mg/kg body weight per day in both sexes} \end{aligned}$$

To continue the illustration of this case-study, the converted LOAEL of 0.19 mg/kg body weight per day for TCE from the Keil et al. (2009) study will be used to derive the health-based guidance value or reference value. As described in chapter 3 (section 3.3.7.3), the risk assessor should apply BMD modelling to the data to derive a POD near the low end of the available data. For the purposes of this case-study, the LOAEL will be used as the POD rather than selecting a model and performing BMD calculations, but the BMD is generally the preferred method.

The next step in the risk assessment process is the application of uncertainty factors, as described in section 3.3.10 in general and section 7.10 with reference to autoimmunity:

- The intraspecies uncertainty factor would be 10, to account for interhuman variability in the absence of more definitive data. Although the database contains multiple studies from autoimmune disease–prone mice, the data used to determine human risk were from a non-autoimmune disease–prone rodent model, and so a standard 10-fold uncertainty factor is used for this case-study. When animal data are from a model that is considered to represent susceptible humans, rather than the general human population, the risk assessor should consider reducing or eliminating the intraspecies uncertainty factor. This is the case for autoimmune disease–prone rodent models, which are considered good models of susceptible humans (see chapter 7 for further discussion).
- The interspecies uncertainty factor would be 10 to extrapolate from experimental animal data to human risk.
- The LOAEL to NOAEL uncertainty factor would be 10 because a NOAEL is not available. The risk assessor could consider reducing the LOAEL to NOAEL uncertainty factor because some of the data at the LOAEL (i.e. increased autoantibodies to single- and double-stranded DNA) represent a relatively early effect in the etiology of TCE-exacerbated autoimmunity and therefore the LOAEL is presumably approaching a NOAEL. For the purposes of this case-study, the uncertainty factor was not reduced. It was considered collectively with the database uncertainty factor and the lack of studies in the lower dose range. Reducing both of these uncertainty factors to 1 or raising both of these uncertainty factors to 10 was considered to underestimate and overestimate the uncertainty, respectively. A default approach for the LOAEL to NOAEL was instead selected.
- The subchronic to chronic uncertainty factor, or use and time factor, would be 1 for a chronic exposure assessment, as the study length was 30 weeks. The application of this uncertainty factor depends on policy, and some institutions, such as the USEPA, are hesitant to reduce the uncertainty factor if the exposure is less than the 2-year study generally considered chronic. Application of this uncertainty factor also depends on the scope defined in the problem formulation stage of the risk assessment (i.e. chronic, subchronic or acute). The animal data suggest that exposure length increases the severity of effects observed and that increasing duration is associated with a lower effect level (e.g. the autoimmune effects in B6C3F1 mice at 30+ weeks were not observed in the 8-week Peden-Adams et al. [2006] study).
- The database uncertainty factor would be 1, because there is a relatively large database for TCE exacerbation of autoimmunity. The lack of studies at lower dose levels could be used to justify a larger database uncertainty factor; however, for the purposes of this assessment, the availability of several studies in multiple rodent strains at 1.4 mg/l (0.19 mg/kg body weight per day) is considered sufficient. As discussed previously, the effects observed at 1.4 mg/l can be considered early markers of autoimmunity and therefore suggest that the dose is approaching a NOAEL. The lack of clear effects in NZBWF1 mice at this level suggests that researchers have provided data on a sufficiently low dose in autoimmune disease–prone mice as well. The LOAEL to NOAEL uncertainty factor addresses the uncertainty associated with the lack of a NOAEL.

To complete the derivation of a health-based guidance value or reference value, the guidance recommends consideration of groups at risk (i.e. children, elderly and genetically susceptible individuals) and then dividing the POD by the total uncertainty factor described above. An additional consideration may be warranted for susceptible life stages because there may be a general increased risk for autoimmunity in older individuals linked to a modulation in

adaptive immunity (see Hakim & Gress, 2007, and discussion in section 7.8 of chapter 7), but there are no animal or human data to inform this potential susceptibility for TCE-induced autoimmunity. In light of the observation that most autoimmune diseases are sex-biased towards females, sex is another important intrinsic factor to consider in association with environmental exposures, including TCE. The animal data on TCE-exacerbated autoimmunity do not suggest a strong sex-related susceptibility, but only female B6C3F1 mice were utilized in the experiment used to derive the reference value. The human data discussed in section C6.3.1.1 provide limited information on potential TCE-susceptible populations by age or sex. There is some evidence that TCE exposure is associated with a greater relative risk for systemic sclerosis for men than for women; however, as discussed previously, this may reflect a greater ability to detect the disease as a result of the 10-fold lower background incidence of systemic sclerosis in men compared with women.

Using the above values, the total uncertainty factor applied for a risk assessment for derivation of a chronic reference value for TCE would be 1000 (10 for intraspecies, 10 for interspecies, 1 for subchronic to chronic, 10 for LOAEL to NOAEL and 1 for database).

For the chronic risk assessment of autoimmunity associated with TCE:

Reference value = 0.19 mg/kg body weight per day ÷ 1000
= 0.000 19 mg/kg body weight per day

The reference value derived from the LOAEL of 1.4 mg TCE per litre (0.19 mg/kg body weight per day) in female B6C3F1 mice is based on increased levels of autoantibodies to double- and single-stranded DNA and an increase in the graded score indicating renal pathology. This reference value is supported by data suggesting a transient increase in autoantibodies to DNA and glomerular antigen in autoimmune disease–prone NZBWF1 mice at the same TCE dose level in drinking-water. The increase in DTH in B6C3F1 mice, also at 1.4 mg/l (Peden-Adams et al., 2006), supports an elevated immune response that may be related to autoimmunity. Numerous studies in MRL+/+ mice support TCE-associated stimulation of autoimmunity at higher doses (Khan et al., 1995; Griffin et al., 2000a,b,c; Blossom et al., 2006, 2007, 2008; Gilbert et al., 2006, 2009; Blossom & Doss, 2007). Therefore, the TCE-induced autoantibodies to DNA and increased renal pathology in B6C3F1 mice represent the most sensitive end-point for autoimmunity in the most sensitive strain and are supported by other animal data.

C6.3.1.3 Is there evidence that the chemical alters immune measures associated with autoimmunity (i.e. autoantibody levels, inflammatory markers, regulatory T cells, lymph node proliferation, etc.) in animal models of autoimmune disease?

Yes. There are a number of studies that demonstrate TCE modulation of immune measures associated with autoimmunity in mouse models of autoimmune disease. Numerous studies present data on autoantibody production in autoimmune disease–prone MRL+/+ mice associated with TCE exposure, and there are some examples in other strains. Multiple studies (Griffin et al., 2000b,c; Blossom et al., 2006, 2007, 2008; Blossom & Doss, 2007) also report TCE-related increases in proinflammatory cytokines (primarily IFN-γ) consistent with a Th1-type inflammatory response in MRL+/+ mice. Many of these data have been described in section C6.3.1.2 above, and therefore the data will be covered only briefly here.

The TCE data in MRL+/+ mice include reports of increased antinuclear antibodies (Khan et al., 1995; Griffin et al., 2000b,c; Cai et al., 2008), anti-histone antibodies (Blossom & Doss, 2007) and antibodies to double- and single-stranded DNA (Khan et al., 1995). Direct exposure to several TCE metabolites is also associated with increased autoantibodies in MRL+/+ mice. TCAH increased antinuclear antibodies and anti-histone antibodies (Blossom et al., 2006); DCAC increased antinuclear antibodies, anti-single-stranded DNA antibodies and anti-cardiolipin antibodies (Khan et al., 1995; Cai et al., 2006). A transient increase in autoantibodies to glomerular antigen was also observed in autoimmune disease–prone NZBWF1 mice (Keil et al., 2009). The increase in antibodies to glomerular antigen was significant at both TCE doses tested at 11 and 19 weeks (not at 16 or 23–36 weeks of age); the increase in antibodies to double-stranded DNA was significant at 19 weeks of age, and at the low dose, only at 32 and 34 weeks of age. The authors concluded that the evidence did not support a role for chronic exposure to TCE in the progression of autoimmune disease in NZBWF1 mice at 1.4 and 14 mg/l. In the same experiment, researchers used a non-genetically prone mouse strain (B6C3F1) to examine the role of TCE in expression of markers associated with autoimmune disease in a strain that does not spontaneously develop autoimmune disease. TCE exposure was associated with a significant increase in antibodies to single- and double-stranded DNA in B6C3F1 mice (Keil et al., 2009). The increases in autoantibodies to DNA in B6C3F1 mice were one of several effects that were collectively identified at the LOAEL for TCE of 1.4 mg/l in drinking-water. Along with kidney pathology, these markers of autoimmunity were used to derive the POD and reference dose in section C6.3.1.2, and therefore a quantitative risk assessment would be a duplicate of the evaluation presented previously and is not presented here.

As described above, there are many examples of TCE-related increases in autoantibody levels in autoimmune disease–prone mice as well as some data from non-autoimmune disease–prone B6C3F1 mice. There is also evidence that TCE exposure results in a proinflammatory status characterized by increased secretion of IFN-γ by T cells. The autoantibody data alone present some evidence of an effect of TCE on autoimmunity. For the purposes of illustration, a risk assessor faced with evaluating a data set restricted to increased autoantibody levels could conclude that there was a potential for TCE to exacerbate or accelerate autoimmunity. However, increased autoantibodies represent a relatively early effect in the etiology of TCE-exacerbated autoimmunity. DNA autoantibodies, antinuclear antibodies, etc. are considered adverse effects, and they are generally associated with pathology at higher doses (renal deposits of IgG, lymphocyte infiltration, hepatocyte proliferation or necrosis). Without data on TCE-related effects such as renal score that are more closely related to these clearly adverse outcomes (i.e. renal pathology), there is greater uncertainty in deriving an effect level on autoantibodies alone. However, even as an exercise, it is difficult to separate these data from the larger database of TCE-related effects reported in animal models of autoimmune disease. Considered in the context of the wider database, the autoantibody data support the human case-study data outlined in section C6.3.1.1 and the relatively strong database from mouse models of autoimmune disease outlined in section C6.3.1.2. Together, this adds to a strong weight of evidence for a TCE-associated increase in autoimmune disease progression.

C6.3.1.4 Is there evidence from general or observational immune assays (lymphocyte phenotyping, cytokines, complement, lymphocyte proliferation, etc.) that the chemical has the potential to modulate autoimmune disease?

Yes. TCE as well as its metabolites TCAH and TCA have been demonstrated to activate CD4+ T cells in autoimmune disease–prone MRL+/+ mice. This was evidenced by increased

expression of activation markers and elevated cytokine levels (in particular IFN-γ) at doses of 0.1–2.5 mg/ml drinking-water. Griffin et al. (2000b,c) reported upregulation of CD44+ and CD54 along with downregulation of CD45RB in CD4+ cells as indicators of activation in T cells from the spleen or lymph nodes of TCE-exposed MRL+/+ mice. Interestingly, Keil et al. (2009) reported TCE-associated T cell activation in non-autoimmune disease–prone B6C3F1 mice, suggesting a general mechanism of TCE action that is not restricted to susceptible strains. A similar pattern of TCE-associated activation of T cells was observed following exposure to TCE metabolites TCAH and TCA using the activation marker CD62L^{lo} in MRL+/+ mice (Blossom et al., 2006, 2007). At similar doses, both compounds also resulted in increased resistance to activation-induced cell death (Gilbert et al., 2006). A series of studies by Griffin and colleagues (Griffin et al., 2000b,c; Blossom et al., 2006, 2007, 2008; Blossom & Doss, 2007) reported increased IFN-γ secretion by splenic or peripheral T cells following stimulation by anti-CD3 or phorbol myristate acetate in MRL+/+ mice. The increase in IFN-γ was consistently observed after 4 weeks of TCE exposure in adults (Griffin et al., 2000b) and following developmental exposure to 4 weeks of age (Blossom & Doss, 2007). An increase in IFN-γ was not consistently associated with longer-term exposure (22–32 weeks) in adults or following developmental exposure to TCE (up to 7–8 weeks of age) (Griffin et al., 2000b,c; Blossom & Doss, 2007). Decreased IL-4 secretion by anti-CD3 activated T cells in MRL+/+ mice was reported after 4 and 22 weeks of exposure to TCE at 2.5 and 5.0 mg/ml in drinking-water (Griffin et al., 2000b); however, IL-4 was not altered in follow-up studies of TCE or the metabolites TCAH or TCA (Griffin et al., 2000c; Blossom et al., 2006, 2007). Increases in IL-2 and TNF-α have also been observed following developmental exposure to TCE at concentrations as low as 0.1 mg/ml in drinking-water (Blossom et al., 2008).

The database of TCE-associated immune changes includes two types of data from general immune assays: lymphocyte phenotyping and cytokine production. By themselves, these data provide equivocal evidence for TCE-related autoimmunity and would not be used to derive a POD (see section 7.7 of chapter 7 for a full discussion of the relative strength and predictability of different assays for autoimmunity). In the absence of the relatively strong database of TCE-induced expression of markers associated with autoimmune disease (primarily from MRL+/+ mice), inflammatory cytokines and markers of T cell activation would suggest immunomodulation, but would not be definitive for autoimmunity. These data would identify a data gap suggesting the need for more conclusive autoimmune-related end-points (e.g. early markers such as increased autoantibodies, and clearly adverse markers such as renal or liver pathology). As such, the data could be used to support the need for additional studies in animal models of autoimmune disease to determine autoimmune potential as well as functional immune assays to test for immunosuppression or immunostimulation. Of course, the equivocal nature of these data is largely due to the fact that we are evaluating the cytokine data out of context and for purposes of illustration. In reality, the cytokine data and evidence for T cell activation are related to the autoimmune end-points, and the data were collected as part of mechanistic studies to characterize the MOA for autoimmune effects associated with TCE exposure. Therefore, consideration of the data on the TCE-associated increase in IFN-γ cytokine and T cell activation is warranted in the context of the full database. The lowest TCE dose evaluated in the MRL+/+ mice was 0.1 mg/ml, or 100 mg/l. Although cytokine levels were not reported in the B6C3F1 mouse studies by Keil and colleagues (Peden-Adams 2006, 2008; Keil et al., 2009), the authors did report a significant increase in activation of T cells at the TCE dose of 14 mg/l. This effect can be used to support the other data for autoimmune-associated effects at 1.4 mg/l, but the T cell activation data are not at the lowest dose level. The data from general immune assays add to the considerable weight of evidence

for TCE-associated exacerbation or acceleration of autoimmunity. The database supporting TCE-associated autoimmunity includes a relatively large number of studies on autoimmune-related effects reported in animal models of autoimmune disease (see section C6.3.1.2), a number of studies reporting autoantibodies and some other immune measures associated with autoimmune disease (see section C6.3.1.3) and strong evidence from human case–control studies (see section C6.3.1.1).

C6.3.1.5 Is there histopathological evidence (thymus, etc.) or are there changes in immune organ weights or haematological changes that suggest that the chemical causes an immune response against self (i.e. immune complex deposition, inflammatory cell infiltrates)?

Yes. The main histopathological evidence of TCE-associated autoimmunity is from studies reporting leukocyte infiltration. In particular, in MRL+/+ mice, TCE has been shown to induce inflammatory cell infiltrates in various organs—i.e. liver, lungs, skin and kidneys at doses of 0.1–2.5 mg/ml in drinking-water (Griffin et al., 2000b; Cai et al., 2008; Gilbert et al., 2009).

TCE-associated hepatic changes also included necrosis and evidence for increased hepatocyte proliferation (Cai et al., 2008) at a TCE concentration of 0.5 mg/ml in drinking-water for 48 weeks. Griffin et al. (2000c) reported lymphocyte portal infiltration in the liver at TCE concentrations as low as 0.5 mg/ml and increased hepatocyte reactive changes at concentrations as low as 0.1 mg/ml for 32 weeks. In kidneys, immune complex deposition was demonstrated in the glomeruli of MRL+/+ mice exposed to TCE for 48 weeks (Cai et al., 2008). Although all of the histopathological changes cited above were reported as part of specific studies of autoimmunity, the inflammatory cell infiltrates may have been detected as part of routine haematoxylin and eosin staining of paraffin-fixed tissue. For the purposes of illustration in this case-study, routine haematoxylin and eosin staining of liver, lung, skin and kidney may have been available to the risk assessor without the database of autoimmune studies described in previous sections. In such a case, the inflammatory cell infiltration data could be used to support the need for additional studies in animal models of autoimmune disease to determine autoimmune potential. In reality, the histopathological data provide support for other autoimmune end-points.

As discussed above, the data set of histopathological evidence in immune tissues following TCE exposure includes inflammatory infiltrates in liver, kidney, lung and skin, hepatocyte proliferation and immune complex deposition in the kidney. The immune complex deposition data alone provide some evidence for TCE-associated autoimmunity. In the context of the larger database, the histopathology data provide mechanistic information suggesting an MOA. The database supporting TCE-associated exacerbation of autoimmunity includes a relatively large body of animal data supporting the connection between TCE exposure and increased progression or pathogenesis of autoimmune disease from several mouse models of autoimmune disease (see section C6.3.1.2), a number of studies reporting modulation of immune measures associated with autoimmunity in animal models of autoimmune disease (see section C6.3.1.3), including general immune assays such as proinflammatory cytokine levels that may support autoimmunity (see section C6.3.1.4), and strong evidence from human case–control studies (see section C6.3.1.1). The specific, autoimmune-related histopathological evidence described above adds to the considerable weight of evidence for TCE-associated autoimmunity.

C6.3.2 Weight of evidence conclusions for hazard characterization

The previous sections were written following the weight of evidence questions in chapter 7 (summarized in Figure 7.1). They provide an illustrative example to outline the process for conducting an assessment of autoimmunity following the guidance from chapter 7. The discussions were written individually to aid the risk assessor in evaluating different levels of evidence for each type of data in the absence of additional evidence. Other than for the purpose of this illustration, the data should not be evaluated separately; rather, the data should be integrated into a single evaluation that brings together the answers to each question and considers the database for a given chemical as a whole. As described in chapters 3–7, the risk assessor should develop the weight of evidence conclusions for TCE-associated auto-immunity based on the answers to all of the weight of evidence questions in chapter 7. The weight of evidence conclusions for autoimmunity should also describe the database in terms of consistency and biological plausibility, including strengths, weaknesses, uncertainties and data gaps. It is also important to note that the discussions of different types of TCE auto-immune-related data in sections C6.3.1.1–C6.3.1.5 (including the quantitative assessment presented above in section C6.3.1.2) are restricted to autoimmune-related effects, and the assessment does not represent a comprehensive risk assessment for TCE-associated health effects or other types of immunotoxicity. For example, a general evaluation of immuno-toxicity may have used the PFC and DTH data from Peden-Adams et al. (2006) to develop additional PODs.

Human data would be preferred for the risk assessment because fewer assumptions are required to derive a reference value from human data. As discussed in section C6.3.1.1, there are very few TCE exposure data with which to evaluate the potential quantitative relationship between TCE exposure and reported symptoms of autoimmune disease in humans. The limited human data are from the Iavicoli et al. (2005) study, which reported an association between occupational exposure to TCE and increased inflammatory cytokines. On average, the exposure levels in the occupational epidemiology studies were 35 mg/m^3 or higher. Assuming an 8-hour working day, this corresponds to approximately 5 mg/kg body weight per day or higher. In these studies, NOAELs were not established. As lower effect levels were observed in the repeated oral dose studies in mice, the latter studies should be con-sidered the most appropriate starting point for developing reference values.

The increased levels of autoantibodies to double- and single-stranded DNA and an increase in the graded score indicating renal pathology in non-autoimmune disease–prone B6C3F1 mice exposed to TCE in drinking-water at a concentration of 1.4 mg/l for 30 weeks (Keil et al., 2009) were observed at the lowest TCE exposure level associated with acceleration or exacerbation of autoimmunity. Therefore, because they represent the lowest effect levels, these data would be used to derive a POD in the quantitative risk assessment of TCE-induced autoimmunity. The procedures and calculations necessary to develop a reference value from the Keil et al. (2009) data are presented above in section C6.3.1.2, including the application of uncertainty factors. Calculations from the LOAEL are used to illustrate the process, although the BMD modelling approach is preferred for a full risk assessment. Section C6.3.1.2 includes other considerations that would be included in a more comprehensive risk assessment, such as a discussion of groups at risk and support for the LOAEL provided by additional studies. Each section on individual data types (sections C6.3.1.1–C6.3.1.5) includes a brief discussion of the consistency and strengths of the database for TCE-induced autoimmunity. The discussion provided in the individual sections would be expanded in a more comprehensive risk assessment. As an example, the risk assessor would go into greater

detail on the evidence supporting or distinguishing effects observed in animal models. An expanded discussion would certainly compare and contrast the human data on severe generalized hypersensitivity dermatitis with the animal data on inflammation, DTH and other hypersensitivity-related end-points relevant to the assessment of TCE-associated auto-immunity.

The weight of evidence discussion would reflect the relatively strong confidence in the data set described above and highlight the support from autoimmune-related effects observed in autoimmune disease–prone mice and non-autoimmune disease–prone B6C3F1 mice (see section C6.3.1.2), studies reporting modulation of end-points associated with autoimmunity in animal models of autoimmune disease (see section C6.3.1.3), the cytokine evidence from general immune assays (see section C6.3.1.4), the autoimmune-related histopathological evidence (see section C6.3.1.5) and strong human evidence, including the association between TCE and systemic sclerosis from human case–control studies (see section C6.3.1.1). These studies add to the considerable weight of evidence for a TCE-associated increase in the progression or pathogenesis of autoimmune disease and support the drinking-water TCE exposure study in non-autoimmune disease–prone B6C3F1 mice (Keil et al., 2009) as the principal study appropriate for derivation of PODs and dose–response assessment of auto-immunity associated with exposure to TCE.

Many studies have been reported on effects of TCE on immune(-associated) parameters. Although unequivocal evidence that TCE can induce or potentiate autoimmune diseases in humans is lacking, most of these effects can be considered indicative of a potential auto-immune disease–inducing or autoimmune disease–stimulating property of TCE. A risk assessment for an autoimmune disease–inducing or autoimmune disease–stimulating property of TCE is therefore indicated. This risk assessment is focused on the risk for long-term (chronic or lifetime) exposure. The goal is to derive a reference value (expressed in mg/kg body weight per day) for comparison with (past, current or expected) exposure estimates.

C6.4 Conclusions

This assessment of TCE-related autoimmunity and autoimmune disease is a demonstration of the application of the risk assessment guidance for the assessment of autoimmunity and autoimmune disease presented in chapter 7 of this document. TCE was selected because of the relatively strong database of autoimmune-related effects in animal models of autoimmune disease as well as the case–control studies exploring the relationship between TCE exposure and clinical disorders linked to autoimmunity. The case-study illustrates the limitations often encountered in evaluating human data (i.e. the number of studies of general solvent exposure in which co-exposure issues and the lack of quantitative exposure measurements limit the conclusions for risk assessment purposes). As with most chemicals for which there are data on the potential association with autoimmunity, the majority of data for TCE are from auto-immune disease–prone rodent models (in this case, the MRL+/+ mouse). However, the POD and reference value were derived from a non-genetically prone mouse strain.

It should be noted that this case-study on TCE is provided with the purpose of illustrating how the risk assessment guidance can be used for assessing the risk of autoimmunity, but it does not represent a comprehensive risk assessment, nor does it represent a final regulatory position.

C6.5 References

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