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Meeting report

Approaches to risk assessment of immunotoxic effects of chemicals[☆]

1. Preamble

A series of scientific meetings dealing with the effects of chemicals on the immune system were organised by the IPCS with several international and national partner institutions over the years. The overall aim was to consider ways of integrating new scientific evidence on this rapidly developing area of work, as it emerges, into risk assessment practices.

The first international seminar on the immune system as a target for toxic damage was held in Luxembourg in 1984 (UNEP, ILO, WHO/IPCS, CEC, 1987). This was followed by an International Workshop on Immunotoxicity of Metals and Immunotoxicology, held in Hannover, Germany, in 1989 (IPCS, 1990). In 1992 a Workshop on Allergic Hypersensitivity Induced by Chemicals was organised in Bilthoven, Netherlands, jointly supported by IPCS and the WHO European Centre for Environment and Health (Vos et al., 1995). Two special joint workshops of IPCS and the Norwegian National Institute of Public Health were held in Oslo in 1995 and 1996, the first on 'Environmental Chemicals and Respiratory Hypersensitisation' (Dybing et al., 1996), and the second on 'Chemical Exposure and Food Allergy/Intolerance' (Lovik et al., 1997). Finally, a scientific symposium on epidemiology and immunotoxicity, jointly organised with the National Institute of Public Health and Environmental Protection of the Netherlands, was held in Bilthoven in 1997 (Van Loveren et al., 1999). In parallel, two Environmental Health Criteria monographs on 'Principles and Methods of Assessing Direct Immunotoxicity Associated with Exposure to Chemicals' (IPCS, 1996), and 'Principles and Methods for Assessing Allergic Hypersensitisation Associated with Exposure' appeared (IPCS, 1999).

Throughout these activities, as well as within the context of a series of inter-laboratory validation studies, the need to harmonise assessment methodologies and to develop a better understanding of the relevance of immunotoxicity test results in animal experiments for humans became evident.

This was the reason for the International Programme on Chemical Safety (IPCS) to sponsor and organise in collaboration with the Free University of Berlin (FU Berlin), the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), and the National Institute of Public Health and the Environment (RIVM) a symposium on 'Approaches to Risk Assessment of Immunotoxic Effects of Chemicals' held from 7–10th December 1999 in Berlin, Germany.

The aims of the symposium were:

- to review human health effects of exposure to immunomodulating chemical agents
- to evaluate the validity of available experimental systems used to assess immunotoxicity and their ability to predict human health risk
- to evaluate means for extrapolation from in vitro and in vivo animal studies to human health effects

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- to evaluate the potential for harmonisation of the test procedures and risk assessment methodologies

International experts were invited to present their current views in these areas of applied immunotoxicology. Groups consisting of speakers and organisers formulated conclusions and recommendations for risk assessment. The following report reflects the opinion agreed upon by the working group members and does not represent views of any national government or international agency.

2. Introduction

The immune response, in general, depends on the successful interactions of the antigen with different cells such as lymphocytes, macrophages and other accessory cells. Exposure to an immunotoxic compound may alter the immune response and result in either immunosuppression, which may cause more severe or prolonged infections or possibly the development of cancer, or immunoenhancement leading to allergic or autoimmune diseases. These adverse effects may be the consequence of a direct and/or indirect influence of the compounds on the immune system.

However, many other factors tend to complicate the assessment of immune competence. In particular, the immune response can be affected by the following:

- host related factors, e.g. genetic background, age, gender, nutritional status, and pathological conditions
- chemical related factors, e.g. dose level, frequency, route and duration of exposure, biotransformation, pharmacokinetics, chemical reactivity

Clinical evidence for consequences of chemical-induced immunosuppression includes increased incidence of infectious diseases and neoplasia. Complications associated with infections have been described following the therapeutic introduction of corticosteroids or anticancer drugs, and they remain a major problem particularly in patients treated with current immunosuppressive drug regimens.

Epidemiological evidence suggests that chemical-induced immunosuppression is associated with certain cancers. For example, non-Hodgkin's lymphoma has been associated with immunosuppressive agents, such as azathioprine, cyclosporin, OKT-3, or tacrolimus and often at a time when these drugs were still at the stage of clinical trials. Except for lymphomas and some virally induced skin cancers, there is no evidence that immunosuppressive, non-genotoxic agents can increase the incidence of neoplasia.

The best-studied example of an environmental immunotoxicant is 2,3,7,8 tetrachloro-dibenzo-p-dioxin (TCDD). Immunological responses induced by polyhalogenated dibenzo-*p*-dioxins and polyhalogenated dibenzofurans (PCDDs and PCDFs) as well as polychlorinated biphenyls (PCBs) in mammals have been observed for the last 25 years at doses varying over a broad range. Despite considerable efforts to elucidate the mechanism of the immunotoxic effects of organohalogen compounds and despite the relevance of this exposure for humans, the mechanism responsible for their actions still remain unanswered. This is even more true for other environmental agents that affect the immune system.

Immunotoxicity is not a well-recognised cause of reduced health and increased disease. In relation to existing environmental factors, one may ask how big this problem is, and how much immunosuppression is required to increase the occurrence of infections and cancer. A growing database from animal testing indicates that there is a potential problem. Also, limited data from exposed wildlife or accident situations with humans support the notion that environmental factors may weaken human immune defence mechanisms. In addition, some studies on health effects from outdoor as well as indoor environment exposures indicate that certain air pollutants may be associated with an increased occurrence of respiratory infections.

Our knowledge of immune-mediated adverse effects of immunostimulating agents has increased since the introduction of potent 'immunoactivating' agents, such as the recombinant cytokines, into the clinical setting. Flu-like reactions, with

mild to moderate hypothermia, chills, arthralgias and malaise, have been described. These symptoms are well known as typical complications following vaccination. Such reactions have also been reported in patients treated with recombinant cytokines, hence the preferred term 'acute cytokine syndrome' to describe the high-grade hyperpyrexia, which is associated with cardiovascular and neurological disorders, was coined. This syndrome is commonly seen with interferons, rIL-2 and monoclonal antibodies, and is often treatment limiting.

Allergic diseases constitute a major health problem in industrialised countries. The main clinical manifestations are skin, respiratory, or gastrointestinal disorders.

The clinical features of allergies are as complex as the mechanisms involved. Anaphylactic (type I) reactions induced by therapeutic agents most often consist of urticaria and angioedema, whereas anaphylactic shock is relatively rare, but life threatening. Contact dermatitis (type IV) occurs with therapeuticals used topically or with occupational or environmental chemicals, which come in contact with the skin.

Autoimmune diseases represent a large group of organ-specific and systemic disorders which are characterised by immune system reactivity to self structures. Although their aetiology is poorly understood, it is thought that they likely result from chronic inflammation following environmental exposures in genetically susceptible individuals. Their occurrence is strongly linked to various genetic (HLA, human leukocyte antigen), physiologic (estrogen) and environmental (bacterial and virus infection) factors. Specific assays to assess chemical or therapeutic agents for their potential to induce autoimmune reactions are lacking. Evidence for considering environmental agents as possible triggers for autoimmune disease includes low concordance of disease in monozygotic twins, clinical case reports with supporting dechallenge and rechallenge data, animal models and epidemiological studies. The environmental agents of concern include selected foods, therapeutic drugs and biologics, consequent to occupational, recreational and/or incidental exposure. Specific examples include drug-associated lupus-like syndromes, L-tryptophan-associated eosinophilia myalgia syndrome, a variety of silica-associated syndromes, and rubella vaccine-associated arthritis. Autoimmune diseases have occasionally been seen with exposure to immunostimulating agents, such as levamisole. They are much more frequent following therapy with recombinant cytokines, such as rIL-2 and the interferons.

3. Hazard identification from experimental data

Adverse effects associated with immune activation are emerging as a challenge in assessing novel chemical and therapeutic agents.

Experimental data from in vivo and in vitro studies are of considerable value, because experiences from human exposure are limited. In evaluation procedures, e.g. for the risk assessment for regulatory purposes, one should be aware of toxic effects on other organs and tissues. Currently, an agreement was reached on the adaptation of a general definition given by the OECD/IPCS (1998). An adverse effect was defined as a change in morphology, physiology, growth, development or life span of an organism, which results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress, or an increase in susceptibility to other environmental influences. This general definition is also applicable to the field of immunotoxicology. However, at present the immunotoxicology data available do not suffice to establish what constitutes an exact level for adversity and its reversibility.

Toxicological data from safety studies in animals and humans are usually the main source of immunotoxicology including histopathology and may be the first indicators for impaired immunologic homeostasis. Since the immune system in humans and animals may differ in sensitivity, antigen recognition and the type of immune reaction, the risk estimate may be complicated after hazard identification from experimental observations alone. Thus, methods and strategies need continuous refinement as the knowledge of immune mechanisms broadens and new thresholds are set for adverse and/or reversible reactions.

3.1. Prediction of immunosuppressive activity

Immunotoxic effects on the immune system of test animals are generally evaluated in context with toxic effects observed in other organs. Body and organ weights give important background information. However, weight changes of immune organs may result from effects on compartments with and/or without immune function.

Pathology is used to flag immunotoxic effects in the revised OECD (1995) test guideline 407 — repeated dose 28-day oral toxicity study in rodents. The revised protocol requires determination of thymus and spleen weights and the evaluation of the histomorphology of thymus, spleen, one draining and one distant lymph node, small intestine including the Peyer's patches and bone marrow. In addition to these standard parameters, the weight of the popliteal lymph node has been found to provide a potential sensitive indicator for immune reactions.

Certain haematology and serum biochemistry parameters are indicative of immune alterations, particularly in combination with evaluation of the T-cell dependent antibody response. However, a high variability of individual and mean values occurs in white blood cell counts and serum concentrations of immune globulins. For practical and efficiency reasons it was the goal to identify test parameters appearing robust enough to indicate immunotoxicity under the conditions of a conventional 28-day test. With the known variability in immune responses five animals per sex and group represent the absolute minimum for immunotoxicological assessment. Eight to ten animals seem to be more appropriate, depending on the strain used.

Phenotyping of leukocytes is of value depending on the antibodies used to characterise lymphocyte subgroups. Information on B- or T-cells, such as CD4 − or CD8 − subsets alone, appears to be of limited value, whereas the evaluation of activation markers in different subsets may provide useful information for hazard identification.

Data from the anti-sheep red blood cell (SRBC) plaque-forming cell assay (PFCA) or its equivalent enzyme-linked immunosorbent assay (ELISA) add important information, because they reflect the type of disordered immune function in response to antigen challenge. Such assays have been 'validated' for the rat. In the United States National Toxicology Program (US NTP) studies, the plaque-forming assay was shown to be a predictive assay also for the mouse. With respect to both tests, PFCA and ELISA, investigators must be aware of different kinetics due to different endpoints measured (i.e. quantitation of B-cells producing antibody versus antibody titer to SRBC in the PFCA and ELISA, respectively).

While not as predictive and reproducible as the plaque-forming assay, other immune function assays can provide valuable information on the mechanism of action of immunotoxic compounds. Lymphocyte proliferation after mitogen stimulation can be performed with human and animal cells and thus, lends itself for use in parallelogram approaches. Additionally, this assay can be carried out with whole blood. In conducting these mitogen stimulation assays, background proliferation needs to be examined to ensure appropriate data evaluation. The natural killer (NK) cell assay represents another approach that should be conducted using the spleen as the source of lymphoid cells, since the cells from mesenteric lymph nodes were found to be inappropriate. However, the NK test is hampered by high individual variances, which reduces the sensitivity of the test system.

3.2. Predictive tests of allergenicity

For the detection of the contact sensitising potential, guinea pig skin assays have been used for decades. Currently, the guinea pig maximisation test is the most commonly used test. One disadvantage of the test is the subjectivity of the read out, which is skin redness. More recently, the local lymph node assay (LLNA) has gained much attention. In the LLNA the test chemical is painted on the ears of mice. Hapten-protein complexes are processed by Langerhans cells, which migrate to draining lymph nodes, where the antigens will be presented to lymphocytes. The proliferative capacity of the lymphocytes in the draining lymph node, which can be measured by tritium thymidine uptake, is an indication of the

sensitising capacity. An advantage over the guinea pig maximisation test is that the LLNA is quantitative and appears to be a reliable alternative to the guinea pig assays. However, further modifications are necessary to improve the accuracy (exclude false positive results due to irritancy) of this test system. Guinea-pig models have been used for decades to identify chemicals responsible for contact (photo) allergy.

Respiratory allergy animal models (i.e. guinea-pig and mouse) that involve inhalation of test substances and subsequent analysis of immune responses and lung functions are currently in use. However, to date there are no validated test systems for systemic allergy. Modified versions of the local lymph node assay, taking into consideration cytokine responses and IgE production in the mice, have shown promise as a screening test.

As for food allergy there is no agreed upon test model. Thus, considerable efforts are necessary to further develop these predictive models and to document their validity.

3.3. Predictive tests of autoimmunity

Chemical- and drug-induced autoimmune reactions are difficult to predict, since there are no appropriate models for their study. At present, the popliteal lymph node assay (PLNA) may provide some indications of autoimmunity-inducing potential. The test compound is injected into the footpad, and the subsequent response in the draining lymph node is taken as indication of activation. This activation may be due to the sensitising capacity of the chemical, or to the formation of neoantigens. Mechanistically, this test is similar to the local lymph node assay (LLNA), albeit by injecting the test compound in the footpads, Langerhans cells are bypassed. A refinement of this test is the use of reporter antigens. Such reporter antigens may be either T-cell dependent (TNP-ovalbumin) or T-cell independent (TNP-ficoll) given at suboptimal doses. The chemicals may reveal responses to these antigens, which will provide further information on the sensitising capacity and perhaps neoantigen inducing capacity of the test compound. Nonetheless, the limitations of these models and our lack of understanding in this area require much more effort be invested in understanding mechanisms for the environmentally-associated autoimmune disorders so that appropriate models may be developed.

4. Epidemiology

To confirm predicted risk and gain information on the actual risk of adverse effects due to actual exposure, there is a need for well-designed epidemiological studies on immunotoxicity.

Particularly for immunotoxicity, epidemiological studies may prove quite difficult. Many of the immune changes in humans after exposure to immunomodulating agents may be subtle and sporadic, which would make health effects difficult to discern. The structure and function of the immune system while manifesting changes may not have any apparent clinical effects on health due to compensatory mechanisms. This implies that exposed individuals may not show obvious health effects, but that effects may be detectable on a population level.

Any epidemiological study should have valid measures of exposures, confounders and health endpoints. Although questionnaires and diaries are important and valuable tools in epidemiology, direct and quantitative biological measures are preferred. A longitudinal study design in which subjects are observed over a sufficient period of time to assess the ultimate health outcomes associated with immunotoxic exposures and alterations in immune functions is preferred. While such studies are most often prospective, retrospective studies using banked specimens from individuals who develop immune related disease would be useful if exposure assessment is objective and quantifiable. It should be noted that a longitudinal study design may especially be effective when aimed at infections as health consequences of immunotoxicity, but is much more problematic in cases where the expected health outcome is cancer due to the long follow up time required.

Two prime biologic measures are identified as being particularly valuable for assessment of immunotoxicity in epidemiological studies. First, the

immune system is most efficiently and informatively assessed for the influence of chemical exposure on hypersensitivity responses by a skin prick test, or, if available, a radio allergosorbent test. For changes in immune function, the system is most efficiently and informatively assessed by vaccination with a neoantigen. It is generally accepted that if children with defined high exposures respond to vaccination with similar titers as non-exposed children, and there is no clinical evidence of increased infections, the agent is not immunotoxic in humans, at least with respect to humoral and T-cell dependent immune responses. Pediatric vaccination programs therefore provide a good opportunity to detect immunotoxic effects within the population, although it is not clear if they fully assess immunodysregulatory mechanisms that could result in autoimmunity. While this approach capitalizes on the newborn/infant, it can also be applied to the adult population. Furthermore, it is most consistent with what has been observed as the most predictive endpoint in animal studies — i.e. the primary antibody response to a T-cell dependent antigen.

4.1. Immunosuppression

The association between immune suppression and increased infections and neoplasia is well documented in humans. Thus potential hazards have been identified. It is known that individuals are exposed to putative immunosuppressive agents in the environment. Hence there is a need for risk assessment.

Epidemiological evidence is sparse and largely restricted to cross sectional studies, which are very difficult to interpret.

An important opportunity exists to examine the relationship between the degree of immune suppression and both infectious and neoplastic disease outcome in the area of therapeutic uses of immunosuppressive agents. Although there is data on the incidence of neoplasm following immune suppression, this has not been related to direct measurement of immune parameters. In addition, the incidence, severity, and duration of infections (both common and opportunistic) could be beneficially studied in this setting. Existing databases — such as the 'General Practice Research Database' (GPRD) and the 'Health Maintenance Organisation' (HMO) data bases in the UK — could be used to do this retrospectively, however, prospective studies may be needed to fully capture data on immune parameters and infectious disease incidence. The design of these prospective studies would be informed by examination of the existing databases.

Exposure to putative environmental immune suppressants is likely to be higher in developing countries and this offers an additional special opportunity to examine effects on infection. Thus it is important to take a global view in selecting sites for epidemiological studies related to this issue. Particular examples are possible risks resulting from exposure to mycotoxins in developing countries, arsenic in drinking water in some places in Asia, and pesticides.

The design of epidemiological studies in immunotoxicity was the subject of a recent meeting in Bilthoven (Van Loveren et al., 1999). The report of this meeting provides useful guidance for investigators.

4.2. Allergy

Allergic reactions to chemicals manifest in an increased reactivity of the skin, the respiratory tract, as well as, increased systemic reactivity. Following topical exposure, contact sensitisation is the usual outcome. Respiratory allergy is not solely restricted to airway exposure but may also appear after topical or oral exposure. The skin is also the target organ where allergic reactions are manifest following oral exposure.

Some chemicals, which accumulate in the skin after systemic exposure or enter the skin via the horny layer, may cause photoallergy. Once photoallergic reactions develop, there is a risk of persistent light reactivity which forces individuals to avoid exposure to UV light even in the absence of the inducing chemical.

In addition, chemicals may cause a dysregulation of the immune system leading to an increased incidence of allergy caused by a naturally occurring environmental factor. However, allergy induced by imbalance of the immune system

depends on many factors like genetic background, health conditions, hygienic conditions and medical practice.

In relation to allergy, there is much information available on the variability of immunological parameters between individuals and groups of populations. During epidemiological studies and surveys databases have been generated that describe relevant immunological parameters in the clinically non-allergic population as well as in populations with various specific allergies and clinical manifestations. Notably, there is also information on parameters like total IgE in relation to exposure to certain pollutants like dust/particles and tobacco smoke. A large variation in total IgE levels between populations in different geographical areas has also been documented. The value (power) of total IgE as a marker of atopy is greatly diminished when used to compare populations in different geographical areas as when used to make comparisons within a limited geographical region. Specific IgE levels to a given allergen will depend upon the level of exposure to the allergen in question, personal factors like heredity (atopic predisposition, HLA type) and environmental factors (socio-economic, birth order, characteristics of the home). The predictive capacity of a specific allergy (test positive) for clinical disease also appears to vary between geographical regions and the different types of diseases. In relation to contact allergy, the major immunological parameter is skin test reactivity (patch testing). Reactivity is influenced by personal factors such as skin properties, reactivity of the inflammatory and immune systems. However, this variability can usually be minimised in its impact by the use of adequate controls. Specific immune reactivity (contact allergy) is largely determined by exposure and accompanying environmental factors (skin irritation and damage), with some influence from genetic factors. It is important to note that environmental chemicals may influence the prevalence of allergy not only by acting as allergens (which sometimes is a minor role), but also by acting as immune response modifiers (adjuvants). This latter phenomenon has been documented experimentally in animals and epidemiologically in humans for diesel exhaust particles.

Test systems, which are clinically applied for diagnostic purposes, are the prick test, the photo patch test and the radio allergosorbent test (RAST). In addition to these commercially available routine test systems, more sophisticated methods can be applied but may have some limitations for use in epidemiological studies. In Germany, Switzerland and Austria, the photo patch test group has performed testing in many facilities. However, the use of the data is limited since photo patch testing cannot differentiate between photo allergic and phototoxic reactions. Evidences for the nature of photoreaction have to come from experimental animal or in vitro studies.

Compounds that may act as allergens include isocyanates (respiratory), anhydrides (respiratory), chemicals in rubber (contact), latex allergens (respiratory), industrial enzymes (respiratory), and metals like nickel (contact).

Factors of concern in the capacity of adjuvants/immune response modifiers are suspended particulate matter (SPM) indoors and outdoors, and chemical and biological substances on the particles. A major route of exposure that is of concern for respiratory allergies is inhalation, but the gastro-intestinal route may also be of importance and its role is perhaps underestimated.

For contact allergy, the major route of exposure is the skin, while the role of the respiratory and gastro-intestinal routes is small.

4.3. Autoimmune disease

Little is known about differences in relevant immune parameters for detection of autoimmunity among populations, however, a number of factors appear to be risks for the development of certain forms of autoimmune disease. These include gender, polymorphic immunogenetic and pharmacogenetic markers, age, race/ethnicity, and possible geographic factors. There are no routine tests at present to identify the at risk populations for autoimmune disease. But approaches that may prove useful and require further investigation as predictive elements of risk include: HLA and other genetic testing, certain autoantibodies, and immune complexes.

Certain autoantibodies are highly specific or even diagnostic for particular diseases. Examples are anti-Sm for lupus, ant-Jo1 for myositis, anti-Scl70 for scleroderma, anti-mitochondrial for autoimmune liver disease, and anti-microsomal or anti- thyroglobulin autoantibodies for thyroid disease.

Data supporting the role environmental agents in induction of autoimmunity include animal models that show some elements of the L-tryptophan-associated eosinophilia myalgia syndrome, a number of drug-induced diseases and mercury-induced renal disease.

While the limited data in the field make it difficult to quantitate risks, based upon clinical reports, animal investigations, in vitro assays or epidemiological studies, there is a growing list of environmental chemicals which may be triggers of autoimmune disease. Specific examples of agents of concern include oral ingestion of a large number of therapeutic drugs and biologics; dietary supplements including L-tryptophan; ingestion of mercury; occupational and incidental inhalation of silica and beryllium compounds; exposures to vinyl chloride and organic solvents.

Much more needs to be learned in terms of the pathogenesis of autoimmunity and the possible genetic and environmental risk factors for the development of autoimmune disease. It is important to take into account a number of possible confounders in epidemiological studies including concurrent or past immunomodulators (UV light exposure) and infectious agents (e.g. HIV).

5. Future perspectives

5.1. In vivo and in vitro studies on the mode of action

For interspecies comparison of immunotoxity data established in one species, it may be important to characterise the underlying mechanisms and disorders of immunoregulation. Current in vivo–in vitro techniques are able to determine the type and role of cytokine mediation in immune reactions and characterise immune responses at cellular and subcellular levels. Further significant information on reactions of the immune system is expected from immune histochemistry, data of reversibility studies, and SAR analysis.

5.2. Cytokines (messenger molecules of the immune system)

Induction and development of immune responses require complex interactions among the involved cells. Release of mediator polypeptides (cytokines) from cells act at the site of origin or in its vicinity (locally) and systemically. Cytokines circulate at low concentrations (pico- or nanogram) in body fluids and their half-life is ~ 10 min. Their responsiveness is regulated by environmental stimuli (endotoxins, cytokines from other cell systems) or by inhibitors (binding proteins, receptors and autoantibodies). Some cytokine expression patterns from in vitro photo-irritation studies and in vivo skin immune reactions are available.

The cytokine protein production can be determined in body fluids by enzyme immunoassays (ELISA) or radio immunoassays (RIA) and, the cytokine amount can be measured in the ELISPOT assay or by flow cytometry (intra-cellular staining; secretion assay); commercial kits are available. The measurement of cytokine messenger nucleic acid (mRNA) by the introduction of reverse transcriptase polymerase chain reaction (RT-PCR) is highly sensitive. This is a technique which was improved by the inclusion of competitive gene fragments (competitive PCR) and by the use of additional fluorescent probes (real time PCR).

The measurement of cytokine profiles during immunotoxic screening requires considerable effort and resources. If cytokine measurements are to be included in immunotoxicological investigations, one may concentrate on selected phases in the immune reaction: (I) initiation; (II) proliferation; (III) differentiation; and (IV) migration. This reduces the number of analyses and thereby saves time and resources.

5.3. Molecular assays

Data from molecular studies using materials from isolated organs, cells or cell lines allow some information on mechanisms. Depending on pathways of signal transduction, intracellular fluxes, phosphorylation cascades or phosophatase activities can be measured. With respect to the interferon pathway, the activation of cytoplasmic STAT proteins and their dimerization characteristics can be measured by electromobility shift assays (EMSA). By the same technique, the activation of NFkB, one central regulator of cytokine gene transcription, can be assessed.

Array systems allow the investigation of many genes at the same time. These high throughput systems may become routine in chemical and preclinical drug toxicity screening. Depending on the type of array system, nitrocellulose filters, chips or other signal transducing devices are employed. Such multi-gene approaches enable the identification of signalling patterns and will contribute to the precise characterisation or prediction of in vivo immune effects.

5.4. Other techniques

From in vivo samples of treated animals, immune effects of a test compound may be studied in terms of cellular and molecular mechanisms by isolating RNA from the respective target cells, assessing the cytokine gene transcription (as mentioned above) and activation of certain transcription factors or cellular oncogenes. Methods to obtain appropriate samples from specific organs and cells need to be standardised.

In order to allow high throughput in screening by immunotoxicity assays, miniature 384 well plates or 'immunotox-chips' are used. Furthermore, to increase the sensitivity and to 'humanise' these systems, cells of transgenic animals or transfected cell lines may allow precise investigations with respect to immune mechanisms involved in the human situation.

5.5. Tissue based assays

Since data from studies on primary cells or cell lines only partially reflect the in vivo situation, complex cell systems (3D artificial organs, perfused tissue samples and organ slices, whole blood) are examined for immune response. However, physiological homeostasis can be maintained only for a few hours.

The whole blood assay (heparinised venous blood) was established to provide an in vitro model to replace the rabbit pyrogen test. The testing endpoint is to induce the expression of a certain cytokine, tumour necrosis factor (TNF-α), in monocytes present in the samples. TNF-α is measured by a conventional ELISA system and the TNF production closely correlates to the monocyte activation status. In comparison to data from studies using monocytic cell lines or macrophages, the whole blood assay is less sensitive but is closely related to the in vivo situation of man. The whole blood assay approach is also used in screening for IgE antibodies in allergotoxicology as well as for measurement of cellular basophilic cell plasma constituent release (histamine or leukotriens) upon addition of a suspected allergen.

6. Harmonisation of test procedures

6.1. The situation in pharmaceutical industry

The status of immunotoxicity testing for new molecular entities (NMEs) in the pharmaceutical industry was elaborated in a survey (Dean et al., 1998). A questionnaire returned by 12 of 15 companies indicated that eleven companies had previously evaluated the immunotoxic potential of NMEs (e.g. immunosuppression, myelotoxicity, allergy (excluding contact hypersensitivity) or autoimmunity)) from different pharmaceutical classes. Nine laboratories (82%) had obtained adverse immune reactions with one or more NMEs. Six laboratories (55%) reported that they had evaluated less than five NMEs and five laboratories (45%) had evaluated both NMEs and biological products.

The decision to evaluate immunotoxicity of a product was overwhelmingly (91%) based on findings during routine toxicity studies, i.e. pathologic values found for haematology parameters, lymphoid organ weights, cellularity, or observations in histomorphology (55%). In some instances, the decisions were based on the chemical class of the test compound, mechanisms of action or clinical indications. Immunomodulators or anti-viral drug candidates were excluded since they are routinely evaluated for immune effects. Immunotoxicity safety data had been requested by regulatory agencies from only seven of the twelve responding companies (64%).

Generally, immunotoxicity testing was performed at some time between preclinical and clinical phases of drug development. Rats and primates were most frequently studied with the studies generally being performed in-house under the responsibility of a toxicologist (64%) or a pathologist (27%). Five companies (45%) indicated that they had observed potential immunotoxicity with an NME during clinical development, which was undetected or not evaluated, in preclinical animal studies for two instances. Three companies terminated the drug development project when immunotoxicity was encountered during clinical trials. Immune effects observed in animals during preclinical drug development had commonly not resulted in termination of the NME development, although such data determined the type of test in the further development. In one of the laboratories, an unexpectedly low frequency of adverse immunological effects was observed (two of 27 NME).

Harmonisation implies the comparison of different test methodologies and their suitability for detecting the functions of the immune system and is therefore important for an adequate risk assessment.

Several multinational efforts have been made to accomplish inter-laboratory validation.

6.2. International multicenter validation efforts

The mouse has been the predominant species in immunotoxicological studies and is still widely used in host resistance models. In addition, the guinea pig and mouse are the species used for prediction of allergenicity. In toxicological chemical and drug safety studies, however, the rat has been the species of choice. Rat immunotoxicity data have now been accepted by regulatory agencies. Three international interlaboratory rat validation studies have been reported. In addition, one validation effort on the local lymph node assay has been performed.

6.3. International Fischer 344 rat study

Nine laboratories participated in this study (White et al., 1994). Cyclosporin A (prototype immunosuppressive compound) was orally administered to male Fischer 344 rats once daily for 14 days according to a detailed protocol and standard operating procedures. Data were collected on standardised forms and submitted to a central laboratory for statistical analysis.

Dose–response trends were similar for the data from the various laboratories. For the NK cell assay and ConA (concanavalin A) mitogen stimulation assay, results were comparative in six of nine (67%) laboratories. The T cell-dependent antibody plaque-forming cell assay and the mixed leukocyte response resulted in comparative data for 100% of the laboratories conducting the assays. The results showed the rat as a useful model for immunotoxicity assessment.

6.4. International Collaborative Immunotoxicology Study (ICICIS)

The ICICIS on the rat was initiated by the International Program on Chemical Safety, a cooperative programme of the United Nations Environment Program, the International Labour Office and the World Health Organisation, the Commission of the European Union and the United Kingdom Department of Health (Dayan et al., 1998). The goal was to investigate whether the 28-day toxicity test (i.e. OECD, 1981 Test Guideline 407) was appropriate to flag immunotoxicity (immunosuppression).

In the initial study with azathioprine, standardised test protocols were not employed. Due to diverse experimental procedures across 20 partici-

pating laboratories, the predictive value of data was reduced.

In the second study, standardised test protocols were employed to investigate the effects of cyclosporin A. With this approach the inter-laboratory variability of data was greatly reduced. The results from 20 laboratories indicated that studies according to OECD TG 407 of 1981 did not identify CYA-induced effects on the rat immune system, while the 'enhanced' pathology as defined in the OECD TG 407 of 1995 did. For the immune function tests, the primary (IgM) immune response to sheep red blood cells (SRBC), as determined by the plaque forming cell assay, was reliable and sensitive. The in vitro mitogen-stimulated lymphoproliferative response assay and the NK cell assay were less sensitive.

6.5. International BgVV co-ordinated studies

Hexachlorobenzene was chosen as the test substance because of its primarily immunostimulatory effects in the rat. Nine laboratories conducted an oral 28-day test according to the OECD TG 407 (1981) on male and female Wistar rats obtained from the same breeder. The test protocol was supplemented with organ weights of the thymus, popliteal lymph nodes and mesenterial lymph nodes. Additional histomorphology was performed on thymus, mesenterial lymph nodes, Peyer's patches (ileum), popliteal lymph nodes and bone marrow. Serum levels of the immunoglobulins (Ig) M, G, and A were determined. Functional tests such as cell counts from spleen, mesenteric lymph node and bone marrow samples were also performed. Leukocyte phenotypes were determined by the flow cytometry using antibodies characterising T- and B-lymphocytes and macrophage populations of spleen and mesenteric lymph nodes. Cells from spleen and mesenteric lymph nodes were examined for proliferation after mitogen stimulation (ConA and PWM), for phagocytic activity, and for a NK cell activity. Spleen cells from satellite groups of rats immunised against sheep red blood cells or keyhole limpet hemocyanin were tested for T-cell dependent humoral response in the plaque-forming cell assay or by ELISA method.

Treatment-related immunomodulating effects were observed in all laboratories. Hexachlorobenzene affected the values for standard haematology parameters, the weight of lymphoid organs (spleen, popliteal lymph nodes), the histology of several lymphoid organs (spleen, mesenterial and popliteal lymph nodes, Peyer's patches, bone marrow), the number of nucleated cells (spleen), the serum concentrations of immunoglobulin (Ig) M, the leukocytic subpopulations, and the lymphoblastic response (proliferation rate) after ConA stimulation. Other immune tests did not show any significant differences compared to control groups.

Cyclosporin A as a test substance with a primarily immunosuppressive effect on T cells was administered orally once daily for 28 days in five participating laboratories (Richter-Reichhelm and Schulte, 1996). The test protocol was identical to that used for hexachlorobenzene.

Data which were indicative of the immunosuppressive effect of cyclosporin A were derived from standard haematology, subpopulations of leukocytes from spleen and mesenterial lymph nodes, values for serum IgM concentrations, organ weight of thymus, histopathology on the lymphoid tissues. Immune response tests on leukocytes from spleen and mesenterial lymph nodes revealed reduced lymphoblast formation (proliferation) after ConA and PWM stimulation, activation of macrophages and NK cells, and reduced antibody formation in the plaque-forming cell assay.

6.6. Interagency Coordinating Committee on Validation of Alternative Methods (ICCVAM)-validation of the LLNA

Recently, the local lymph node assay (LLNA) was peer-reviewed by ICCVAM (Anon., 1999) and was recommended as an alternative test method to guinea-pig tests, for the identification of contact sensitises. The data submitted indicate that the LLNA does not accurately predict weak sensitisers (false negatives) and does indicate some strong irritants (false positives). However, when comparing the LLNA with currently accepted methods, the LLNA appears to provide an equiv-

alent prediction of the risk for human allergic contact dermatitis.

7. Risk assessment

Because of the complexity of the immune system and its responses, animal experimentation is essential in considering testing procedures sensitive enough to predict risk of immunotoxicity in humans. While in vitro systems may be predictive of hazard, they are not adequate as stand alone methods for risk assessment. They are, however, useful in studying mechanism(s) of toxic action and may therefore add to the strength of the extrapolation of risk from animal experiments to humans.

Among immune reactions triggered by chemicals and drugs in man, allergies are the main immunotoxic effects. Immune suppression occurs and is therapeutically induced to treat undesired immune reactions. Adverse immune responses seen in laboratory animals may lack similar observations in humans since there are quite a number of factors that affect immune responses and tend to complicate risk assessment:

1. host related factors such as genetic background, age, gender, nutritional status and health condition
2. chemical- or drug-related factors such as dose, frequency, route and duration of exposure, biotransformation, pharmacokinetics, chemical reactivity, etc.

There is ample evidence that chemical-induced immunotoxic effects follow conventional dose–response relationships. Accordingly, standard quantitative risk assessment procedures can be applied, provided there are sufficient relevant quantitative data about the dose–response relationship of the effect being considered. As such, it is important to remember that adequate exposure assessment is a critical component of risk assessment.

In order to improve the predictability of experimental data on adverse immunoreactions in the risk assessment for the human situation, the parallelogram approach was proposed. Specific attention needs to be given to the type of data generation and interpretation of observations. Items of concern include: (I) selection of relevant toxicological endpoints; (II) adequacy of in vitro tests for the complexity of the immune system in vivo; (III) comparison of different target cells, e.g. antibody response of animal spleen cells versus that of peripheral blood lymphocytes in man; (IV) selection of adequate species, gender, and exposure routes; (V) data from supporting in vitro tests, structure activity relationship (SAR) and mechanistic studies to characterise the underlying mode of action; (VI) interpretation of interspecies differences.

For the process of risk assessment, functional tests will likely provide information on no-adverse effect levels and are therefore valuable. However, caution is needed in determining the relevance of slight effects on immune parameters in view of the functional reserve capacity of the immune system. In those cases, host resistance infection models can be very helpful for risk assessment, as they are tools to elucidate the actual consequences of disturbances in immune function; effects observed using such infection models have surpassed the reserve capacity of the immune system. The fate of the pathogen, and the associated host pathology, may serve as indicators of the health implications of the immunotoxicity of the test chemical. Pathogens used in these host resistance models are chosen so that they are good models for human disease.

If the test findings indicate that immunotoxicity is the critical effect, these effects on the immune system should be considered in the risk assessment process like any other toxic action. Should the immunotoxicity be identified as resulting in allergic sensitisation the risk should be characterised and risk management should take into account appropriate steps to protect both the general population and those already sensitised.

Study reports submitted for risk assessment must include all relevant immunotoxicity findings, even those that are not statistically significant, and a critical assessment of their significance must be made. Recognising the need for better risk communication, risk assessors should include in their reports all information relevant to the assessment, which may include indications of effects on the immune system even those that are not of

obvious significance at the time. The appropriate perspective should be placed on these findings.

8. General conclusions

It was concluded that there is sufficient, although limited evidence, to indicate that immunotoxicity is a potential risk to human health. In principle, the interaction of xenobiotic agents with the immune system may result in hypersensitivity to the agent or agent-induced immunosuppression or autoimmunity.

All types of chemicals, including pharmaceuticals, environmental contaminants, industrial chemicals, food additives and pesticides are considered to be candidates for immunotoxicity assessment. While additional toxicological information has recently become available for certain industrial chemicals prior to registration, little formal information about immunotoxicity is available about many chemicals and environmental contaminants to which humans are exposed.

As there are different types of chemicals that may represent a risk as well as several different classes of immunotoxicity, it is recognised that a flexible testing approach is needed to accommodate these variables rather than to rely on a fixed testing strategy. Currently, there are accepted test protocols for testing chemicals for immunosuppression and contact sensitisation

In vivo and in vitro experimental investigations have shown some advances in detecting immunosuppression and in detecting contact hypersensitivity, but there is still an urgent demand for adequate testing procedures for respiratory and gastro-intestinal allergy as well as for autoimmunity.

The procedures in the 'enhanced' OECD test guideline 407 would probably suffice to identify the immunosuppressive potential of most chemicals but it has not been fully evaluated for this purpose, some experts consider that the antibody response to sheep red blood cells (SRBCs), as measured by the plaque-forming cell assay (PFCA) or enzyme-linked immunosorbent assay (ELISA), should also be performed as a routine test. In addition to the specific results of such testing, attention should be paid to the health of animals during dosing and the occurrence of any infections. For the substances of concern there may also be value in applying the haematological and histopathological procedures of OECD 407 to F1-generation animals euthanised at the time of weaning in reproduction toxicity studies in order to screen for potential developmental immunotoxicity.

These test parameters, however, are not designed to detect contact or other forms of hypersensitivity, or autoimmunity.

As indicated above, while there are established procedures to assess the potential for contact sensitisation, there are no agreed procedures or validated techniques to predict respiratory and gastrointestinal allergies, or chemical-induced autoimmunity.

It is important to emphasise the need for specific guidelines for immunotoxicity testing because the procedures and endpoints differ from conventional toxicity testing. There is a particular need for validation and harmonisation of immunotoxicity test procedures for immunosuppression, especially for examination of quantitative dose–response relationships and the association between changes in immune function and host resistance to infections and neoplasia.

At present, a scientifically sound and specified risk assessment for prediction of any adverse effect on the multifunctional immune system remains a difficulty.

Animal tests are suitable for hazard identification and attempts to establish a risk. However, risk assessment for the human population based on these animal data remains unsatisfactory. In addition, the predictive ability of these models is not 100%, due to certain groups of chemicals which confound the models.

Structure activity relationships, especially when evaluated by proven computational predictive programs or expert systems (e.g., deductive estimation of risk from existing knowledge (DEREK), computer automated structure evaluation (CASE), toxicity prediction by computer assisted technology (TOPKAT), and others), may also suggest the nature of subsequent optimal tests. The relevance of effects at high doses should

be considered in relation to the magnitude of potential exposure to humans. If data suggestive of immunotoxicity need to be assessed, attention should be paid to the fact that they are not due to stress and/or other general toxic actions. The importance of the dose–response relationship should be considered as well as the bioavailability and plasma concentration of the test agent.

The symposium reflected the current view of the multiple experimental methodologies and their value for a reliable risk assessment of immunotoxicants. Human health effects taken from all sources such as clinical data banks, epidemiology and case reports of clinical drug testing were considered to be one major part in that process. It was recommended to better use these data and to refine the methods to create a sufficient database.

These approaches — if followed by practising clinicians, investigators, regulatory agencies, journal editors and reviewers — may accelerate our understanding of a possible new arising of epidemic environmental diseases and assist in deciphering environmental triggers for many currently recognised conditions.

In any case, risk assessment should take into account all available information and evaluate this in light of exposure conditions as well as different genetic, age, and geographic variables within the population. With this approach a number of areas that require more intensive research were identified and consequently recommendations for future investigation formulated.

9. Recommendations

In immunotoxicology, standardisation, validation and harmonisation of test and assessment procedures are essential.

There is need to accept a grading system for adverse immune effect levels. Databases and registries of exposure (type and route) to hazardous agents associated with adverse immune reactions (type, frequency, severity and relapse) need to be established.

Epidemiologic studies on patients already clinically surveyed (organ transplant patients undergoing immunosuppressive therapy) and certain occupationally exposed groups need to identify immunotoxic reactions by laboratory and clinical tests for comparison with results from laboratory animal studies.

Computer-based SAR systems need to be used for predicting immunotoxicity. Industries are encouraged to share currently available information they have on alerts related to immunotoxicity. Additional investigations or environmental studies must be based on information on the immune status and exposure from general safety toxicology testing.

Immunohistochemistry is of high value in the assessment of immunotoxic agents as identification of biomarkers is relevant for exposure.

Data from reversibility studies should be considered when assessing immunotoxic potential of chemicals and drugs. An antigen challenge assay, e.g. PFCA or ELISA equivalent should be added to repeated dose studies according to OECD guidelines. Interim data from long-term dose–response studies may provide insight on the development of immunotoxic effects and the influence of age factors.

Because of high variability in values of some haematology parameters, eight to ten animals per group might be more appropriate to be examined instead of the five animals recommended in some OECD guidelines. If flow cytometry analysis is carried out, the appropriate set of antibodies for activation markers and specific subtypes of lymphocytes needs to be chosen.

Relationships between defined immune functions and host resistance and other immunomodulatory effects (e.g. ultraviolet radiation, stress, life style factors) should be taken into consideration for risk assessment.

Standardised sets of allergens for skin testing are required and there is need to define genetic risk factors for allergy. Models need to be developed for the prediction of allergy induced by food components (including mixtures, emulsifiers and additives). There is a need to develop and validate assays to identify systemic allergic reactions affecting the respiratory and gastrointestinal tract.

Case cohort studies need to identify exposure conditions leading to chemical- or drug-induced autoimmunity. There is need to develop models to

predict autoimmunity and discriminate idiosyncratic and allergic immune reactions. A refinement and validation study should be performed to determine the value of the popliteal lymph node assay (PLNA) to predict autoimmune responses.

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