BIOMARKERS, 1999, VOL. 4, NO. 2, 135–157

MEETING REPORT

Report of the Bilthoven Symposium: Advancement of Epidemiological Studies in Assessing the Human Health Effects of Immunotoxic Agents in the Environment and the Workplace

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Summary

A scientific symposium Epidemiology of Immunotoxicity was held in Bilthoven, the Netherlands, on November 12–14, 1997. This symposium was stimulated by publication of the report of a 1994 WHO/IPCS Task Group meeting on principles and methods for assessing direct immunotoxicity associated with exposure to chemicals (WHO, 1996), a report by the World Resources Institute (WRI 1996) that raised concern about the immunosuppressive effects of pesticides on exposed populations in developing countries, and a workshop on 'Environment and Immunity' organized by the European Union (EU 1997). A common theme among these reports was the need for well designed epidemiological studies on immunotoxicity. Experts in epidemiology, clinical immunology, and immunotoxicology who participated were asked to reach consensus on the most useful approaches to assess immunotoxic effects in humans. The symposium demonstrated the benefits which can be derived from 'cross-fertilization'–a meeting of the minds and sharing of ideas–i.e. the best product in terms of design, conduct, and interpretation of a complex scientific issue.

The meeting concluded that epidemiology is an essential method for assessing immunotoxicity in humans. Every epidemiological study should have valid measures of exposures, confounders and health endpoints. Although

1354-750X/98 $12.00

questionnaires and diaries are important and valuable tools in epidemiology, direct and quantitative biological measures are preferred. When possible, a longitudinal study design in which study subjects are observed over a time sufficient to assess the health outcomes associated with immunotoxic exposures and alterations in immune functions is preferable. 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. A longitudinal study design may be most suitable where infections are the health consequences of immunotoxicity, but is much more time-consuming and costly when the expected health outcome is cancer.

Two prime biological measures were identified as particularly valuable for assessment of immunotoxicity in epidemiological studies. First, the immune system is most efficiently and accurately assessed for the influence of chemical exposure on direct hypersensitivity responses by the skin prick test, or antigen-specific IgE ELISA or RAST tests. Second, for suppression of immune function, the system is best assessed by vaccination with an antigen to which no prior exposure has occurred. It was generally accepted that if children, with defined high exposures, respond to vaccination with similar titres as non-exposed children, and there is no clinical evidence of increased numbers of infections, the agent is most likely not immunotoxic in humans. A strong recommendation is therefore to make a greater use of (paediatric) vaccination programmes. While this approach focuses on the children, it can also be applied to the adult population. These measures are also most consistent with observations in animal studies as the most predictive endpoint–i.e. the primary antibody response to a T-cell dependent antigen.

Studies of childhood infectious diseases and malignancies may be emphasized in developing countries, whereas studies of allergic and malignant diseases may be emphasized in developed countries, reflecting the most critical health concerns in the respective types of countries.

1. Introduction

Humans and animals are exposed to a variety of agents in the environment that may modulate the immune system. Such agents comprise chemicals including industrial chemicals such as pesticides, air pollutants, natural toxicants and ionizing and ultraviolet radiation.

Immunotoxicology has been defined as the study of adverse effects on the immune system resulting directly or indirectly from occupational, environmental, or therapeutic exposure to chemicals, biological materials, and, in certain circumstances, physical factors, collectively referred to as agents. It encompasses studies of altered immunological events associated with exposure of humans and wildlife species including immune disregulation (suppression or enhancement), allergy, and autoimmunity. In the former case, the systemic or local (e.g. skin, lung) immune system acts as passive target for the agent, and the result may be an increased incidence or severity of infectious diseases or neoplasia. In allergy, the immune system responds to chemical (hapten)–host protein conjugates or high molecular weight compounds. The most likely health consequences of the latter include respiratory tract allergies (e.g. asthma, rhinitis), or allergic contact dermatitis. Autoimmunity may occur either as a result of an agent-induced alteration in host tissue, endocrine function, or immune disregulation. Direct

immunotoxicity leading to enhancement or immunosuppression may also have an impact on immune responses to antigens that are not related to the immunotoxic chemical, and thus have an impact on allergies and autoimmunity, by exacerbation of these responses. The present report is focused on the effects of direct immunotoxicity on infectious diseases, allergies, and cancer.

The potential for adverse health effects in humans due to alterations in the immune system has been a matter of increasing scientific and public concern. In humans, a number of agents have been shown in volunteer studies or after accidental exposure to have immunomodulatory properties, based upon various tests. The true biological impact of those changes has not been documented as stringently, however. That modest immunomodulation may be of clinical importance in humans is evidenced by stress-related decreases in vaccination titres, and increased Herpes simplex symptoms after ultraviolet radiation. The full impact of drug-induced immunodeficiency is appreciated from the increased incidence of (particularly opportunistic) infections and certain types of neoplastic diseases seen with the use of immunosuppressive agents for control of transplant rejection reactions.

There have been marked efforts both in basic research undertaken within this area as well as development and incorporation of appropriate test methods to assess potential immunotoxicities in laboratory animals, wildlife species, and humans. Regarding testing methods, appropriate selection has been complicated by the need to employ distinct tests based upon the site examined (e.g. systemic, lung, skin) and the immunopathology of concern (i.e. hypersensitivity, immune disregulation, autoimmunity, inflammation). Moreover, our increased understanding of the molecular, cellular, and genetic events responsible for mounting appropriate immune responses, as well as for the immune mediators involved in organ-specific necrotic and apoptotic processes, has provided opportunities for the utilization of more streamlined and informative tests for risk assessment and the development of novel preventive or therapeutic measures.

Altered immune regulation (i.e. suppression or unintended stimulation of the local or systemic immune response) may have implications for infectious diseases, allergies, cancer, and autoimmunity and, as such, has been afforded considerable attention. Initially, efforts were made by immunologists working in the area of toxicology to identify agents which could potentially alter the immune system using various *in vitro* or rodent models (i.e. hazard identification). Subsequently, studies were conducted to develop more predictive test methods, to determine mechanisms of action, or to identify affected human populations. Based on such studies, a number of agents (see appendix) have been shown to alter regulation of the immune system.

Many of the immune changes seen in humans after exposure to immunodulating agents may be subtle and sporadic, and effects on health may be difficult to discern. The structure and function of the immune system may manifest changes, but 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 at a population level, for example as an increased prevalence of common cold, influenza, otitis media. These effects may occur especially in subpopulations that are more vulnerable to the risk of exposure to immunotoxic agents, such as children and the elderly. In addition, it should be recognized that the immune status of populations is extremely

heterogeneous. Age, race, gender, pregnancy, stress and the ability to cope with stress, coexistent disease and infections, nutritional status, tobacco smoking and other life style factors, medication, and seasonal differences contribute to this heterogeneity.

In the environment, exposure will occur to a mixture of immunomodulating agents, typically at low levels and by more than one route. Typically, in the human population quantitative exposure data and dose–response relationships are either non-existent or very limited. Better exposure data and well validated biomarkers of exposure and effect are necessary for effective epidemiological studies.

In 1994 a WHO/PCS task group meeting on principles and methods for assessing direct immunotoxicity associated with exposure to chemicals (WHO 1996) recommended investigating the relationship between alterations in immune function and human health, and in particular developing biomarkers of exposure, effect, and susceptibility. These biomarkers would be monitored in epidemiological studies to evaluate subtle alterations in immune status induced by chemical exposure. The need for this information was underscored in a report by the World Resources Institute (WRI 1996) that raised concern about the immunosuppressive effects of pesticides on exposed populations in developing countries, where infectious diseases are by far the most important causes of illness and death. Immune alterations due to relatively high exposure to pesticides could combine with other factors that influence host defences, e.g. insanitary living conditions, unprotected water supplies, undernutrition, etc. (WRI 1996). In addition to altered resistance to infectious diseases immunotoxic agents may also exacerbate allergic diseases (e.g. air pollution has been considered as one possible cause of the increased prevalence of asthma). The need for further development of design for epidemiological studies of immunotoxicity was emphasized by a workshop on environment and immunity organized by the European Union (EU 1997), especially on air pollution, ultraviolet light, environmental chemical exposure, and combination effects of immunotoxic agents.

These initiatives led to the organization of the Scientific Symposium Epidemiology of Immunotoxicity that was held in Bilthoven, The Netherlands, from November 12–14, 1997. This meeting, chaired by R. F. Vogt (USA), brought together scientists of various disciplines–epidemiologists, clinical immunologists, and immunotoxicologists–to discuss the most useful tests available to assess immunotoxic effects in humans, to delineate the most significant problems in better understanding the links between exposure to environmental immunotoxic agents and adverse health effects, to specify the most promising epidemiologic approaches, including questionnaires, and to assess the health consequences of immune dysregulation.

In general, the clinician is concerned with the individual patient, already suffering from a disease. The toxicologist is concerned with mechanisms by which chemicals exert effects, as a basis for risk assessment, with the aim of prevention of disease. The epidemiologist is concerned with issues at the population level, also aimed at risk assessment. The multidisciplinary approach to address these issues ensured that different important aspects that have a great impact on the issue were taken into account, and the symposium was the first meeting of its kind bringing these disciplines together.

While addressing these issues, a distinction was made between developed and developing countries. The distinction is critical and valid for a number of reasons

relating to exposures, national capabilities for controlling the use of chemicals, health status, and available resources. It should be mentioned that rather than the two categories of developed and developing countries, there is in fact a wide range from highly industrialized countries, to countries such as those of the former Soviet Union, where in general medical standards are well developed, but socio-economic developments are less sophisticated, to those countries at the other side of the spectrum where socio-economic standards and medical resources are scarce.

Deliberations were preceded by the following overview lectures and case studies:

- Immunotoxicology from the immunotoxicologists' point of view (M. I. Luster, USA)
- Immunotoxicology from the clinical immunologists' point of view (I. L. Bernstein, USA)
- Public health: infections (A. Hall, UK)
- Public health: allergies (J. Devalia, UK)
- Organochlorines, immune status, and susceptibility to infections in Inuit (P. Ayotte, Canada)
- Pesticides in Moldavia (L. Kovtiuh, Canada)
- Moderate immune suppression due to stress (R. Glaser, USA)
- Air pollution and allergy (T. Nicolai, Germany).

Subgroup discussions were structured around three areas: immunological assays, epidemiological study design, and epidemiological questionnaires, each concerning developed and developing countries. Specific topics were exposure to air pollutants (inflammation, allergies and infectious diseases), and systemic exposure to immunotoxicants (perinatal exposure to persistent organochlorines and adult exposure to pesticides, mycotoxins, and radiation). The Scientific Committee of the symposium served as the authors to formulate the summaries and conclusions from the discussions during the meeting (R.R. and H.S.K. *in absentia*), and drafts of the document were commented by participants.

The meeting was sponsored by:

- National Institute of Public Health and the Environment (The Netherlands)
- International Programme on Chemical Safety (UNEP-ILO-WHO)
- World Resources Institute (Washington, DC)
- European Union
- European Science Foundation
- National Institute of Environmental Health Sciences (USA)
- Environmental Protection Agency (USA)
- National Institute for Occupational Safety and Health, Centers for Disease Control (USA)
- American Crop Protection Association (Washington, DC)

II. Considerations for the design of epidemiological studies of immunotoxicity

The design of an epidemiological study is a crucial factor in assessing the usefulness of a particular study for risk assessment of immunotoxicity in human populations. Considerations of epidemiological studies should address not only the specific design of studies (cross-sectional, case-control, etc.), but also the many

other factors required to achieve meaningful results. Any epidemiological study should consider the many determinants that have an impact on the immune system, and define which of these are confounders that may distort the association between the exposure of interest and the health outcome, and need to be accounted for. Questionnaires and diaries are important and valuable tools in epidemiology. If possible, validated direct biological measures of exposure and effect will provide additional strength to the study. It should be mentioned in this respect that biological measures are not always easy to interpret in terms of health, and that only those that are interpretable are valid. Important considerations in designing and conducting epidemiological studies include the following.

Longitudinal and prospective studies in comparison to cross-sectional studies. A common design used in epidemiological field studies is cross-sectional, in which all persons or a sample of persons in a population are evaluated at a single point in time. Information on exposure and disease are ascertained at the same time, thus allowing different exposure groups to be examined for differences in the prevalence of health effects or the distribution of biomarker values. An option may be to select two groups of individuals that are virtually identical except for a single variable, usually exposure. However, it is very seldom possible to select two groups that are virtually identical with respect to other variables than the endpoint that is studied. The many confounding variables that influence the immune system (discussed below) make the selection of two groups that are comparable except for exposure status extremely difficult to identify. Generally, it is more efficient to have one large group with a great range of exposures and end point values. Cross-sectional studies provide little information about the natural history of exposure-related effects. In cross-sectional studies cases of long duration are over-represented and cases of short duration are under-represented. Obtaining information on the date of diagnosis is helpful to assess the selection bias, but does not resolve the inherent problem. Especially to this end case-control studies on overt health outcomes with short incubation periods (e.g. otitis media, childhood pneumonia), may be useful, because rapid retrospective exposure assessment is reasonably representative of the exposure circumstances at disease induction. Cross-sectional and case-control studies are generally the simplest and least expensive approach for assessing exposure-related health effects at the population level.

Prospective or cohort studies in which study subjects are assessed over a time period long enough to assess the ultimate health outcomes associated with immunotoxic exposures and immune changes (the latter being possible even within one individual) are preferred. However, such studies are usually costly and difficult to carry out, and a decision to perform such a study should be based on information to support such a study. Such information may stem from an earlier cross-sectional study or case-control study, in addition to toxicological information that may be available. Longitudinal approaches will demand multi-disciplinary teams with practicing expertise in epidemiology, immunology, exposure assessment, and other relevant disciplines. Retrospective studies using banked specimens from individuals may be quite powerful if exposure assessment is adequate. The ultimate epidemiological approach may be an intervention study. Such studies are done by randomization of sufficient numbers of individuals to remove the potentially immunotoxic exposure, and provide the best means to control for both known and unknown confounding factors. It is obvious that such an approach is seldom feasible.

In practice, the most useful epidemiology study design is one which collects valid information on exposure and disease. Individualized segments of the study design should reflect a multi-tiered approach: diaries; questionnaires; and laboratory measurements. The use of diaries and questionnaires can be applied in a cost-effective way in a large population size (thousands); the use of laboratory measurements will increase the cost and the need for technical expertise and will result in a decrease in the sample size. A combination of diaries, questionnaires and laboratory measurements is considered essential to detect subtle differences and for a meaningful contribution to our understanding of human immunotoxicity.

Valid measures of exposure, health effects, and immunological markers. While any epidemiologic study should have valid measures of exposures, confounders and health endpoints, variability in the immune status and the wide range of 'normal' values for the human indices of immune function makes these issues especially crucial in seeking differences attributable to immunotoxic exposures. Exposure misclassification is an especially troubling prospect and can lead to either false positive or false negative results. Questionnaires and diaries are important and valuable tools in epidemiology, but only if they are well validated. Direct biological measures may be valuable for both exposure and health assessments whenever possible, but also only if they are well validated. Environmental measurements of exposure may not always represent individual exposure levels. Laboratory measurements concerning the immune system may not always provide useful information on the risk of health effects.

Best use of self-reported data. Information from diaries and questionnaires may be best applied in the initial stage of large population studies (thousands), where these tools are the most cost-effective way to obtain preliminary information and determine whether further studies with laboratory analyses or clinical evaluation are warranted. It should be emphasized that self-reports of exposures and conditions or symptoms need to be validated, otherwise they form a poor basis for research.

Importance of controlling for confounding variables and effect modifiers. If confounding factors and effect modifiers are not recognized and effectively addressed in the study design, population selection, and statistical analysis, they may severely compromise the assessment of immune function in population studies. Factors that cause measurable alterations in immunological parameters include age, race, gender, pregnancy, stress and the behaviour used to cope with stress, coexistent diseases or infections, nutritional status, life-style, tobacco smoking, and many medications. Besides these variables, normal periodic influences, ranging from daily to seasonal, also affect the immune system and may cause random noise in these biomarkers of effect. Even when the magnitude of many such effects is minor, they may introduce enough bias or imprecision in population studies to introduce false differences or obscure real effects from low level immunotoxic exposures.

Opportunities to study individuals as exposures change. Immunotoxicologists should be alert for opportunities to monitor populations as exposures change. For instance, good opportunities for study have occurred as chemical exposures due to air pollution have been reduced in the eastern part of Germany. In such instances it is important to obtain baseline data rapidly. Designs that include experimental intervention are preferred when possible. Occupational exposures often provide the best opportunity for studying high-level exposures, but consideration should be

given to the fact that employed workers are not representative of the population at large.

Using existing information from health and exposure registries. Epidemiological studies of immunotoxicity should make use of existing historical information such as health registries to develop an overall picture of health and exposure in different populations.

Focusing on studies of newborns and young children. The developing immune system is especially vulnerable to immunotoxic agents, hence newborn children are especially important scientifically for human immunotoxicity studies. Their sensitivity, ease of identification, short history of environmental exposures, and lack of adult personal confounders favour more clear-cut assessment of immunotoxic human exposures. Childhood vaccination programmes offer a singular opportunity for simultaneously assessing immunotoxicity and improving public health. The establishment of cohorts monitored from birth through early childhood would be especially valuable, since exposure sources can be tracked as they change from *in utero* to almost exclusively indoor to increasingly outdoor as children grow. In developed countries, assessing exposures in pregnant women from their first positive pregnancy test and following the health status of their growing children could be especially productive. Vaccination studies in newborns and young children may provide the greatest opportunity to study immunotoxicity in humans.

Vaccine responses as indicators of immune function. Vaccination is probably the greatest opportunity for monitoring immune status on the population level, as well as conferring tangible public health benefits. Paediatric vaccination programmes (such as against diphtheria, pertussis, tetanus, poliomyelitis, measles, meningitis and tuberculosis) provide a propitious opportunity for repeated assessment of immune function. Vaccines may also present an opportunity to examine waning immune function in older populations. Decreased antibody responses to Hepatitis B vaccine in adults has been used very effectively to identify stress-related immune suppression. It should be mentioned that rather than antibody titres, cellular immune responses may be a better indicator for immunocompetence, at least with certain vaccines. Albeit more laborious than antibody titre measurements, *in vitro* T-cell responses may be measured for this reason. For populations vaccinated against tuberculosis, skin testing with PPD may be an alternative.

Choosing the most beneficial studies. The ultimate choices of where and when epidemiological studies are to be carried out, as well as how they should be designed, must be based upon the potential benefit of study results on the health of the population to be studied. In addition, they should be based on the feasibility of such a study, i.e. there should be sufficient contrast with respect to exposure. For these reasons studies of childhood infectious diseases and malignant diseases may be emphasized in developing countries, whereas studies of allergic and malignant diseases may be emphasized more in developed countries, where these diseases are more critical health concerns. It should be recognized, however, that when investigators choose to focus on one health effect (e.g. infectious disease instead of allergy), other health effects may be overlooked.

In the context of a cost-benefit analysis, studies to address immunotoxicity from environmental pollution in developing countries may have relatively low priority in light of concerns such as adequate nutrition and access to clean water. However, as emphasized in a previous report (WRI 1996), these very factors may

themselves increase the burden upon the immune system, so that potential immunotoxic effects from environmental pollutants become more important public health problems in developing than developed countries.

Specimen banks and ethical considerations. Human samples collected for epidemiological study are an invaluable resource and should be preserved for future analysis as new laboratory techniques are developed and new health and exposure information becomes available. Concern over ethical considerations has made specimen banking very difficult, and review boards may even compel the destruction of specimens after the analyses approved by informed consent have been completed. Study designs should explicitly include provisions for specimen banking with full compliance with ethical principles (CIOMS 1991, 1993).

Stress-related effects as a biological calibrator for immune suppression. An important observation has been made in the investigation of stress that directs the further development of immunotoxicity testing in humans, both in volunteer studies and in epidemiological studies. Studies on the effects of stress have revealed subtle, but physiologically significant, declines in immune responsiveness that can be correlated with impaired host defence to common infectious agents. The best indicators of such impairment in these studies were diminished primary responses to vaccine antigens (e.g. Hepatitis B and influenza), and elevation of antibodies to Epstein–Barr virus (EBV) suggesting at least partial reactivation of latent EBV infection. These studies provide a reference frame for designing and interpreting studies of immunotoxic exposures, and indicate that primary immune responses may be an important laboratory measurement that may be included in epidemiological designs.

III. Questionnaires

Questionnaires are a prime tool in epidemiological studies. Four major areas which data from questionnaires would address in epidemiological studies of immunotoxic exposures can be identified:

Demographics
Exposure Assessment
Health Effects
Confounders

General points for consideration are:

Questions posed in questionnaires. It should be realized that the list of desirables for inclusion in questionnaires is often long. However, response to the questionnaires may decline with the expansion of the list. Certain types of questions may have a more negative effect on response than others. Careful selection of questions to be included is therefore required.

Validating questionnaires. Questionnaires should include internal consistency checks and be validated whenever possible by testing in reference populations with known exposures or health effects. Since repeated utilization provides some indication of validity, questionnaires with a long history of use by organizations like NIAID, CDC/NIOSH, WHO and ATS should be considered as templates for composing questionnaires for specific studies. Translating questions into different languages can be challenging, and consideration should be given to re-validating previously-validated questionnaires that have been translated from other languages.

To be used effectively, questionnaires must be considered along with validated laboratory tests at the earliest phase of study design. Indeed, one of the goals in selecting laboratory tests should be to provide independent assessment of questionnaire responses. To this end, the relevance and reliability of the laboratory tests should be addressed. In relation to the feasibility of the integration, it should be mentioned that extensive questionnaires in addition to extensive laboratory tests will have a negative impact on the participation of the selected individuals in the epidemiological study.

Questionnaires and study design. Questionnaires are generally considered most valid for use in longitudinal and prospective studies where they can be administered concurrently to the exposures being assessed. They are considered weakest for use in retrospective case-control designs in which recall bias can be a major issue. Yet, in retrospective studies often questionnaires are the only alternative, laboratory tests seldom being possible retrospectively.

Standardizing questions and responses for future comparisons. Since the need for prospective and longitudinal studies was emphasized throughout the conference, considerable importance was placed on designing questionnaires and coding responses for consistency with and relevance to potential follow-up studies. For example, disease-related questions should be referable to a standard coding system such as the International Classification of Diseases (currently ICD-9).

Consideration for differing cultural factors. The use of questionnaires must include respect for specific cultural constructs of the participating populations as well as general ethical standards. In some cultures certain questions may be considered more intrusive than blood sample collection. Consequently, study and questionnaire design should involve public health professionals from the respective cultures at the earliest stages. Differences in languages, dialects and idiomatic usage must be understood and addressed. The degree of literacy may vary widely and must be considered in any use of questionnaires.

Confidentiality and informed consent. Confidentiality must be ensured, both on ethical grounds and in order to encourage the most truthful responses. Informed consent must be obtained with the same sensitivity to cultural factors as the other considerations discussed earlier.

Use of local public health professionals. Studies should engage local public health personnel at the earliest planning stages so they can help in designing questionnaires. Interviews should be conducted by those familiar with the communities from which study participants are drawn, and the interviewers should be trained at least in part by local public health professionals. Familiarity with local patterns of infectious disease is essential to selecting questions relevant to the population studied.

Diaries and self-reported symptoms. Diaries are considered problematic in many ways, but could be of use in carefully-selected settings. For instance, diaries could help document ingestion of foods that might be correlated with pesticide exposures. Diaries could also be beneficial for assessing variation in health status over time, such as those that might vary with changing levels of air pollution or seasonal flux in allergens. Whether elicited from questionnaires or diaries, preferably self-reported symptoms are corroborated by clinical evaluation or medical records. This is, however, often not possible for logistic reasons. In addition, the cost of medical evaluation of minor, yet possibly significant health effects, cannot be ignored.

Early life experiences. Since evidence suggests that early life experiences (including perinatal exposures) may have a strong impact on future morbidity and mortality due to infectious disease, allergy, and asthma, questionnaires should try to include assessment of these early events. Recalls of early life experiences are as likely to be altered by recall bias as health effects, yet there is no way to accurately validate them.

Assessing the entire family unit. Because of the importance of many factors that are a function of the entire family unit (e.g. birth order, number of siblings, occupational exposures, premature death of family members), questionnaires should be designed to characterize these factors fully.

Differences between developed and developing countries. Administering questionnaires in developing countries is generally more difficult than in developed countries, and the conclusions may be less reliable. Health endpoints are often under-reported in developing countries, in part because symptoms may be so common as to be disregarded, and in part because health care can be scarce. Nutritional status is often compromised, which can be a major confounder in assessing immune function. Illiteracy is often more common. Meagre infrastructural resources may cause greater operational difficulties; for instance, transportation may be less convenient, telecommunications less widely available, and training and recruiting facilities less suitable.

Areas of strength and weakness in using questionnaires. Questionnaires were generally considered weakest for purposes of exposure assessment, although they may be more relevant for certain types of exposure such as indoor air pollution and diet (particularly if food pesticide levels are known). Questionnaires were considered more suitable for identifying confounding variables and major (often acute) health-related life experiences, and they were considered strongest for tabulating demographics. For different exposures it should be noted that such confounders require the same level of exposure assessment as the exposure of interest.

Questions related to demographics and family history. The questionnaire should include a complete history of all immediate family members living and deceased, including gender, date of birth, occupational history, residence history, ethnicity, socio-economic status, education, fertility histories (pregnancies and stillbirths), and marital status.

Questions related to exposure assessment. Depending on the type of exposure under study, questions should elicit information on residence history, indoor air exposures, outdoor air pollution, overall diets, specific sources of food and water, histories of breast feeding, occupational exposures, school-related exposures, accidental exposures, and exposures to pets and farm animals. Assessment of perinatal exposure requires specially designed questionnaires and protocols. Food frequency questionnaires or diaries may be helpful if food contaminant surveillance data are available for pesticides.

Questions related to health effects. A multi-step questionnaire with precise questions is preferable. In areas with ready access to health care, questions may be phrased 'have you ever seen a doctor for …', or, perhaps to be more specific, 'has a doctor ever told you that you have …'. It should be mentioned however, that physicians may misclassify diseases. Children should be assessed for their history of viral infections, diarrhea, respiratory diseases, allergies, and asthma. Several existing questionnaires should be used as models for eliciting health effect

information (e.g. the ISAAC, and the WHO infectious and respiratory disease questionnaires).

IV. Selection of laboratory tests and other biomarkers

General considerations

As indicated in the introduction, there are different classes of toxicants to which populations are potentially exposed. Regardless of the specific type of toxicant (i.e. chemical nature) to which the population is exposed, and effects may be different in nature (i.e. acute, delayed, reversible), the approach to evaluate adverse effects is basically the same, although attention must be given to local immune responses (e.g. skin, lung) versus systemic responses.

Limited facilities and differing socio-economic factors impose severe restrictions on the degree and variety of immune system assessments which can be performed. This applies both to developed and developing countries, but for obvious reasons may especially restrict possibilities in developing countries. For instance, investigations of the immune system which require specialized sample handling, such as lymphocyte proliferation assays, can only be utilized if the infrastructure allows for such assays. In some cultures, blood samples are difficult to obtain.

Specific considerations

When selecting appropriate laboratory tests, considerations should include: (a) the types of pathology (immune suppression, autoimmunity, allergy, inflammation, or lymphoproliferative diseases) that might be encountered; (b) results from immunotoxicity assays conducted in animals; (c) lessons from diagnostic tests and procedures used in patient care; (d) differences in the resources and needs between developed and developing countries; and (e) the feasibility of sample collection in field studies. For these laboratory tests, it is necessary to have information on intra- and inter-individual variability. In addition, values provided by laboratory tests should be interpretable in terms of health, and need to be corroborated by clinical evaluation referable to a standard coding system such as the International Classification of Diseases (currently ICD-9).

In epidemiological studies, they need not to be seen as stand-alone tools, but rather in combination with information gained from questionnaires and diaries. While most studies will need to be modified based upon the endpoints that need to be assessed, the following provides a consensus of tests that should be considered for health effects. Laboratory tests of exposure assessment (analytical toxicology) are not addressed, as this is a separate and very specialized field, but the need for objective measurements of exposure, as discussed in the design section, is re-emphasized here.

Physical examinations. Physical examinations should be included whenever possible. Depending on the research question, emphasis should be placed upon possible signs of immune system alteration, infection, inflammation, or cancer. Specific searches for conjunctivitis, rhinitis, skin eruptions, organomegaly, onchyomycosis, lymphadenopathy, and swollen joints should be conducted.

Basic laboratory tests. A few simple, widely-available and inexpensive laboratory tests may be included in most studies where blood samples are obtained. These

laboratory tests may include a complete blood count (CBC) with differential, which will yield lymphocyte, monocyte, eosinophil, basophil, and neutrophil granulocyte count; serum immunoglobulin; serum transferrin, which will provide an estimate of nutritional status; serum C-reactive protein or erythrocyte sedimentation rates, which serve as a screen for inflammatory processes. However, these tests should not be considered as either sensitive or specific for immunotoxicity.

Antibody responses to vaccines as tests for immune competence. Primary immune responses to antigens, particularly vaccines, were identified as the best measure of a functioning immune system. Antibody response to a primary vaccination is recommended as the most convenient and comprehensive measure of immunocompetence, as it measures both B-cell and T-cell function. Specific changes in immune function would be reflected as a decrease in specific antibody titre. The primary immune response is also among the most predictive assay for immunotoxicity in laboratory studies.

Secondary (recall and booster) responses are usually not as sensitive to immunotoxic damage as primary responses. In cases of severe immunosuppression, however, decreased responsiveness to recall antigens has been a useful marker.

In children, the standard vaccinations against childhood infections, such as diphtheria, pertussis, tetanus, poliomyelitis, meningitis, and measles, provide an opportunity for assessing primary immune responses by measuring serum antibodies. In adults, the serum antibody response to Hepatitis B vaccination is often available to assess primary responses, although the increasing use of this vaccine in the general public will render it unsuitable for assessing primary responses in some individuals and makes a baseline (pre-vaccination) determination essential. Hepatitis B is also endemic in some populations. Responses to influenza vaccines involve a mixture of primary and secondary reactions which may be difficult to resolve but may still be useful in some settings. Influenza vaccines linked to a marker epitope may be useful. Serum antibody responses to pneumococcal vaccines are also worth consideration.

Antibodies to Epstein–Barr virus (EBV). The immunosuppresive effects of stress have been related to the reactivation of latent EBV infection as revealed by changes in the isotype, titre and specificity of anti-EBV antibodies. With proper attention to methodological considerations, assays for anti-EBV antibodies may also be useful in detecting effects of low-level immunotoxic exposures.

Delayed skin-test reactions as tests of cell-mediated immunity. Cell-mediated immunity can adequately be assessed by delayed-type hypersensitivity (DTH) responses, such as the tuberculin skin test. However, DTH skin tests are somewhat difficult to administer consistently, and reading the reactions involves more subjectivity than serum antibody measurements. The system of administration of the MultiTest antigens has overcome some of these problems, and has been used very often to assess the immune status. Yet, these commonly used antigens are more often associated with secondary rather than primary reactions. The subjectivity and uncertainty of prior antigen exposure limit the usefulness of DTH skin reactions.

Tests for allergy. The skin prick test or determination of specific IgE by an *in vitro* method (RAST and similar ELISA-based tests) is the most efficient means of evaluating the modulating effect of a chemical on the allergic response to an allergen. Total IgE antibody levels are considered to be less informative for specific hypersensitivity responses, but may be useful in identifying excessive allergy stimulating Th2 responses associated with chemical exposure.

Tests for immune-mediated respiratory disorders. For respiratory disorders, a lung ventilation test, often accompanied by exercise challenge, is often performed. Allergy tests for appropriate antigens, discussed above, may reveal an atopic aetiology. Because of the local nature of respiratory disorders, considerations should be given to analysis of induced sputum or nasal lavage (broncho-alveolar lavage is considered too invasive for routine field use but may be appropriate in some clinical settings). The secretions should be analysed for total protein and lactic dehydrogenase (LDH), inflammatory interleukins such as IL-5, chemokines such as IL-8, and cell differential counts to enumerate neutrophils, eosinophils, and macrophages. Further characterization of these leukocytes, including histochemical assessment and assays for phagocytic function of neutrophils and macrophages, may be indicated. Chemical analysis for mast cell- and eosinophil-derived proteins such as mast cell tryptase (MCT) and eosinophil cationic protein (ECP), respectively, can help elucidate local atopic disorders. Sputum IgM and IgA levels may reveal secretory deficiencies. It should however be noted that the distributions of most data provided by nasal lavage or by analysis of sputum are quite broad, and reference ranges have not been well defined for many demographic groups comprising different genders, ages, and races. Moreover, the biological significance of differences that show statistical significance is usually unclear, and the health implications of borderline or even outlying results are mostly unknown, and more insight is required before it can be recommended to include such type of testing in epidemiologic investigations.

Lymphocyte phenotype determinations. Lymphocyte phenotypes may be identified by differences in cell surface receptor expression commonly measured by flow cytometry. Lymphocyte phenotyping has been very useful in specific applications such as assessing HIV infection and diagnosing lymphoid malignancies. This type of analysis is often used clinically to detect residual disease in remission states of leukaemias and lymphomas, so it may be appropriate in longitudinal studies focused on detecting emerging haematological malignancies possibly related to environmental exposures. In addition, it has been applied in epidemiological studies of effects of PCBs in humans, indicating that background levels of PCB exposure might influence the human foetal and neonatal immune system. However, the general use of these tests in immunotoxicological studies is not warranted. The distributions of most data provided by flow cytometric analysis are quite broad, and reference ranges have not been well defined. In addition, the biological significance of small or even big differences is unknown. Even if modern flow cytometers are equipped to analyse large numbers of samples, lymphocyte phenotyping is relatively expensive and presents logistic difficulties since samples must be analysed relatively soon after collection.

Obviously it is not always feasible to conduct the tests that are previously described in all possible circumstances. The immune system is most efficiently and accurately assessed for influence by chemical exposure on hypersensitivity responses using the skin prick test, or alternatively, an antigen-specific IgE ELISA or RAST test. For alterations in immune function, the system is most efficiently and informatively assessed by vaccination with an antigen to which no prior exposure has occurred. It is recommended therefore that a greater use of vaccination programmes be made. This approach capitalizes on the newborn; but it can also be applied in an adult population. It is most consistent with what has been observed in the animal studies as the most predictive endpoint–i.e. the primary

antibody response to a T-dependent antigen. We stress that these two most valuable tests should be included in the proposed study whenever possible.

V. Specific study examples

Although epidemiological studies of potential effects of exogenous agents on the immune system, leading to impairment of resistance to infectious diseases, or exacerbation of allergy or autoimmunity are relatively scant, a number of study examples are given below.

Air pollution. Some air pollutants can modulate the immune system. Examples are nitrogen dioxide and ozone, which have been investigated in laboratory animals predominantly. Nitrogen dioxide depresses humoral and cell-mediated immunity. It produces morphological changes in alveolar macrophages with decreased phagocytosis, a reduction in the local T-lymphocyte population, suppression of splenic T- and B-cell responsiveness, and decreased Ig levels. Exposure of children to nitrogen dioxide is associated with an increase in lower respiratory tract infections. Ozone also depresses humoral and cell-mediated immunity. Alveolar macrophages show decreased phagocytosis, decreased lysozyme activity and decreased superoxide anion radical production, and T- and B- cell populations in the lungs are also decreased. Host resistance to infections is reduced. In addition to these effects on resistance to infectious diseases, oxidant gases, by virtue of their effect on the immune system, may have an impact on allergic responses in the respiratory tract. Increased atmospheric concentrations are associated with an increase in episodes of asthma attacks in children, which may in part be due to this effect on the immune system.

The potential immunodulating effects of air pollutants can be studied by establishing cohorts to determine the incidence of infectious diseases. As an example, one could establish a birth cohort and follow prospectively for the occurrence of infectious diseases (e.g. respiratory infections) which establishes immune status by means of vaccination responses. If possible, the severity of infections should be assessed. Serology should also be evaluated. In view of their importance, there should be frequent checks on breast feeding and nutritional status (individual biomarkers are needed unless it can be shown that the population is fairly homogeneous on nutritional factors–e.g. vitamin A), in addition to confounding exposures. Skin prick testing at 5 years or older will provide valuable information on immune status in childhood; however, this may not be acceptable in some countries.

The feasibility of doing a study depends on having a valid plan for exposure assessment. Exposure monitoring, outdoor and indoor, at periodic intervals, is necessary to provide a basis for validation of exposure classification. Exposure monitoring will validate exposure and help to understand exposure variability. Validated biomarkers of exposure are important in this respect.

Epidemiological studies on the incidence of allergic diseases, e.g. asthma, may also identify effects of immunomodulation by atmospheric pollutants. Similar considerations to infectious disease studies apply. Antigen specific IgE at age 5 is relevant.

Occupational exposures. Even if significant efforts are undertaken to limit occupational exposure and standards for occupational exposure exist, there are occupational exposures to defined immunotoxicants or mixtures of

immunotoxicants, among others pesticides. An occupational cohort study, designed around selection factors, exposure assessment, and assessment of confounding exposures, is an appropriate method for studying health effects of such exposures. The ideal situation is to focus on one exposure. However, it should be noted that there is often exposure to more than one chemical, such as is the case for pesticides in big agricultural farms. If possible, urinary biomarkers can be used to classify exposure. Disease endpoints are infections, allergies and cancer (retrospective cancer incidence assessment). Case-control studies for cancers are only feasible when recall bias can be thwarted as in a country with standardized and accurate historical records of pesticide use. There should be initial cross-sectional assessment of workers' immune status. Clearly, if correlations between exposure and immune status were observed, intervention and prevention strategies should be initiated. It is essential to have access to complete medical histories; this may be easier in countries with developed public and occupational health systems. Sero-conversion can be used as a measure of infectious disease and also compared with general health parameters.

A cohort of new employees could be used to help in the assessment of the incidence of allergy. Allergy can also be evaluated in cross-sectional studies, provided a dose–response relationship can be obtained. If such studies showed no differences related to exposure, it is considered unlikely that there will be differences related to environmental exposures to the same agent(s) in the general population and, as such, occupational exposed groups can serve as a 'sentinel' for the community.

Mycotoxins. A number of mycotoxins (e.g. aflatoxins, ochratoxins, trichothecenes) are immunotoxic in laboratory animals. Exposures are typically low in developed countries and high in developing countries in tropical regions. Hence, studies should focus on such developing countries. There are good biological markers for some mycotoxins (e.g. aflatoxins) so that exposure can be measured reasonably well. An appropriate type of epidemiological study is a randomized intervention whereby immune function is evaluated and the exposure is reduced and immune function re-evaluated. Compliance with reduced exposure is monitored by means of biomarkers.

Cancers can be studied by prospective cohort studies. Individual biomarkers of exposure and other determinants (e.g. Hepatitis B) are essential. Exposure must be monitored continuously because there is high individual variability (e.g. an individual can be in the highest quartile of the population one year and in the lowest quartile the following year). It should be noted that such prospective cancer studies, in which exposure is continuously monitored, would be extremely resource intensive, and may not always be feasible.

VI. Follow up: Application of the output of the meeting

Initial follow-up activities to apply the outcome of the meeting may be the initiation of a series of workshops or concerted actions, where multidisciplinary groups, considering various themes that are focused on types of exposure (e.g. an immunotoxic agent, or combination of such agents, in a specific part of the world) discuss the weight of the evidence, the epidemiological design that is best suited to answer the question posed, and address the specific questionnaires, diaries, and laboratory tests that should be used. Ultimately, appropriate epidemiological

studies should be undertaken to examine possible effects of non-therapeutic immunotoxic chemical exposure on human health parameters. Such studies may be resource-demanding and this is particularly relevant to the situation in developing countries where exposure to potential immunotoxic agents is significant. There are considerable advantages in undertaking collaborative joint projects between developed countries with greater resources and developing countries. Studies should be undertaken to:

- Assess the immunotoxic effects in populations exposed to (a) air pollutants, (b) pesticides, (c) PCBs, (d) metals (e.g. lead), (e) UV radiation, and (f) mycotoxins. In these studies there must be particular attention paid to possible increases in susceptibility to pulmonary diseases, leukaemia, neurological effects, reproductive abnormalities, and prenatal effects. Particular attention should be focused on the developing immune system and vaccination programmes as they provide a public health benefit to the individual as well as information on potential immunotoxicants in a sensitive population.
- Identify populations susceptible to chemicals because of genetically based immune suppression.
- Develop and validate new biological measures and markers.

Such studies could be multicentre collaborative efforts involving two or three countries, developed and developing. Expertise and resources could be shared and training and technology could be provided to participating developing countries after an assessment of the study design. Collaborative studies of this type will enhance national expertise and capability in developing countries. To organize and run studies efficiently an advisory group should be established to coordinate the 'network' and facilitate the collaboration between investigators from developed and developing countries and the investigations in these countries. This will give active support to 'capacity building' with an emphasis on shared resources, joint projects and greater access to available information.

Sources of funding are critical and potential national and international funding agencies (e.g. UNEP, UNICEF, the World Bank, ESF, EU) and donors must be identified. Bilateral aid agreements between developed and developing countries are another possible source of funds.

VII. Recommendations

- Initiate concerted actions or workshops of multidisciplinary groups that address weight of evidence, possible epidemiology approaches, and laboratory investigations of specific immunotoxicology problems.
- For epidemiological studies of immunotoxicity, longitudinal study design in which study subjects are assessed over a time period long enough to assess the ultimate health outcomes associated with immunotoxic exposures and immune changes may be preferred. Such studies are difficult to perform and costly, and execution thereof should be based on sound scientific information that warrants such a study.
- Information from cross-sectional or case-control studies may be used as a basis to perform longitudinal studies. In addition, in designing epidemiological studies, careful consideration should be given to findings in animals regarding the type of immunopathology.

- Every epidemiological study should have valid measures of exposures.
- Occupational exposures often provide the best opportunity for studying high-level exposures.
- Questionnaires and diaries are important and valuable tools in epidemiology. Whenever possible, they should be supplied by validated direct biological assessments.
- For laboratory tests, information on intra- and inter-individual variability should be obtained.
- Efforts should be made to make values provided by laboratory tests referable to a standing coding system such as the International Classification of Diseases (currently ICD-9).
- The immune system is most efficiently and accurately assessed for influence by chemical exposure on hypersensitivity responses using the skin prick test, or alternatively, an antigen-specific IgE ELISA or RAST test.
- For immune function alterations, the immune system is most efficiently and informatively assessed by antibody responses to vaccination with an antigen to which no prior exposure has occurred.
- It is recommended that a greater use of paediatric vaccination programmes is made. In this respect it is of importance to understand determinants that influence vaccination titres.
- Established cohorts should be maintained for future evaluation.
- Specimens should be banked to enable future evaluation as technology develops.
- Studies of childhood infectious diseases and malignant diseases may be emphasized in developing countries, whereas studies of allergic and malignant diseases may be emphasized more in developed countries.
- Studies should be undertaken to assess the immunotoxic effects in populations exposed to air pollutants, pesticides, PCBs, metals, mycotoxins, and UV radiation.
- Immunotoxicologists should be alert for opportunities to monitor populations as exposures change.
- Such studies could be multicentre, collaborative studies involving two or three countries, including developed and developing countries. Expertise and resources should be shared.

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Appendix

Chemical	Human	Animal
Immunomodulating chemicals (not therapeutic)—*in vivo* evidence		
Pesticides		
Atrazine		x
Captan		x
Cyclohexamide		x
Diuron		x
Diquat		x
Paraquat		x
Maleic hydrazide		x
Propanil		x
Organochlorines		
Chlordane	x	x
Chlordecone		x
DDT		x
Dieldrin		x
Endrin		x
2,4-D		x
Pentachlorophenol	x	x
Organophosphates		
Azinophos methyl		x
Butiphos	x	x
Diazinon		x
Dichlorvos		x
Dimethoate		x

Appendix (*cont.*)

Chemical	Human	Animal
EPN		x
Fenitrothion		x
Fenthion		x
Malation		x
Methyl parathion		x
Parathion		x
Carbamates and thiocarbamates		
Aldicarb		x
Barban		x
Carbaryl		x
Chlorpropham		x
Methyldithiocarbamate		x
Sodium methylthiocarbamate		x
Monolite		x
Zineb		x
Pyrethoids		
Alphacypermethrin		x
Supercypemethrin		x
Deltamethrin		x
Metals		
Lead	x	x
Cadmium		x
Vanadium	x	x
Berylium	x	x
Metalic salts, organometals		
Gold salts	x	
Mercuric chloride		x
Methylmercury		x
Tributyltin		x
Tributyltin chloride		x
Tributyltinoxide		x
Triphenyltin hydroxide		x
Di-*n*-octyltinchloride		x
Di-*n*-butyltinchloride		x
Solvents		
Trichloroethylene	x	x
Benzene	x	x
Toluene	x	x
Xylene	x	x
Carbon tetrachloride		x
Halogenated aromatic hydrocarbons		
Polychlorinated dibenzodoxins	x	x
2,3,7-8-tetrachlorobienzo-*p*-dioxin	x	x
Polychlorinated dibenzofurans	x	x
Polyhalogenated biphenyls	x	x
Hormones		
Diethylstilboestrol	x	x
Ethinyl oestradiol	x	
Nitrosamines		
Dimethyltrinitrosamine		x
Gases		
Nitrogen dioxide	x	x
Ozone	x	x
Polycyclic aromatic hydrocarbons		
Benzo(*a*)pyrene		x
7,12-dimethylanthracene		x
Dibenz(*a, h*)anthracene		x
3-methylcholanthrene		

Appendix (*cont.*)

Chemical	Human	Animal
Mycotoxins		
Aflatoxins		x
Ochratoxin A		x
Citrinin		x
Patulin		x
Wortmannin		x
Trichlorthecenes		x
Miscellaneous		
Asbestos	x	x
Benzidine	x	x
Hexachlorobenzene	x	x
Substances of addiction		
Tobacco smoke	x	
Ethanol	x	x
Cannabinoids	x	x
Cocaine	x	x
Morphine		x
Methadone		x
Therapeutic agents not primarily for immunosuppression		
Anti-inflammatory agents		
Aspirin		x
Indomethacin	x	
Adrenocorticosteroids-prednisone	x	
Antibiotics and antineoplastics		
Actinomycin D	x	
Adriablastine	x	
Bleomycin	x	
Mitomycin C	x	
Mithramycin	x	
Antifungal agents		
Griseofulvin	x	
Tranquilizers		
Chlorpromazine	x	x
Clotiapine		x
Erphenazine		x
Promethazine		x
Haloperidol		x
Antidepressants		
Desipramine		x
Imipramine		x
Diazepam (benzodiazepine)		x
Anti-parkinsonism		
L-dopa		x
Antiepileptics		
Phenobarbital		x
Phenytoin	x	x
Anaesthetics		
Halothane	x	x
Physical and other factors		
UVB	x	x
Stress	x	x

This list was not discussed during the symposium, but was prepared by members of the organizing committee to provide examples of chemicals to which immunotoxicity has been attributed, and that thus may pose a hazard to human health. The criteria according to which immunotoxicity was defined may not have been similar for all chemicals mentioned. It is not within the scope of this report to describe the immunotoxic data concerning all individual chemicals in the list. The evidence in humans exposed to non-therapeutic chemicals often concerns accidental exposure or occupational