

## FORUM

# A Proposed Testing Framework for Developmental Immunotoxicology (DIT)

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Received April 26, 2004; accepted August 30, 2004

A group of thirty immunotoxicology experts from the U.S. and E.U. representing government, industry, and academia met in May 2003, in Washington, D.C., to reach consensus regarding the most appropriate methods to assess developmental immunotoxicology (DIT) for hazard identification, including under what conditions such testing might be required. The following points represent the major conclusions from this roundtable discussion: (1) the rat is the preferred model; (2) any DIT protocol should be based on immune assays already validated; (3) DIT methods should be incorporated into standard developmental and reproductive toxicity protocols to the extent possible rather than a 'stand-alone' protocol; (4) the approach to address DIT potential should be similar for chemicals and drugs, but the experimental design should be flexible and should reflect the specific questions to be answered; (5) it is possible to utilize a study design that assesses all critical windows in one protocol, with the results leading to further study of specific effects, as warranted; (6) animals should be exposed throughout the treatment protocol; (7) the triggers for DIT may include structure-activity-relationships, results from other toxicity studies, the intended use of a drug/chemical and/or its anticipated exposure of neonates and/or juveniles.

**Key Words:** developmental immunotoxicology; DIT; immune system; developmental and reproductive toxicology; risk assessment; roundtable; study design; testing methods.

While immunotoxicology has evolved to the point where guidelines exist within many regulatory frameworks, there is still a lack of consensus on the most appropriate experimental approaches and assays available to assess developmental

The views expressed in this document are those of the authors and do not necessarily reflect the views of policies of the U.S. Environmental Protection Agency or the U.S. Food and Drug Administration. As well, no official support or endorsement by these agencies is intended or should be inferred.

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immunotoxicology (DIT). From a scientific perspective, the interest in DIT has been predicated around the hypothesis that the developing immune system demonstrates greater susceptibility to chemical perturbation than the adult system. In light of some experimental data, DIT may be operationally defined by greater susceptibility, manifested as a *qualitative* difference (i.e., a chemical affects the developing immune system differently than the adult immune system; for example, demonstrates different profiles of immunotoxicity, or affects different immune parameters), a *quantitative* difference (i.e., a chemical affects the developing immune system at lower doses than it would affect the adult immune system; for example, demonstrates a similar profile of immunotoxicity or affects similar immune parameters, but shows a shift in the dose-response), or a more *persistent* effect (i.e., demonstrates a different pattern of recovery after exposure in younger animals as opposed to adult animals). From a regulatory perspective, there is concern that existing guideline immunotoxicity studies conducted exclusively in young adult animals would not detect this greater susceptibility. Moreover, although there is no question that there has been increasing interest in designing appropriate developmental toxicity protocols over the last five or more years, the integration of parameters reflecting the immune system has been quite minimal to date. This is best illustrated by the fact that immune organs are still not routinely included as potential target organs in most developmental toxicity protocols.

It is with this background that ILSI HESI convened a panel of international experts from academia, government, and industry in May 2003 for a roundtable discussion on DIT. It is generally accepted that development of the immune system extends well beyond the early neonatal period in rodents (Holsapple *et al.*, 2003; Landreth, 2002; Luster *et al.*, 2003). Furthermore, the U.S. Environmental Protection Agency, in its Guidelines for Developmental Toxicity Risk Assessment (U.S. EPA, 1991), defines developmental toxicology as 'the study of adverse effects on the

**TABLE 1**  
**ILSI Hesi Roundtable Discussion Points**

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1. What are the current regulatory expectations for developmental immunotoxicology testing guidelines?
  2. What may be the triggers that would cause regulatory agencies to require a developmental immunotoxicology study?
  3. To what extent can developmental immunotoxicology endpoints be built into existing developmental/repro protocols?
  4. What role can pathology play to assess developmental immunotoxicology?
  5. What is the appropriate study design to evaluate developmental immunotoxicity?
    - What species and strains (inbred vs. outbred) are most appropriate?
    - What age should animals be at assessment?
    - What is most the appropriate route of administration?
    - What considerations/methods need to be addressed in order to ensure adequate exposure of the primary test subject (i.e., the offspring)?
  6. What are the most appropriate immune assays/endpoints (e.g., functional, pathology, or host resistance endpoints) to evaluate alterations of the developing immune system? Will different assays be needed to assess different developmental windows of vulnerability?
  7. What additional research is needed to move the science forward so we can more effectively assess these risks and how do we prioritize this research?
  8. What is the regulatory thinking on allergy and autoimmunity in children?<sup>a</sup>
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<sup>a</sup>Time at the roundtable did not permit discussion of the final question.

developing organism that may result from exposure prior to conception (either parent), during prenatal development, or postnatally to the time of sexual maturation.’ It is with this definition of developmental toxicology in mind that roundtable participants worked to reach agreement regarding the most appropriate methods to assess DIT, including under what conditions such testing might be required. A series of pre-determined discussion questions were identified (Table 1) and key observations and points of consensus were captured during the discussion. A summary of these points was prepared and distributed to all roundtable participants to ensure accuracy and completeness. The consensus conclusions of the roundtable discussion are addressed in this article. It is anticipated that this summary will contribute significantly to efforts to develop an appropriate DIT guideline protocol.

### Triggers

A key issue in determining the need to conduct DIT studies is the identification of appropriate ‘triggers,’ that is, cause(s) for concern. Several general triggers were identified during the roundtable discussion.

1. **Structural alerts.** There are two general considerations with respect to chemical structure: structure-activity relationships (SAR) identified using computational methods and chemical class effects. There appears to be no public database which could be used for computational determination of immunotoxic potential. However, if a compound belongs to a class known to be

associated with immunotoxicity (Dietert *et al.*, 2001), this could be taken to indicate the need for DIT studies.

2. **Findings in toxicology studies.** A wealth of toxicology data is generated to support the safety assessment of pharmaceuticals and industrial or agricultural chemicals. These studies are generally conducted in adult rodent and non-rodent species, and include screening level assessments of a range of endpoints across multiple organ systems, following various durations of exposure via the route that is expected to be most relevant to humans. A typical toxicology database will also include standard studies that assess effects of prenatal and/or postnatal administration of the material to animal models. Treatment-related perturbations to immune system structure or function may be identified. These could be observed as adverse clinical findings (e.g., susceptibility to disease) that indicate inadequacy in immune system function; alterations in immune system functional endpoints (e.g., T-cell dependent antibody response; TDAR) in a guideline immunotoxicity study in adult animals; alterations in lymphoid organ weights, macroscopic findings, or histopathology of spleen, thymus, or lymph nodes; alterations in differential white cell counts or serum globulin/immunoglobulin levels; or cellular alterations that are nonspecific indicators of an effect on the immune response (e.g., increased numbers of macrophages in lung tissue or an increased incidence of inflammatory dermal lesions). Thus, compounds that are immunotoxic in adults should be considered likely to be immunotoxic to the developing immune system in general, although qualitative and quantitative differences may exist, as discussed above.

3. **Findings in humans.** Occasionally, evidence of immunotoxicity may be observed in humans, either due to intentional (e.g., drugs) or unintentional (e.g., foods or environmental chemicals) exposure. These findings, such as increased infections, are probably rare and would likely only be definitively detected in controlled clinical trials. However, if such effects are observed, and follow-up toxicology studies confirm immunotoxic potential, consideration should be given to conducting DIT studies.

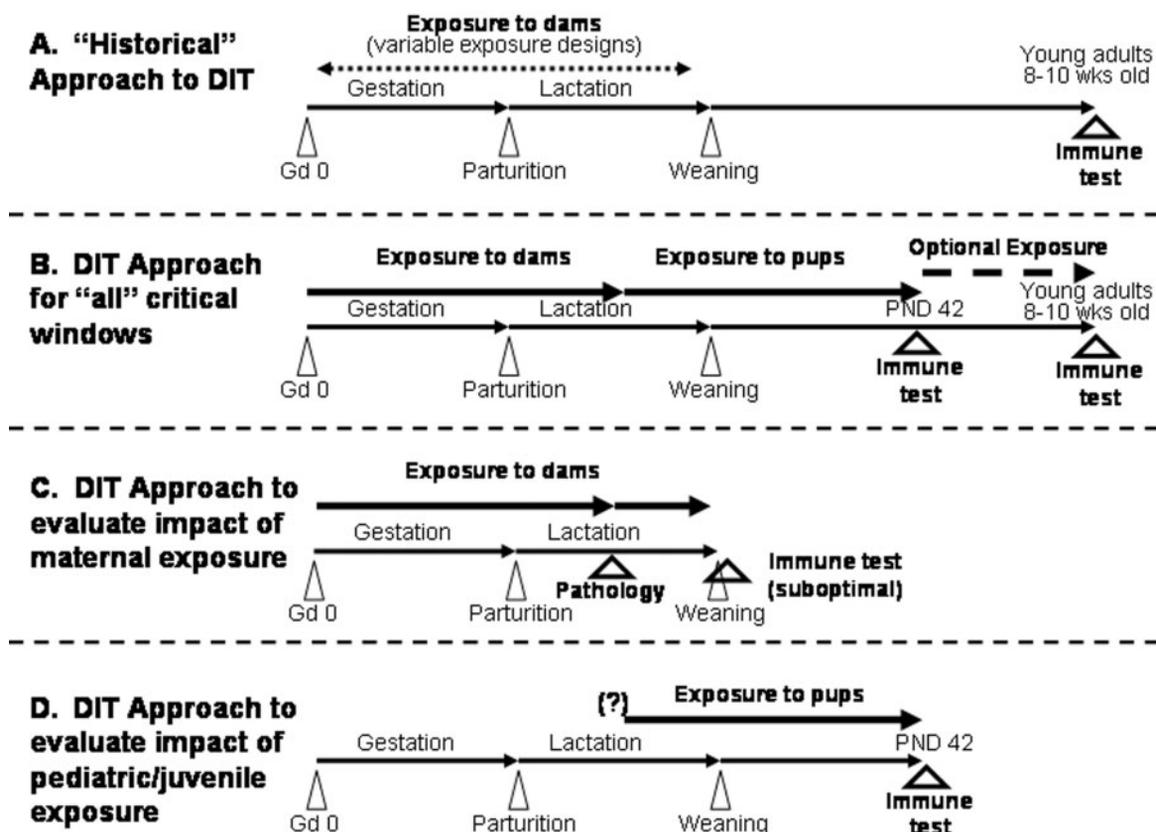
4. **Intended use.** If a compound has been identified as causing immunosuppression, under most circumstances, inclusion of immunotoxicity determinations in developmental toxicology studies should be considered. The labeled indication often represents only a subset of real world use (which can include off-label use as well), and this fact should be taken into consideration in determining the need for DIT studies. Another issue to consider is the ability of the test compound to cross the placenta and/or be secreted in milk. If fetal/neonatal exposure appears to be so low as to be of no real consequence, this could be taken to indicate that DIT studies would be of little use in assessing risk. Characteristics of the intended patient population should be considered as well. For example, if a drug is intended to be used to treat HIV infection, immunotoxicology studies are usually needed because of the apparent increased vulnerability of these patients to drug-induced immunosuppression. In this circumstance, DIT studies would be needed for a comprehensive evaluation of risk,

especially if the drug is intended to be used to prevent perinatal transmission of HIV infection.

With respect to use of a drug in pediatric patients, there is currently no general expectation that juvenile animal toxicology studies need to be conducted if there is sufficient confidence in the established hazard assessment database (U.S. FDA, 2004). However, this issue is typically addressed on a case-by-case basis, and if a compound is a demonstrated immunotoxicant in mature animals, consideration should be given to conducting an appropriate immunotoxicology study in a juvenile animal model in order to estimate relative risk.

### *A General Protocol for Assessment of Developmental Immunotoxicology*

Historically, evaluation of developmental immunotoxic effects has most often involved *in utero* exposure of pups, with dams being exposed at a variety of times during gestation and/or lactation and immune evaluation occurring when pups had attained adulthood (Fig. 1A). The period of non-exposure prior to immune evaluation may make this protocol more applicable for the evaluation of persistent immune effects, but leaves open the possibility that biologically significant effects may occur at earlier times and could be missed due to recovery during the period of non-exposure.



**FIG. 1.** Approaches to developmental immunotoxicology (DIT) testing. (A) Depicts the 'historical' approach to DIT. In most studies to-date, the dams are exposed, and the impact of this exposure on the developing immune system is assessed in the offspring when they become adults. The specific design for exposure has been variable and is frequently limited to a single exposure on one day of gestation. The rationale for assessing the immune status in adults is that the immune parameters have been validated in adults. (B) Depicts an approach to DIT that addresses all critical windows of immune system development. In this proposed protocol, exposure is initiated in dams on gestational day (GD) 0 and continues in dams through lactation. The pups are exposed starting sometime in pre-weaning, and exposure is continued until postnatal day (PND) 42. The specific design for initiating exposure in pups is complicated and must consider a number of points, including the age at which pups can be effectively gavaged, the impact of a bolus gavage dose versus exposure via lactation and the impact of exposure via both direct dosing and lactation. The rationale for PND 42 is that most immune parameters have reached near-adult status at that time. An optional exposure beyond PND 42 is depicted, which would allow the critical window of adulthood to be assessed. (C) Depicts an approach to specifically evaluate the impact of maternal exposure on the developing immune system. In this proposed protocol, exposure is initiated in dams on GD 0 and continues through lactation. If the impact of this exposure is assessed post-weaning (i.e., PND 21), then it must be recognized that the immune tests would be suboptimal relative to adults. If the impact of this exposure is assessed pre-weaning (i.e., PND 10), then it must be recognized that these pups are functionally incompetent (i.e., in terms of immune status) and the assessment would be limited to pathology. (D) Depicts an approach to specifically evaluate the impact of pediatric/juvenile exposure. In this proposed protocol, there is no exposure in the dams and exposure is initiated in the pups sometime before weaning. The specific design for the exposure is depicted as a (?) and must consider some of the points described above with the exception being that there is no contribution for exposure via lactation. In this protocol, the impact of exposure is assessed on PND 42, as described in (B) above.

Roundtable participants were asked to consider the extent to which DIT endpoints could be included in existing reproductive and/or other developmental protocols. Though not specifically discussed at the roundtable, incorporation of DIT endpoints into a standard prenatal developmental toxicology (teratology) study is not feasible because fetuses are evaluated prior to birth and exposure often occurs only during a specific stage of development (e.g., gestation days 6–17). Thus, other stages of immune system development would be missed. Consensus was achieved among roundtable participants that it may not be technically possible to add more endpoints to a developmental neurotoxicology (DNT) protocol (U.S. EPA, 1998c) because, logistically, this protocol is already quite complex. Participants did agree, however, that addition of DIT endpoints to standard reproductive/developmental toxicology protocols is generally possible. The feasibility of the latter approach has been previously demonstrated in studies described by Smialowicz and colleagues (2001). Such a protocol would assess the potential immunotoxic impact of a chemical during all critical windows of immunological development (Fig. 1B). In this proposed design, exposure of dams would begin prior to or at the time of conception, and would continue either through lactation or until such time as the pups could be directly dosed. Direct dosing would continue post-weaning until the pups were evaluated either on postnatal day (PND) 42, or when pups were 8–10 weeks old (young adults) if dosing into adulthood was desired. It should be noted that the purpose of the suggested protocol is not risk assessment, but rather, hazard identification. If one desires, modifications of this design could be made to assess the exact window(s) of sensitivity; for example, whether observed developmental immunotoxic effects are induced during *in utero* exposure (Fig. 1C) or during neonatal/juvenile exposure (Fig. 1D).

When considering the proposed DIT protocol, assurance of offspring exposure to the test compound must be addressed. Roundtable participants agreed that pups in DIT studies should be exposed throughout the entire developmental period for the purpose of identifying potential adverse effects. While exposure cannot be specifically controlled *in utero* (placental transfer is a function of the chemical being tested), offspring can be exposed to compounds either via lactational exposure or through direct oral dosing. Participants also agreed that the decision to direct-dose pre-weaning pups may depend on the compound. If data on the test chemical indicate that lactational exposure does not occur, then direct-dosing may be necessary to assure exposure throughout the treatment protocol. Participants also agreed that the criteria for pre- and post-natal exposure should be consistent across developmental toxicity guidelines/protocols (e.g., developmental neurotoxicity) and should be predicated on the need to ensure exposure of the young during the critical window of organ system development under assessment.

Several important considerations were discussed; specifically, limitations on our ability to quantify internal dosimetry under different dosing regimens and across different windows of susceptibility. Roundtable participants agreed that an understanding

of the maximum tolerated dose (MTD) in pups was important because, depending on the chemical, the MTD may be higher or lower than that observed in the adult since the developmental status of the gastrointestinal tract and general metabolic processes, and/or potential target organ systems may have significant impact on the absorption, distribution, metabolism, and elimination characteristics of a compound in these young animals. It was questioned whether there was a need for a dose range-finding study in young animals. While consensus was reached on the ability to direct-dose rat pups beginning at PND 7, some investigators reported experience in oral dosing as early as PND 4. Participants acknowledged that determination of plasma levels in rat pups was technically challenging and consensus was not specifically reached on whether acquisition of toxicokinetic endpoints in pups was critical. For risk assessment purposes, it was felt that an understanding of these parameters was important, but this specific information may not be required for hazard identification (although it was considered beneficial).

Roundtable participants also agreed that, if a study was conducted assessing all critical windows of exposure at once as suggested in the proposed DIT protocol (Fig. 1B) and that study were negative, then there would be no need to conduct further immunotoxicity testing solely in adult animals. In this instance, carrying exposure out to PND 42 in the proposed DIT protocol prior to functional evaluation would likely identify a similar response as would be seen if a standard immunotoxicology study were conducted in adult animals only.

#### *Methods to Assess Developmental Immunotoxicology (DIT)*

Although researchers have exposed animals *in utero* to chemicals or drugs, the majority of studies evaluating DIT to date are based on the measurement of immune status in the adult (Barnett, 1996; Dietert *et al.*, 2000 (Fig. 1A)). This is not surprising, as most procedures developed for assessing the effects of chemicals and drugs on the immune system have undergone extensive evaluation in adult rodents only (Luster *et al.*, 1988, 1992). As demonstrated by Luster *et al.* (1988, 1992, 1993), the most effective and sensitive methods of detecting immunotoxic alterations in adult B6C3F1 mice are those that assess immune function (e.g., the primary humoral immune response). Moreover, the EPA's immunotoxicity testing guidelines (U.S. EPA, 1998d) requires assessment of a functional parameter (i.e., the primary antibody response to a T-dependent antigen), together with measurement of immune organ weights as the first tier of testing. EPA also recommends that histopathology of immune system organs be assessed in subchronic and chronic studies. FDA, in their immunotoxicity testing guidelines (U.S. FDA, 2002), also recommends the use of functional assays to assess the immune system of adult animals.

Roundtable participants suggested that, when possible, methods to assess DIT should be added onto existing reproductive/developmental toxicology protocols. It was agreed that histopathologic evaluation of immune organs could easily be

incorporated into existing reproductive/developmental protocols. Currently, neither EPA (U.S. EPA, 1998a,b) nor FDA (International Conference on Harmonisation, 1998) recommends histopathology of immune organs in guideline reproduction and developmental studies. It was agreed that histopathologic evaluation of immune organs could easily be incorporated into existing protocols. Despite this general agreement, there was much discussion around exactly what role pathology should play in assessing DIT. Some participants suggested routine histopathology was not sensitive enough to detect all potential immunotoxic effects, as noted in the recent article by Germolec *et al.* (2004). These participants argued that this may be particularly true for the developing immune system, and that more sensitive methods that assess immune function may need to be utilized. For example, thymus weight and limited histopathology of the thymus failed to detect the developmental immunotoxicity of lead, whereas immune functional assays indicated lead-induced alterations (Bunn *et al.*, 2001b). However, other participants felt that histopathology could detect immunotoxic effects, although more defined methods (specifically immune markers, grading, and image analysis) may need to be identified.

Roundtable participants emphasized the importance of capitalizing on already evaluated/validated assays and endpoints for assessing DIT. To this end, roundtable members agreed that an assay assessing the T-cell dependent antibody response (TDAR) was ready to be included in a DIT protocol. Either the plaque-forming cell (PFC) response (Holsapple, 1995) or an ELISA (Temple *et al.*, 1993) may be used to measure the antibody response to a T-cell dependent antigen (e.g., sheep erythrocytes (SRBC)). Several other assays (i.e., phenotypic analysis of immune cells by flow cytometry, macrophage function, and natural killer cell activity) previously discussed at the NIEHS/NIOSH workshop (Luster *et al.*, 2003) were also discussed. These assays were deemed to require further evaluation and validation before their utility in assessing DIT could be determined. Additionally, host resistance assays were not considered appropriate for a DIT screen (Holsapple, 2002), as these assays are considered to be a final tier of testing and are typically conducted only when data from a primary screen suggest alterations in immune parameters.

The question of whether different assays were needed to assess different developmental windows of vulnerability was also discussed. Participants acknowledged that required tests would likely vary by chemical, based on the specific questions that each agency (EPA versus FDA) needs to have addressed. Data on whether test procedures optimized in adult animals are useful in assessing the functionally immature immune system of young animals, however, are limited. Results of a study by Ladics *et al.* (2000) suggest that it may not be possible to measure an antibody response in rat pups due to the immature status of their immune cells, whereas data in rat weanlings (i.e., PND 21 or older) indicate that an antibody response to SRBC of sufficient magnitude can be measured with the PFC assay. Roundtable members agreed that in rats, PND 30 SRBC responses were

similar in magnitude to adult levels. Furthermore, although reports indicate that the delayed-type hypersensitivity (DTH) response can be assessed in weanling rats (Bunn *et al.*, 2001a), participants agreed that data are lacking as to whether cell-mediated immune (CMI) assessments in younger animals are feasible. The degree of variability that occurs in the antibody response over different ages (i.e., stages of development) of a rat was also discussed; however, participants acknowledged there were too few data available to address this issue.

Roundtable participants discussed whether inclusion of a TDAR, immune organ weights, and histopathology of immune system organs in the proposed DIT protocol was adequate for assessing DIT. Although data are limited, there are some examples of developmental immunotoxicants (i.e., lead and dioxin) that have been reported to affect only the CMI system (Bunn *et al.*, 2001a,b; Gehrs and Smialowicz, 1999; Miller *et al.*, 1998). It is important to emphasize, however, that the preferential alteration of CMI versus humoral immunity in the developing immune system was identified in a previous DIT workshop as an important data gap needing further investigation (Holsapple, 2002). Nevertheless, it was recommended at this roundtable and in previous DIT workshops that a CMI assay, such as the DTH, be considered in any proposed DIT protocol (Holsapple, 2002; Luster *et al.*, 2003). For measurement of CMI, roundtable participants suggested that a 'validated' DTH or T-cell responses to anti-CD3 be evaluated. Previous results (Bunn *et al.*, 2001a) have suggested that it is possible to avoid the use of a separate group of animals when assessing both the humoral and CMI responses.

#### *Data Interpretation*

It is important to emphasize that the roundtable discussion did not address the relevance of results from a DIT test to human clinical outcomes. As previously mentioned, the proposed DIT protocol is intended for hazard identification, not risk assessment. Furthermore, questions regarding the clinical relevance of results from developmental animal studies are not unique to DIT, but rather, should be addressed for all developmental and reproductive testing protocols (e.g., DNT). Nevertheless, some discussion regarding the applicability of results from animal studies to human health risk assessment were touched upon, although not resolved, at the roundtable.

While immunotoxicology screening tests have undergone a series of validation exercises, and it is established that immunosuppression can lead to an increased incidence and/or severity to infectious and neoplastic diseases, interpreting results from immunological tests in DIT studies, or even from epidemiological studies conducted in children for quantitative risk assessment purposes, is problematic. This is particularly true when the immunological effects, as might be expected to occur from inadvertent exposures, are minimal-to-moderate in nature. Thus, it is important that a scientifically sound framework be established that allows for more accurate and quantitative interpretation of such data in the risk assessment process. Although experimental

animal models provide an opportunity to establish more reliable exposure estimates and conduct more informative immune tests than human studies, extrapolating findings across species still requires the application of certain estimates or assumptions to account for differences in the integrity of the host's anatomical and functional barriers and the overall immunocompetence of an individual which can be affected by genetics, age, gender, use of certain medications, nutritional status, and environment (Morris and Potter, 1997).

### *Conclusions and Future Needs*

The roundtable addressed a number of research needs previously identified in other forums. These include a recommendation that all critical windows be addressed in one protocol and that the results trigger subsequent studies, as warranted; the need for flexibility in any approach to address DIT potential and to understand the role of exposure in study design; and the recognition that immunotoxicity is most appropriately addressed by functional tests. Several assays were discussed for inclusion in a DIT study based upon their common application in clinical immunologic evaluations and immunotoxicity testing in adults. These include macrophage function, complement analysis, and surface marker analysis. In addition to the research needs identified for these specific assays, there was some emphasis placed on the need to more fully examine the relationship between adverse immune system responses in animals and estimations of human immunologic risk.

Participants in the DIT roundtable emphasized a number of important research efforts currently underway in the area of DIT, which examine immune system responses following developmental exposures to known immunotoxicants. For example, an American Chemistry Council-funded research project involving two laboratories is evaluating whether the developing immune system shows differential susceptibility to chemical perturbation compared to the adult immune system (Dietert, 2003; Dietert and Lee, 2003; Dietert *et al.*, 2003, 2004; Matulka *et al.*, 2003). As well, studies are being conducted under the auspices of NIEHS to better understand the effects of endocrine disruptors on immune system development (Guo *et al.*, 2002; Karrow *et al.*, 2004). Inter-laboratory comparisons of such data will assist in further defining the standardization of methodologies used in DIT assessment.

Methods for immune system hazard characterization currently focus on assessing immune suppression, and to some extent hypersensitivity, in adult animals. However, there is no established assessment model for the induction of autoimmunity or immune system over-stimulation—both important potential immunotoxic responses. Furthermore, the inclusion of an assessment of CMI response in a DIT test guideline was recognized at the roundtable as desirable, yet there remains some uncertainty regarding the status of validation for this methodology.

Some methodological aspects of standardized DIT testing need to be further developed and supported through research.

For example, while it is widely recognized that pharmacokinetic data could be useful in establishing dose levels for a study, there is no consensus regarding what those preliminary pharmacokinetic studies should entail. Also, concerns about ensuring exposure of the offspring via direct-dosing need to be further explored to determine the most relevant and scientifically valid manner in which to approach this issue. Additionally, the manner in which studies can be combined should be examined in order to reduce the number of animals used in testing.

Further research is also needed to more effectively assess risks to developing humans. Foremost is a need to establish quantitative models to allow for accurate extrapolation of results from DIT screening studies to potential health effects (e.g., infectious disease, leukemia, etc.) in children. As well, more information is needed to determine whether an effect on an immune endpoint from an adult study can be applied to set safe levels in juveniles; specifically, information on expression of immune endpoints in juveniles is lacking. It is also not known whether juvenile humans are more sensitive to immune system perturbations than adults, although animal data have clearly identified age-related sensitivities for some chemicals (e.g., lead and dioxin). Additionally, the role of other factors in susceptibility to immune system perturbation is not known. These include polymorphisms in genes that are associated with immune system responses and the role of stress. Evaluations of the public health implications of immune system suppression will assist in further characterizing potential immunotoxic risks.

### ACKNOWLEDGMENTS

The authors thank the Immunotoxicology Technical Committee (ITC) at the International Life Sciences Institute, Health and Environmental Sciences Institute (ILSIHESI) for hosting the May 2003 *Developmental Immunotoxicology (DIT) Roundtable* in Washington, DC, and supporting the preparation of this manuscript. The authors also acknowledge their fellow roundtable discussion participants: Peter Bugelski (Centocor, Inc.), Miklos Csato (F. Hoffmann-La Roche AG), Rodney Dietert (Cornell University), Ellen Evans (Schering-Plough Research Institute), Dori Germolec (NIEHS), Helen Haggerty (Bristol-Myers Squibb Company), Danuta Herzyk (Glaxo-SmithKline), Dennis Hinton (U.S. FDA, CFSAN), Catherine Kaplanski (Merck Research Laboratories), Thomas Kawabata (Pfizer Global Research & Development), Hervé Lebrec (3 M Pharmaceuticals), Cynthia L. Mann (ExxonMobil Biomedical Sciences, Inc.), Barbara Mounho (Amgen, Inc.), Laurie G. O'Rourke (Novartis Pharmaceutical Corporation), Don O'Shaughnessy (D. O'Shaughnessy Consulting, Inc.), Marc Pallardy (Université de Paris), Richard W. Pfeifer (Wyeth Research), Lynnda Reid (U.S. FDA, CDER), Mary Jane Selgrade (U.S. EPA), Kimber L. White, Jr. (Medical College of Virginia), Daniel Wierda (Eli Lilly and Company), and Michael Woolhiser (Dow Chemical Company). The input of all involved was incorporated into the preparation of this manuscript. Finally, the authors thank Norbert Kaminski (Michigan State University) and Robert Luebke (U.S. EPA) for their careful review of the document as part of ILSI HESI's peer review process.

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