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Immunotoxicogenomics: The potential of genomics technology in the immunotoxicity risk assessment process

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Abstract

Evaluation of xenobiotic-induced changes in gene expression as a method to identify and classify potential toxicants is being pursued by industry and regulatory agencies worldwide. A workshop was held at the Research Triangle Park campus of the Environmental Protection Agency to discuss the current state of the science of “immunotoxicogenomics”, and to explore the potential role of genomics techniques for immunotoxicity testing. The genesis of the workshop was the current lack of widely accepted triggering criteria for Tier 1 immunotoxicity testing in the context of routine toxicity testing data, the realization that traditional screening methods would require an inordinate number of animals and are inadequate to handle the number of chemicals that may need to be screened (e.g., high production volume compounds) and the absence of an organized effort to address the state of the science of toxicogenomics in the identification of immunotoxic compounds. The major focus of the meeting was on the theoretical and practical utility of genomics techniques to 1) replace or supplement current immunotoxicity screening procedures, 2) provide insight into potential modes or mechanisms of action, and 3) provide data suitable for immunotoxicity hazard identification or risk assessment. The latter goal is of considerable interest to a variety of stakeholders as a means to reduce animal use and to decrease the cost of conducting and interpreting standard toxicity tests. A number of data gaps were identified that included a lack of dose response and kinetic data for known immunotoxic compounds and a general lack of data correlating genomic alterations to functional changes observed in vivo. Participants concluded that a genomics approach to screen chemicals for immunotoxic potential or to generate data useful to risk assessors holds promise, but that routine use of these methods is years in the future. However, recent progress in molecular immunology has made mode and mechanism of action studies much more practical. Furthermore, a variety of published immunotoxicity studies suggest that microarray analysis is already a practical means to explore pathway-level changes that lead to altered immune function. To help move the science of immunotoxicogenomics forward, a partnership of industry, academia and government was suggested to address data gaps, validation, quality assurance, and protocol development.

INTRODUCTION

EPA's 28 day repeated dose oral toxicity testing guidelines (U.S. EPA, 2000) mandate weighing and preserving the spleen, thymus and lymph node from all animals, and microscopic evaluation of lymphoid tissues from control and high dose animals for evidence of pathological changes. In combination with general biochemical indicators and complete blood count data, these endpoints constitute the only means to flag potential immunotoxicants for functional evaluation according to the OPPTS guidelines for immunotoxicity testing, with the exception of separate tests for contact sensitizing potential. Immunosuppressive environmental chemicals and drugs often, but not invariably, alter lymphoid organ mass or structure; however, statistical studies of results obtained in the testing of 50 environmental chemicals indicated that the predictive value of these observations is low as stand alone tests (Luster et al. '92). As such, there is not widespread agreement on which, if any, of the general toxicity guidelines measures provide suitable triggers for further testing using functional assays as described in the EPA OPPTS Immunotoxicity Testing Guidelines (U.S. EPA, 1998). Even if one assumes that these screening methods reliably detect potential immunotoxicants, relying on this approach will require an inordinate number of animals and may prove inadequate to handle the number of chemicals that may need to be screened, particularly high production volume compounds.

Evaluation of xenobiotic-induced changes in gene expression as a potential method to identify and classify potential toxicants is being explored by industry and regulatory agencies worldwide as a means to screen and prioritize chemicals for functional evaluation. The recent growth of the technology lead the U.S. EPA to organize an Agency genomics taskforce, which released a white paper addressing the potential impact of toxicogenomics on risk assessment and regulatory activity at the Agency (USEPA, 2004). Although the majority of research interest has focused on nonlymphoid tissues, immunotoxicologists have begun to investigate the use of toxicogenomics to detect and characterize chemical modulation of the immune response. A workshop was held at the Environmental Protection Agency in Research Triangle Park, NC to address the potential application of genomics techniques as an alternative and/or adjunct to traditional screening methods for immunotoxicity. The use of genomics techniques as potential screening tools for immunotoxicity and as a technique to identify mode or mechanism of action was discussed, as was the use of genomics data in the risk assessment process. Expert practitioners from other toxicology disciplines provided current state-of-the science overviews of toxicogenomics in nonlymphoid organ systems, summarizing the positive and negative aspects of a variety of techniques, data reproducibility, prerequisites for successful application of these techniques, and the predictive value of genomics data for functional changes. In addition, meeting participants met in breakout sessions to address specific issues regarding the utility of genomics in screening, identification of mechanism or mode of immunotoxicant action, and applicability of results to risk assessment. The meeting agenda and presentations are available at http://www.epa.gov/nheerl/immunogen_workshop/. The questions addressed by breakout groups, group chairs and group participants are available in the supplemental data file.

Use of genomics techniques to screen for potential immunotoxic effects

Current goals of toxicogenomics, which would also be relevant to immunotoxicology, include hazard identification by comparing microarray results with analyses of structure activity relationships or animal bioassays, risk characterization by coupling genomic data with population exposure assessment or cross-species comparisons (Dr. William Mattes, Gene Logic, Inc., Gaithersburg, MD. Studies such as the multi-site collaborative project, begun in 1999 and sponsored by the ILSI Health and Environmental Sciences Institute Genomics Committee (http://www.hesiglobal.org/Committees/TechnicalCommittees/Genomics/), provide a template that immunotoxicologists may apply to reach these same goals. The ILSI-

sponsored effort suggest that 1) biological pathways can be identified consistently across platforms in response to chemical treatment; 2) direct gene comparisons are challenging; and 3) genomic data alone are insufficient and should be anchored to a phenotypic marker. Quality assurance and control are critical to the generation of reliable data and must be addressed at the individual lab or facility level, and across labs and facilities if comparable data are to be obtained. Multi-institution projects that have compared gene expression on identical non-immune system samples, in multiple laboratories, on the same and different platforms, indicate that significant variability may be observed. To date, a complete understanding of sources contributing to this observed variability is lacking, but likely sources include such factors as operator, instrumentation, analysis tools, and gene annotation errors. Nevertheless, although detected changes in individual genes may vary, alterations in the biological pathways in which these genes are involved are usually consistent. Issues of cost, experimental design, dose and exposure kinetics, cross-species extrapolation and data quality and interpretation remain as major concerns that must be resolved before studies from multiple laboratories can reliably be compared and used to assess the utility of genomics techniques in evaluating chemical-induced alterations, including those of the immune system.

There is no universally accepted trigger to initiate immune function testing. However, expression profiling has been used successfully in other organ systems to identify and even to characterize toxicants (Steiner et al., 2004), and immunotoxicologists have employed gene expression analysis to identify modes of chemically-induced immunotoxicity (Pruett et al., 2004). The breakout groups concluded that while it is possible that immunotoxicologically definitive microarray results could be obtained in acute short term immunotoxicity studies, generalized toxicity associated with acute high dose exposures may confound data interpretation. Data are not available to support or refute this concern. Multiple doses/times of exposure below the maximum tolerated dose (MTD) and below the level which induces toxicity will be useful in distinguishing specific vs. generalized toxicity, as well as toxicity vs. adaptive changes. To date only a few laboratories have systematically investigated gene expression as a potential screening tool for immunotoxicants. Application of these methods was exemplified by the presentation of concurrent analyses of immune function and gene expression in the spleen and thymus of B6C3F1 mice exposed to the prototypical immunotoxicants 2,3,7,8-tetrachlorodibenzolo-*p*-dioxin, cyclophosphamide, cyclosporin A or dexamethasone (Rachael Patterson, Environmental Immunology Laboratory, NIEHS, RTP, NC). The primary goals of these studies were to examine differential gene expression in the thymus and spleen following chemical exposure, to correlate those changes with functional immune endpoints and known mechanisms of action, and to compare the genomic responses in thymus and spleen. Animals were exposed to vehicle or immunotoxicant for 5 days and subgroups were dedicated to functional or genomic analysis. RNA isolated on day 6 from the thymus and spleen was analyzed using a custom Illumina Sentrix Array Matrix (SAM). Genes modulated by all four chemicals in thymus included: upregulation of Apoe, caspase 1, interferon gamma (IFNγ) inducible protein 16, pre-B cell enhancing factor 1, lymphocyte antigen 6a, and downregulation of cyclin D3, CDK2, T-cell transcription factor, T-cell activation linker, and IFNγ receptor. Transcriptional effects induced by the four chemicals were more different than similar. Many of the genes differentially expressed in thymus are known to play a role in apoptosis, responses to biotic stimulus, immune cell proliferation and activation, and inflammation. The findings were consistent with observed alterations in immune function. Genomic analysis revealed several gene expression changes that may be commonly associated with xenobiotic-induced immune system perturbations, as well as distinct gene profiles that may be related to chemical-specific cellular targets and modes of action. The experimental design used in these studies may serve as a prototype for others to follow when evaluating transcript profiling as a screening method for immunotoxicity.

One of the most likely immune mediated adverse effects of chemical exposure is hypersensitivity. Dr. Lucy Gildea (The Procter and Gamble Company, Miami Valley Innovation Center, Cincinnati, OH) described the development of an in vitro approach to detect potential contact allergens by following gene expression in cells exposed to a known strong sensitizer or irritant. Changes in gene expression by dinitrosulphobenzene-activated dendritic cells generated from CD14+ adherent human mononuclear cells identified 29 candidate target genes important in the response to sensitizing agents (Ryan et al., 2004). Further evaluation using a range of weak, moderate and strong chemical allergens, and some irritants, narrowed the list of target genes and prioritized expression patterns for these genes. Genes associated with sensitizers included receptors for cytokines and chemokines (e.g., CCL23) and cell surface receptors/membrane proteins (e.g., NOTCH3, CD1E). Validation of the method awaits further testing, although initial results suggest that ultimately, differentiation of irritants and sensitizers may be accomplished without animal testing. Based on the progress made to date, the breakout groups suggested that comprehensive arrays may be useful to detect sensitizing chemicals, although widespread application of the technique is in the future. Furthermore, if sufficient data can be obtained on the genomic profile of sensitizers, then it might be possible to differentiate respiratory and contact sensitizers. While there may be potential for predicting either respiratory or contact hypersensitivity, optimal experimental designs would need to be established, including tissue(s) to be examined and timing of analysis after sensitization. Although the local lymph node assay (LLNA) is a sensitive method for detecting contact sensitizers, it requires the use of radioisotopes and separate groups of animals. Development of reliable array-based detection methods could facilitate analysis of sensitizing potential in a 28 day study, eliminate the use radioisotopes, and meet the ICCVAM goals of developing assays that meet the criteria of Reliability, Repeatability and Reproducibility. Furthermore, linking positive results of array-based screening for hypersensitivity potential with histopathology might be helpful in validating this screening method.

Once lists of candidate genes are identified, an ideal screening method should give comparable results in humans and laboratory animals, allowing for direct comparison of laboratory animal data and clinical endpoints in exposed populations. The utility of genomics as a screening technique in humans was demonstrated by microarray analysis of peripheral blood samples obtained from welders before and after exposure to welding fume, and compared gene expression to nonexposed controls (Dr. David Christiani, Harvard School of Public Health, Harvard Medical School). Transient gene expression alterations induced by short term metal particulate exposure could be detected in whole blood total RNA. The association of genes clustered in the functional pathways related to inflammatory and immune responses in particulate-exposed individuals suggests that respiratory exposures have systemic consequences that may be evaluated using gene expression changes detectable in circulating human mononuclear cells (Wang *et al*. 2005).

The studies presented at the meeting and discussed above are an important first step in linking changes in gene expression with changes in immune function. What must follow are studies that incorporate both functional analysis and gene expression, across platforms and with various classes of immunosuppressive agents, in order to develop a better understanding of the predictive value of expression data for changes in immune function, and the factors that are critical to the successful use of immunotoxicogenomics as a screening tool. To this end, the increased inclusion of genomics as an endpoint in screening studies should be encouraged, and a database should be compiled from immunotoxicology studies that include genomics data. Both primary (thymus and bone marrow) and secondary (spleen, and blood) sources of lymphoid cells should be evaluated if possible. Peripheral blood is the only source of easily obtainable lymphoid cells in humans, and while the relative proportion of circulating lymphocytes differs in mice and humans, these cells provide the most convenient means of cross-species comparisons. The breakout groups concluded that it is also important to evaluate

effects of chemical exposure in animals at immunologic steady state and in immunized animals. Immunization stimulates a cascade of transcriptional and translation activity and may therefore maximize the likelihood of detecting chemical effects on expression of genes that are up- or downregulated during the innate and adaptive phases of the response. However, the “optimal” study design to detect adverse affects remains a significant question and a panel of genes that are predictive of responses when the immune system is challenged, and are representative of effects on cell-mediated, humoral-mediated and innate immune responses, will first have to be developed and validated.

Initial studies need to incorporate multiple doses, including low environmentally relevant doses, and evaluation of both early and late time points after exposure, to ensure that the association between changes in gene expression and functional endpoints are linked. (Toxicogenomic evaluations in other tissues suggests that early time points may show the largest number of gene changes.) The use of tissues from standard toxicity testing designs for genomic screening is attractive from an animal use standpoint. However, there is little evidence that this would be a cost savings versus the use of more traditional methods for evaluating immunotoxicity, as genomics studies are still comparatively expensive. Both positive and negative controls should be included in database building and validation studies. In order to ensure that the data is useful for risk assessment purposes there should be a formal effort to examine the comparison between in vivo and in vitro studies, using multiple rodent species and both rodent and human cell lines. In deciding whether to conduct in vitro or in vivo exposure studies, it was noted that cells may dedifferentiate in vitro and lose interaction with neighboring cell types that might be important in expression of immunotoxicity. However, genomics-based toxicity data in other organ systems suggest that some chemical signatures can be interpreted correctly by transcript profiling of exposed primary cells (Calvo *et al*., 2002).

It was recommended that some type of publicly available database be generated through either industry, academic and/or government partnerships, with rigorous criteria for study inclusion. Data should be taken from studies using standardized platforms and protocols. Issues such as analysis strategies (fold-induction versus other indicators of regulation) and reproducibility will need to be addressed. It was suggested that while academic and government laboratories might be efficient at generating the samples to be utilized in genomic analysis, contract laboratories or centers specializing in the conduct of gene arrays would be the best places to generate consistent, high quality data for use in a database to identify the genes critical to predicting immune toxicity.

Use of genomics techniques to determine mode or mechanisms of immunotoxicant action

The workshop addressed the use of genomics techniques to investigate mode or mechanism of immunotoxicant action. The number of genes that would be necessary to differentiate the complexity of the immune system could be in the hundreds, but may be significantly greater. However, it was suggested that rather than focus on individual genes, the data should be evaluated at the pathway level. Using this strategy, it may be possible to exclude responses not specifically targeting immune function, such as apoptosis, necrosis and acute phase responses. While these endpoints may ultimately result in alteration of immune function, they could result from mechanisms not specific to the immune system.

Genes and regulatory cis-elements were identified that were expressed in immune tissues (spleen, thymus, lymph node and peripheral blood mononuclear cells) from stimulated and non-stimulated mice (Dr. John Hutton, Director of Biomedical Informatics, Cincinnati Children’s Hospital). From a hierarchical clustering of 8734 expressed sequences, 680 genes were overexpressed in lymphoid tissues (Hutton *et al*., 2004). After subtracting genes with high expression in nonlymphoid tissues, 360 genes were identified with preferential expression

in immune tissues. These genes were associated with immune or defense responses, receptors or cell signaling, apoptosis, transport, adhesion or chemotaxis.

As an alternative to using an identified suite of genes to probe for mode or mechanism of action, genomics techniques may be used as a discovery tool to identify unanticipated pathways which impact the immune system. For example, Dr. Steven Pruett (Dept. Cellular Biology & Anatomy, LSU Health Sciences Center, Shreveport, LA) determined that acute ethanol exposure suppressed macrophage mediated resistance to bacterial peritonitis in B6C3F1 mice. Microarray analysis of these cells determined that ethanol inhibits Toll-like receptor signaling by preventing receptor clustering, a key initial event in pathogen recognition and destruction. These studies suggest that simultaneous monitoring of diverse pathways, and integrating these data with immune-specific measures, will lead to a more comprehensive understanding of mechanisms of immunotoxicity.

While commonly expressed changes in signature genes may indicate common inflammatory mechanisms, and these common events may be useful to screen chemicals for asthma-inducing potential, multiple effector pathways may be responsible for the phenotype. Although similar exposure regimens to ovalbumin (OVA) or trimellitic anhydride (TMA) resulted in similar asthmatic phenotypes (Dr. Jean Regal, Dept of Biochemistry & Molecular Biology, University of Minnesota), these prototypical respiratory allergens evoke unique gene activation and mechanistic pathways in the effector phase of asthma (Greene, *et al*., 2005). Differentially expressed genes in the lungs of BALB/c mice included arginase 1, Gatm and Ddah2, that were phenotypically linked to changes in arginase enzyme activity. These results suggest that OVA may operate through an airway remodeling pathway, while TMA causes bronchodialation and production of reactive nitrogen species.

Ultimately, application of genomics techniques to understand mode or mechanism of action will require the melding of genomic analysis with the outcome of functional studies. These proof of concept studies were not adequately discussed; however, the breakout groups suggested that tissues could be collected in the course of on-going functional immunotoxicity studies and shared with investigators who have access to genomic core facilities, allowing gene expression and immune function to be analyzed in the same animals.

Use of immunotoxicogenomics data in the risk assessment process

The EPA Genomics Task Force White Paper identified risk assessment as one of four areas that are “very likely to be influenced” by genomics data, generated within or submitted to the Agency (US EPA, 2004). Before genomics data can be used in immunotoxicity risk assessment, an additional issue that must be overcome is a lack of agreement on how relatively small, but statistically significant changes in the immune response in laboratory animals correlate to potential alterations in humans. In addition to changes observed in vivo, the complexity of the risk assessment equation is likely to be significantly amplified when one must also consider relatively small changes in numerous genes or proteins. To date, immunotoxicology risk assessment has not been performed using array data. However, toxicogenomic data have occasionally been included in the submission package for evaluation of pharmaceutical products. Genomics data are less common in toxicity studies; there has been a single case where toxicogenomic data was submitted as part of a pesticide re-registration package and considered as part of the general weight of evidence for evaluation of mode of action of Acetochlor in the induction of nasal olfactory epithelium tumors in rats, as part of the cancer risk assessment. The potential of use of genomic analysis to characterize risk based on mode of action is exemplified by studies that distinguished a carcinogen from a noncarcinogen (Dr. Douglas Wolf, U.S. EPA, Environmental Carcinogenesis Division, RTP, NC). Formaldehyde and glutaraldehyde both induce hyperplasia, squamous metaplasia, inflammation and apoptotic bodies following nasal instillation in F344 rats. However, formaldehyde induces fewer and

different apoptosis-associated genes, along with greater DNA repair genes than gluteraldehyde in the nasal epithelium (Hester *et al*. 2005). Furthermore, the use of genomics to evaluate parallel modes of action in human and animal cells for arsenic and dimethylarsinic acid might be useful to predict interspecies mechanism(s) and responses (Sen *et al*. 2005). Currently, a case study is being conducted within EPA using dibutyl phthalates to understand whether incorporation of toxicogenomics data would provide a more robust characterization of mode of action, tissue concordance, interspecies extrapolation and/or low dose extrapolation. The Food and Drug Administration (FDA) is accepting toxicogenomics data, on a voluntary basis, to better characterize the toxicity of therapeutics. FDA has also used toxicogenomics information for label changes of certain anticancer drugs. With respect to immunotoxicity, gene array data derived from dendritic cells is being explored as part of a hazard assessment strategy for allergic contact dermatitis Ryan *et al*., 2004). Given the pivotal role of dendritic cells in initiation of all types of immune responses, results of these studies may have applications to other types of immunotoxicity. Genomic array data may also hold promise as a means to determine whether a particular pathway that controls the toxicity of a chemical in an animal model is present or absent in humans.

SUMMARY AND CONCLUSIONS

Expression profiles generated by microarray analysis should reflect changes in functional assays or outcomes (e.g., the Local Lymph Node Assay to predict contact dermatitis; the antibody response, NK assay, cell surface markers and histopathology to predict immunosuppression). To clarify the connection between changes in gene expression and functional endpoints, it is critical to define an appropriate chemical set to evaluate and to choose the appropriate cell type(s) as a source for mRNA. The association (concordance) between genomic and traditional immunotoxic endpoints still needs to be established, although our understanding of the immune system at the molecular level should eventually allow us to do this. Genomics has been used successfully to study mechanisms of action (specific pathways associated with immunotoxicity of a specific chemical). However, other than studies conducted at the National Toxicology Program, genomics techniques have not been evaluated as a screening tool for immunosuppressants. At present, autoimmunity, because of its complexity, does not lend itself to interpretation using arrays. Additional studies will be required in order to establish the relevance of genomic alterations observed in vitro or in laboratory animals to humans as well as wildlife. This would include testing a variety of known positive and negative chemical entities and various controls. Specific genes or groups of genes will need to be identified that are predictive of immunotoxicity. Consideration should be given to both primary (thymus, bone marrow) and secondary (spleen and blood) lymphoid organs before and after immunologic challenge (immunization). Peripheral blood as a source of immune system cells was considered to be valuable, as this approach will allow comparison with humans. Consideration should be given to both early and late time points as well as multiple rodent species and wildlife species.

A recurring theme in the presentations and breakout group discussions was that the use of genomics techniques to screen chemicals for immunotoxic potential or to generate data useful to risk assessors holds promise, but that routine use of these methods is years in the future. Nevertheless, participants agreed that it is a goal worth pursuing and that financial resources devoted to research would be a worthwhile investment. Recent progress in molecular immunology has made mode and mechanism of action studies much more practical. A variety of published immunotoxicity studies suggest that microarray analysis is already a useful method to explore pathway-level changes that lead to altered immune function. Regardless of the investigator's intent (screening or mechanistic studies), quality assurance and appropriate statistical analyses are critical for generating reliable, reproducible data. Speakers and breakout groups agreed that establishing a publicly available immuntoxicogenomics database is critical

to moving the science forward. Such a database could be developed by including microarray analysis of multiple immune system tissues as part of immunotoxicity studies that include more traditional endpoints, providing a solid link between changes in gene expressions and functional data. It is also important to evaluate a set of known positive and negative immunotoxicants in conjunction with immune system challenge (immunization or sensitization) to clarify true changes in function from adaptive responses. Finally, it was suggested that building partnerships among industry, academia and government laboratories would provide a critical boost to developing practical and reliable protocols.

In summary, while genomics may, at some point, prove to be a predictive tool for the screening of compounds that target the immune system, the basic data needed to establish the relevant genes and the most efficient study design are not yet available. As suggested by the data on distinguishing contact sensitizers from nonsensitizers, it may be possible to utilize a relatively small number of genes to predict certain immune effects; as responses to pathogens or neoplastic cells may involve multiple cell types, second messengers and target tissues, any strategy will need to take into account the complex nature of the immune system.

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