6 The Promise of Genomics and Proteomics in Immunotoxicology and Immunopharmacology

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GENOMICS IN IMMUNOTOXICOLOGY

Introduction to Genomics and Single Nucleotide Polymorphisms

Genomics

There are a number of examples in which histopathology and the functional immunotoxicity tests recommended by regulatory guidance documents would not detect known

immunotoxicants.[1-3] Furthermore, we are no better able today than we were 20 years ago to quantitatively estimate the effect that a particular amount of suppression of immune parameters will have on resistance to infections or cancer.[1,2] In addition, very few mechanisms of immunotoxicity have been fully characterized. The incorporation of genomics and proteomics in immunotoxicology studies has the potential to impact all of these issues. Genomics and proteomics have stimulated the development of systems biology, and this field is remarkably consistent with the goals of immunotoxicology, "Systems biology studies biological systems by systematically perturbing them (biologically, genetically, or *chemically*); monitoring the gene, protein, and informational pathway responses; integrating these data; and ultimately, formulating mathematical models that describe the structure of the system and its response to individual perturbations."[4] Progress toward analogous goals in other areas of toxicology[5-9] suggests that these goals are feasible and that the results will be useful. Thus, in spite of valid concerns about difficulties in determining the functional significance of complex changes in gene expression, it seems that there are reasons to proceed with the use of microarray technology in immunotoxicology.

Genomics includes the study of DNA sequences of organisms, including coding and non-coding DNA sequences and their location on chromosomes. Quantitative measurements of gene expression, as indicated by the quantities of specific mRNA, are also generally regarded to fall within the definition of genomics, but the term transcriptomics is also used. A wide variety of microarrays for measuring gene expression are now available commercially. These range from arrays focused on one biological process to arrays that incorporate virtually the entire genome of a mouse, rat, or human. They consist of a substrate divided into equal sized segments. To each segment multiple copies of a unique oligonucleotide sequence (referred to as probes) are attached. In most cases, sample preparation for quantitative analysis of gene expression is relatively simple, and processing involves isolation of cellular mRNA, production of cDNA, production of cRNA from the cDNA, and fragmentation of cDNA to generate segments within the size ranges that can more effectively bind to complementary probes on the microarray. During cRNA synthesis, a label (often biotin) is incorporated, and the quantity of cRNA binding to the probe in a particular region of the microarray is measured after addition of fluorescent-labeled avidin. The fluorescence is quantified using an optical reader, and the results are normalized to account for background fluorescence.

A wide range of software is available to analyze microarray results for expression of individual genes and sets of genes in particular biological pathways. Some of the more popular packages (e.g., Genespring) can perform multiple analyses including: identification of genes for which expression is similarly altered, categorizing altered gene expression to indicate which pathways or broad areas of cellular function are likely to be affected, as well as statistical analysis to indicate which changes are significant. A number of open source (free) software packages are available as well.[10]

In general, quantitative gene expression results obtained with microarray analyses correspond well to results obtained using other methods.[11] However, quantitative data from microarrays are typically less reliable for genes that are very highly or very poorly expressed.[12] In addition, microarrays are much less sensitive than real time PCR and RNAse protection assays.[13] However, the ability to analyze most of the mRNA spe-

cies (the transcriptome) in human, mouse, or rat cells in a single experiment can be remarkably useful.

Databases for microarray data have been established, such as the gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). A standardized format for reporting microarray data has been developed, and it is referred to as minimum information about microarray experiments (MIAME) (http://www.mged.org/Workgroups/MIAME/miame checklist.html). However, the cost of microarray experiments remains a major impediment in the use of this technology by most investigators. For other investigators, the cost precludes the use of sufficient numbers of replicates to allow optimum statistical analysis. Many investigators have chosen to deal with this issue partly by pooling samples from multiple animals for microarray analysis. However, a recent study provides objective evidence that this approach causes a substantial number of meaningful changes in gene expression to be missed.[14]

There are some additional practical issues with regard to microarray studies of the immune system that deserve further consideration and discussion. For example, most target organs affected by toxicants are bounded by a defining membrane, and they are comprised of a predominant cell type. In contrast, the immune system consists both of distinct organs and widely distributed cells found throughout the body. In most cases, a variety of cell types are present in lymphoid organs, and functional changes would depend on the cell type in which particular changes in gene expression were measured. For purposes of immunotoxicity screening and for exploring mechanisms of action, determining which organ and cell type(s) to evaluate may require a substantial initial investment by the research community as a whole, but the results could be incorporated in a database that would allow rational selection of the cell type(s) for study in future experiments.

Single Nucleotide Polymorphisms

Genetic biomarkers of susceptibility, which are considered DNA sequence variations, determine a large part of an individual's variability of response to chemicals or drug treatment as well as in the development of many common diseases. These variations fall into two categories; genetic polymorphisms or genetic mutations. A *polymorphism* is a DNA sequence variant that has a frequency of 1% or greater in a population. If the frequency of a sequence variant is less than 1%, the variant is regarded as a *mutation*. There are several types of polymorphisms in the genome including repeat polymorphisms and insertions or deletions but most of the DNA sequence variation is in the form of single nucleotide polymorphisms (SNPs), which result from single base changes occurring at a frequency of approximately once per 300 base pairs. The human genome contains approximately 10 million SNPs, however, the vast majority do not alter gene structure or function and, thus, are unlikely to be associated with phenotypic changes. Those that do affect phenotype can be referred to as *functional* polymorphisms. Functional SNPs are most likely located in the coding region (cSNPs) where they affect protein structure or function or the regulatory/promoter regions (rSNP) where they affect expression levels, timing, or location. Although SNPs in non-coding regions of the genome have no influence on phenotype, they can be used as markers in genetic association studies. Only about 1% of identified SNPs alter an amino acid in a protein

and less than that affect expression. However, considering the large number of SNPs that exist in the human genome, an individual is likely to have many SNPs that affect gene expression.

The potential applications of SNP studies include gene discovery and mapping, disease association-based candidate polymorphism testing, pharmacogenetics, diagnostics and risk profiling, homogeneity testing, and the prediction of response to environmental stimuli.[15] There are advantages of employing SNPs to identify the genetic components of complex human diseases. SNPs are frequent, stable and distributed throughout the genome. They also exhibit linkage disequilibrium (LD) and haplotypic diversity that can be used for SNP mapping, which is useful for the identification of the variants that are associated with traits. Haplotypes are a group of neighboring SNPs in a region of a chromosome. Since the risk associated with any individual variant is small, haplotype analyses are a more effective approach to evaluate the combined effects of these neighboring variants. LD is the nonrandom association between alleles at different loci due to their proximity on the same chromosome. SNPs in LD with the true causal allele can also be used to identify susceptible individuals.

Linkage analyses are useful in the identification of genes responsible for monogenic traits such as cystic fibrosis. Although this approach can search susceptibility genes over the entire genome, the detection power is low, as it detects only genes that are highly penetrant. Association studies using SNPs, on the other hand, provide a powerful method to identify variants that may increase the risk of complex diseases because they have greater statistical power to detect genes with small effects. While several experimental designs are available for assessing potential associations between SNPs and disease, the case-control association study, due to its relative simplicity, is the most widely used design for detecting common disease alleles with modest risk.[16] Most case-control studies use a candidate-gene approach, which evaluates associations between specific genetic variants and a disease. These susceptibility variants are hypothesized to directly influence an individual's likelihood of developing disease. SNPs, with frequencies of at least 5% or greater in the general population, are more likely to be useful in candidate gene studies.[17]

Overview of Results Relevant to Immunotoxicology

Genomics

Microarray technology has been used primarily in immunotoxicology to evaluate the effects of pharmaceuticals. For example, genes coding for IL-1 and for pro-inflammatory chemokines were found to be induced by the anti-fungal drug, amphotericin B, in a monocyte cell line and freshly isolated human peripheral blood mononuclear cells.[18] Dexamethasone, a widely used anti-inflammatory drug, decreased the expression of a number of inflammation-related genes, but unexpectedly increased the expression of others (e.g., immunoglobulin Fc receptors and receptors for several cytokines and chemokines) in human monocytes.[19] Montelukast, a leukotriene antagonist, affected a variety of immune-related genes in a complex manner, as would be expected for a compound that inhibits an endogenous immune/inflammatory mediator.[20] Such inves-

tigations have been informative and have identified some genes not previously known to be regulated by the drug in question.

One of the more interesting and potentially useful applications of microarray technology is illustrated by its use in a fifth generation pharmacokinetic/pharmacodynamic model for the effects of pharmacological dosages of synthetic glucocorticoids.[21] This approach expresses the mechanisms by which glucocorticoids act to modulate gene expression in mathematical terms, using differential equations to express changes over time in gene expression, protein synthesis, and protein degradation. It has revealed groups of genes that are co-regulated and exhibit remarkably similar changes in expression over time. This approach will likely yield important information about the side effects of glucocorticoids and a deeper understanding of the immunological effects, including changes in lymphocyte and neutrophil trafficking, which can be predicted by the model.[22] This type of comprehensive approach should ultimately be useful in identifying genetic programs associated with resistance to infection and with septic shock syndrome and allow prediction of the effects of immunotoxicants on these important outcomes.

A number of studies have also involved the use of microarrays as tools for the evaluation of mechanisms of immunotoxicology. For example, toxic effects of ricin,[23] hexachlorobenzene,[24] and nickel,[25] revealed an unexpected role for inflammatory processes. These findings illustrate the value of microarray analysis in identifying inflammation as a mechanism involved in the toxicopathology following exposure to a particular compound and highlight the underestimated importance of inflammatory processes in immunotoxicity.

Recent results also demonstrate the utility of microarrays in detailed mechanistic studies in immunotoxicology. The immunosuppressive and anti-inflammatory effects of acute ethanol exposure have been extensively documented in humans[26,27] and in animal models.[28] Recent studies suggest that this may be mediated in part by inhibition of toll-like receptor (TLR) signaling.[13,29] However, it was not clear which aspects of signaling were particularly important in the effects of ethanol, and the complexity of TLR signaling and consequent responses made traditional reductionist approaches impractical. Thus, we utilized the more global evaluation of effects that can be obtained by microarray analysis. The results strongly suggested that a self-amplifying signaling loop involving Type I interferons is an important target of ethanol and may explain much of the suppression of pro-inflammatory mediators induced through TLR 3 in mouse peritoneal macrophages (Table 6.1).[13] There was no reason to suspect that this self-amplification loop was a central target of ethanol, and it is not clear if this would have been detected using other methods.

A similar approach involving pathway-specific gene arrays has been used to identify genes that may be involved in the induction of apoptosis in the thymus by dioxin.[30] The role of immunosuppression in decreased resistance of mice to tumor cells following administration of Δ-9 tetrahydrocannabinol was indicated by a microarray study.[31] In another informative study, microarray analysis indicated that suppressor of cytokine signaling 2 (SOCS-2) is upregulated by dioxin in B lymphocytes and that this may well explain some of the immunosuppressive effects of this compound.[32] Again, the complexity of the biological system under investigation in each of these cases probably precluded the use of a strictly reductionist approach to evaluate individual proteins that

TABLE 6.1.

Molecule	Effect of Ethanol
MAP Kinases, NF-κB, and AP-1	Decreased activation
GIF-1, Nmi, STAT-1*	Decreased gene expression
ISGF3-γ*	Decreased gene expression
IRF-7*	Decreased gene expression
Type I Interferons*	Decreased gene and protein expression
Chemokines	Decreased gene and protein expression

*These components are part of a self-amplifying signaling loop in which activation of initial transcription factors leads to low level IFN production, which activates STAT-1. Along with other components, STAT-1 forms ISGF3, a transcription factor that drives expression of IRF-7, which in turn leads to increased expression of Type I interferons, which decreases further STAT-1 activation and thereby diminishes the amplification of the signaling loop. The decrease in ISGF3 function is also apparently sufficient to decrease expression of several chemokines (most notably CXCL9), which are dependent on it. These results are derived from a previously reported study. [13]

might be involved. Thus, the use of microarrays would seem to be an excellent early opportunity for initial mechanistic studies of immunotoxicants.

Single Nucleotide Polymorphisms

In pharmacology and toxicology, the focus of SNP studies has been on their role in chemical/drug detoxification and metabolism, including pharmacogenetics, and to a lesser extent receptor binding or expression of biological mediators. The primary goal of these efforts is to identify the genetic bases for interindividual variations in sensitivity or resistance to a drug or chemical. Chemical/drug responses are complex and, in addition to genetic factors, are complicated by dose, drug interactions, environmental factors, and diet. Therefore, individual genes may have only a small effect on the response. Pharmacogenetic studies have focused mostly on metabolizing enzymes.[33] In addition to metabolizing enzymes, there are reports showing the influence of cytokine and HLA polymorphisms on drug responses. For example, the IL-10 (-1082) and TGFβ1 (+29) variants were reported to be associated with resistance to combined antiviral therapy.[34] The polymorphic TNF-α2 microsatellite and TNFα (-308) allele are associated with a risk of chemotherapy-induced pulmonary fibrosis and severe carbamazepine hypersensitivity reactions, respectively.[35,36] Major histocompatibility complex (MHC) genes, (HLA-B57, HLA-DR7, and HLA-DQ3) are associated with adverse reactions to Abacavir, a reverse transcriptase inhibitor used in HIV/AIDS treatment.[37,38] Recently, TNFα -857C/T SNP was found to be a genetic marker for predicting the response to Etanercept in rheumatoid arthritis patients with individuals possessing the T allele responding better to therapy.[39] Other examples of therapeutically relevant polymorphisms can be found as well.[40–42] Applications of SNPs in pharmacology not only improve drug efficacy and reduce drug toxicity, but also dramatically change the approaches in pharmacological interventions.

Efforts to incorporate SNP studies into environmental/occupational epidemiology investigations have focused on examining hypothesis-driven associations between exposures and specific polymorphisms. Most common human diseases such as asthma,

TABLE 6.2

Disease	SNPs	References
Asthma (including occupational)	TNFα -308 GSTP1 Ile105Val	78 79
Alcohol and chemical-induced Hepatitis	TNFα -308, -238 IL-1β +3953, -511	80 81
Cancer (benzidine-induced)	GSTP1 Ile105Val	82
Chemical-induced neurotoxicity	IL-1α -889 TNFα -308	83 84
Chronic beryllium disease (CBD)	TNFα -308 HLA-DPB1(Glu69)	85 44
Chronic obstructive pulmonary disease (COPD)	TNFα -308 TGFβ codon 10	86 87
Coal workers' pneumoconiosis (CWP)	TNFα -308	88
Silicosis	IL-1RN +2018 TNFα -238, -308	89 43

cancer, or cardiovascular diseases are multigenic and multifactorial in nature involving interactions between genetic, physiological, and environmental factors. Therefore, in addition to exposure assessment and genetic factors associated with chemical metabolism, SNPs associated with mediators involved in disease initiation and progression need to be considered. In this respect, genetic markers related to the immune system have been identified in several exposure-related diseases such as TNFα -238, -308 in silicosis and HLA-DP Glu69 in chronic beryllium disease (CBD).[43,44] In silicosis, for example, proinflammatory cytokines, such as TNFα and IL-1 have been implicated in the formation of fibrotic lesions. A strong association was found between disease severity and the frequency of the TNFα -238 variant.[43] Recent studies investigating the contribution of HLA alleles to disease processes revealed an association between HLA-DPB1 (Glu69) variation and CBD.[44] Workers with CBD and sensitization were found more likely to be homozygous DPB1 (Glu69) compared to workers without disease or sensitization. Vaccine efficacy to hepatitis B was also reported to be influenced by the IL-1β +3953 variant and UVB exposure found to suppress hepatitis B virus antibody responses in individuals with this variant.[45,46] Genetic modifiers are known for a number of common complex diseases where immune mediators and environmental factors play a role. Table 6.2 provides further examples of associations between SNPs and environmental/occupational diseases of an inflammatory nature.

Future Directions and Challenges

Genomics

The utility of microarrays in the pharmaceutical industry has been questioned recently.[47] Undoubtedly, initial expectations expressed by some investigators were so high that there has been some disappointment that these expectations have not been realized. However,

recent results for other target organs of toxicity, such as liver, clearly demonstrate the potential power of this methodology.[5,6,8] For example, microarrays have been used to identify "signatures" of particular chemical toxicants in the liver.[8] However, the use of microarrays (along with proteomics and other approaches) to understand how cells of the immune system respond to toxicants and how this affects their functions has not progressed as rapidly as in other tissues, such as liver. This may reflect the diffuse nature of the immune system and the multiple cells types that must be considered. Technical issues such as purification of a particular cell type before assessing gene expression are important in immunotoxicology research.

The current practice of using a T-cell-dependent antibody response or natural killer cell function is based on analyses suggesting that these parameters would suffice to identify the vast majority of immunotoxicants.[1,2] However, it is clear that there are exceptions[1,2] and that some immunotoxicants are not effectively identified by these functional tests or by histopathology.[3] Thus, it remains possible, particularly if the cost of microarray analysis decreases, that microarrays will eventually prove to be the most practical method available to identify immunotoxicants.

Single Nucleotide Polymorphisms

In recent years, epidemiology studies have often incorporated tests to identify genetic variants that influence disease susceptibility or drug responses. New pharmacogenetic approaches can offer personalized treatment by targeting predictive diagnostics and therapeutics to the genetic profile of a patient, in addition to providing novel insights into adverse drug reactions and drug efficacy. The practice of identifying individuals reacting differently to a specific therapy could be extended to xenobiotic exposures since responses to environmental and occupational stimuli are also influenced by the genetic background. In this respect, recent advances in toxicogenetics and pharmacogenetics will help to identify individuals or populations at risk, define many common disease subtypes more precisely and also help to understand disease mechanisms.

PROTEOMICS IN IMMUNOTOXICOLOGY

Introduction to Proteomics

The first section of this chapter discussed the discipline of genomics, which considers the total nucleotide sequences of an organism, including structural genes, regulatory sequences and noncoding DNA segments. Rapidly growing interest in functional genomics has led to recognition of the need for enhanced proteomic analyses, including the total protein product of an organism's genome. Proteomics thus emerged as a new discipline that focuses on the study of expression, structure, biochemical activity, localization, interactions, post-translational modifications, and cellular roles of as many proteins as possible.

There are fundamental differences in the information that can be collected by proteomic versus genomic profiling. The proteome (total set of proteins expressed

by an organism) is considerably larger than the genome (total complement of genes), since multiple proteins may be encoded by a single gene. For instance, an average of more than 10 proteins are encoded by each gene in humans.[48] The protein products of individual genes may correlate poorly with respective mRNA transcripts, meaning that gene expression level may not be a reliable predictor of total translated protein. Actual protein levels may be transcriptionally controlled (e.g., Met, Arg and Leu biosynthetic pathways) and correlate well with gene expression, or post-transcriptionally controlled, as in golgi-associated coat protein I (COPI) or ribosome protein complexes.[9] Post-translational modifications of proteins are then common (over 100 known), may be of considerable functional importance, and cannot be predicted by genome sequence. These include glycosylation, phosphorylation, methylation, and oxidation/reduction reactions, and may occur in multiple splice variants per gene. Further complicating interpretation of global protein expression changes, each protein participates in an estimated 5–10 interactions with other proteins.[49] The 30,000 to 60,000 estimated genes in the human genome therefore encode millions of proteins when post-translational modifications and alternative splicing are included, with multiple millions of potential interactions. A major strength of proteomics therefore is the potential to reveal information about relative protein abundance, important functional post-translational modifications, effects of alternate splicing on protein structure or function, and protein-protein interactions that cannot be obtained in a typical genomic analysis (for an in-depth review, see[50]).

Proteomic evaluation initially requires protein separation, which is most commonly accomplished through two-dimensional gel electrophoresis (2-DE). Separation in the first dimension is by isoelectric potential, and then in the second dimension by size and mass. Individual proteins are then rapidly available for extraction from the gel, followed by enzymatic digestion and analysis. Protein analysis and identification are most typically accomplished by mass spectrometry, amino acid composition analysis, N-terminal sequencing or immunoblot analysis.[51] Presently, mass spectrometric analysis represents the single most valuable component of proteomic analysis—for which J. Fenn and K. Tanaka won the 2002 Nobel Prize in Chemistry.[52] While it is true that protein content of a sample cannot be amplified in a manner comparable to genomic mRNA sample amplification by PCR, mass spectrometers are capable of resolving proteins at remarkable 10^{-18} M concentrations, obviating the need for amplification.[53] After gel separation and digestion, the peptide fragment samples are initially ionized for determination of mass-to-charge (m/z) ratio, which corresponds to the molecular weight of the fragment. Proteases digest proteins at predictable points in a polypeptide, which allows calculated molecular weights of digested protein fragments to be compared to protein sequences derived from the human genome, and correlated to statistical identification algorithms that calculate the probability of a match (for a detailed description, see[54,55]). Different mass spectrometer-based protein identification methods exist for different specific needs and are summarized in the following paragraphs.

A modified version of 2DE and gel image analysis, with silver staining, autoradiography, and protein identification and measurement of peptide mass, uses matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as a rapid and sensitive technique for identifying peptides. MALDI-TOF-MS applies well to protein detection in biological fluids.[56] A second advantage of this technique is

ability to detect low-level proteins and peptides in tissue sections, by using the mass spectrometer to determine molecular weight of proteins in the surface layers of the tissue (for a detailed description, see [57]).

Fluorescence difference gel electrophoresis (DIGE) is a protein identification technique used for differential quantification of several proteins simultaneously. DIGE utilizes differential fluorescent protein labeling with succinimidyl esters of several cyanide dyes, with subsequent 2D gel electrophoretic separation of labeled proteins. In this way, the isolated proteins can be identified through mass spectroscopy for relative abundance and presence of post-translational modification.[58] Multiplexed proteomics (MP) allows similar determination of protein expression levels as DIGE, as well as altered functional post-translational attributes of the proteins such as glycosylation, drug-binding capabilities, or drug-metabolizing capabilities.[59]

Affinity capture-release electrospray ionization mass spectrometry (ACESIMS) is another recently introduced technique for quantification of proteins, and to date has most often been applied to clinical enzymology.[60] The product conjugates of the enzymatic reaction between the synthetic substrate and targeted enzyme are captured by immobilized affinity reagents, purified, released into solution, and analyzed by ESI-MS.

Isotope-coded affinity tagging (ICAT) offers advantages for quantifying proteins and identifying peptide sequences of individual proteins within complex mixtures.[61] Cysteine thiol groups in proteins are conjugated, followed by enzymatic digestion of the protein into peptides, and then analysis of the relative quantities of the conjugated peptides by mass spectrometry.[60] Two variations of this technology exist for specific applications with regard to increased sensitivity and speed of identification of gel-separated proteins: nanoscale liquid chromatography-electospray ionization mass spectrometry (LC-ESI-MS) which provides quantitative information, whereas nanoscale liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) is typically utilized to evaluate qualitative information based on the peptide molecular mass and amino acid sequence.[62]

A great stride in the technological advancement of high-throughput proteomics research has been the development of protein and antibody microarrays. Protein arrays are similar in theory to gene arrays, and typically utilize an ELISA-based membrane, gel, microwell, or slides in which hundreds or thousands of protein capture agents (proteins or antibodies) are spotted or immobilized onto the surface of the substrate. These immobilized ligands are incubated with labeled proteins that are suspected of interacting with them. Capture of a labeled protein suggests an interaction with the immobilized ligand.[63] This methodology is used to quantitatively or semi-quantitatively evaluate proteins present in serum or cell suspension samples through radioisotopic or chemiluminescent detection methods.[64] Most currently available protein arrays are still fairly low-density, thus more complex arrays are being developed to take into consideration the complexity of the proteome, especially with regard to post-translational modifications in health and disease.

Goals of proteomic profiling by the above techniques include biomarker development, detection of signatures of chemical toxicity, and identification of novel drug targets. Regarding drug targets, there is a huge proteomics interest in the pharmaceutical industry, because both disease processes and treatments commonly manifest at the

protein level. Use of serum proteomics for profiling circulating cytokine levels may prove to be particularly useful as part of immunopharmacology or immunotoxicology studies.[55]

An Overview of Results Relevant to Immunotoxicology and Immunopharmacology

Use of proteomic methods to support immunology-related research is increasing rapidly. This includes evaluation of proteins in serum or cell suspensions to characterize pathway-specific alterations in expression, as a result of infectious or toxic immunological alterations.[64] Related to infectious processes, Mullick and colleagues recently used protein arrays to demonstrate a link between deficient C5 complement component and dysregulated inflammatory cytokine response in *Candida albicans* infected mice.[65] The C5 deficiency and impaired host resistance were related to higher levels of circulating cytokines TNF-α, IL-6, monocyte chemotactic protein 1 (MCP-1), MCP-5, and eotaxin. Xu and colleagues made similar use of proteomic microarrays to examine mechanisms by which neuronal cells undergo apoptosis following exposure to HIV-1.[66] Putative upregulated host factors released from virally infected macrophages and related to inappropriate induction of neuronal cell apoptosis, included IL-5, IL-6, MCP-3 and granulocyte macrophage-colony stimulating factor (GM-CSF). Regarding immune-mediated mechanisms in bacterial diseases, Zhou and coworkers used cytokine antibody arrays in TLR2- and TLR4-deficient macrophages to show that most cytokines induced by *Porphyromonas gingivalis*, its LPS or its major fimbrial protein (FimA) signal through TLR2, while most of cytokines induced by live *P. gingivalis* signal through both TLR2 and TLR4.[67]

As might be expected from the preceding examples, protein microarrays are also proving valuable for characterization of non-pathogen driven immune responses underlying important human diseases. Leukotriene B4 (LTB4) is a product of arachadonic acid metabolism that has been implicated in enhanced inflammation related to atherosclerosis, however specific mechanisms through which LTB4 acts to increase inflammation have remained poorly defined. Huang and coworkers[68] used proteomic cytokine analysis to broadly examine cytokine production in LTB4-exposed primary human monocytes, and found that the pro-inflammatory chemokine MCP-1 was linked to atherogenicity.

Proteomic studies are also being used to increase basic understanding of normal immune system regulation and function, thus increasing the database against which toxicity studies can be compared. For instance, it has been recognized that some cytokines, for example, interferons gamma (IFN-γ) or alpha (IFN-α), are multifunctional cytokines that induce diverse protein products from different classes of immune and non-immune cells. Rosengren therefore used a differential proteomic approach to search for new IFN-α-regulated proteins in human CD4+ T cells. Two new INF-α-inducible proteins were identified, soluble N-ethylmaleimide-sensitive factor attachment protein alpha (α-SNAP) and cleavage stimulation factor-64 (CstF-64).[56] The mechanistically focused protein array, phospho antibody for proteomics-1 array (PAP-1), has been used for probing the serine phosphoproteome of antigen receptor-activated T lymphocytes.[69]

These authors identified novel serine kinases activated by cytokines and chemokines in T cells using PAP-1, and verified MAP kinase dependent IL-16 secretion in T-cell receptor-activated T cells.

Immunopharmacologic studies suggest abnormal proteomic patterns induced by drugs may be predictive for increased risk of idiosyncratic reactions. In particular, the incidence of severe drug reactions in hospital patients is approximately 7%, and these adverse events are often difficult to predict or explain, especially with multiple drug therapy or in chronic disease state.[70] Therefore, use of toxicoproteomics in early drug development may highlight "danger signals" and suggest unacceptable adverse drug effects that might have previously only been detected later in human clinical trials using traditional toxicological studies.[71]

Early proteomic studies demonstrated alterations in liver function and protein expression following xenobiotic exposure, information that led to formulation of a prototype database to predict adverse outcomes based on a wide range of dose and time exposures.[72] The utility of such protein-based diagnostics is clearly expected to improve as new proteins are associated with disease or toxicity. For this reason, the U.S. National Cancer Institute was among the first to develop a protein expression database, to study relationships between cellular activity and protein expression as they may relate to melanoma, leukemia, and cancers of the breast, prostate, lung, colon, kidney, ovary, and central nervous system.[57,73] Similar proteomic array-based data management, and establishment of structured databases, will be needed to assist immunopharmacology and immunotoxicology studies aimed at detecting associations between altered immune protein production or activity and drug, chemical or disease effects on immune system function.

Future Directions and Potential Impact

The proteomic technologies discussed above have melded organic chemistry, mass spectrometry techniques, and array-based assays to provide high throughput, efficient, and broad-spectrum tools in the areas of molecular biology and medicine. Future development of these methodologies should focus upon increasing sensitivity and specificity of peptide identification, especially in regard to low-abundance and membrane-bound proteins.[74–76] Additionally, it will be important to develop standards by which researchers may store and report proteomic data to enable comparisons across platforms and research groups.[64] The Human Proteome Organization (HUPO; http://www.hupo.org) has developed the Proteomics Standards Initiative in order to establish guidelines for reporting and presentation of proteomics data.

CONCLUSIONS

Genomics, including gene expression profiling (transcriptomics) and evaluation of SNPs, have been used in immunotoxicology studies to a limited extent. The primary value of high throughput microarray technology has been the identification of potential mechanisms of immunotoxicant action. Further implementation of genomic and proteomic

methodology may serve to move immunotoxicology into the arena of systems biology. This approach could be beneficial with regard to mechanistic and screening applications and could also reveal new information about immune function.

Similarly, data already available indicate that SNPs will be quite relevant in immunotoxicology and immunopharmacology. Findings that SNPs of immune-related proteins have an impact on the development of beryllium disease and silicosis suggest that SNPs may explain much of the daunting diversity of human responses to environmental and pharmaceutical agents.

Proteomics involves the use of a wide array of methods, some of which can be conducted in a high throughput mode and are remarkably sensitive. Because proteins are the ultimate mediators of function in cells and organisms, proteomics will be a necessary component of any systematic effort to understand the mechanism of action of drugs or chemicals on the immune system. In conjunction with gene expression analysis, such studies can reveal whether the test article acts at the level of transcription or translation (or post-translationally). The development of "user friendly" mass produced methodologies, such as protein microarrays, is not as far along as in the case of genomics, but the potential uses of proteomics are similar to those mentioned already for transcription profiling.

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