Immunotoxicology and Immunopharmacology

Third Edition

Edited by

Robert Luebke

Robert House

Ian Kimber

TARGET ORGAN TOXICOLOGY SERIES

Series Editors

A. Wallace Hayes

John A. Thomas

Donald E. Gardner

CRC Press
Taylor & Francis Group

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DEDICATION

The third edition of *Immunotoxicology and Immunopharmacology* is dedicated to the memory of Professor Dr. Jef Vos. Jef was one of the founding fathers of immunotoxicology, and among the first to recognize that environmental agents may have adverse effects on the immune system. In his long career at the National Institute of Public Health and the Environment in the Netherlands (RIVM), he guided the development of many young scientists and lead established colleagues by example. His reputation as a first-rate scientist and his warm personal manner won him respect and admiration far beyond RIVM. His friends and colleagues are saddened by his loss, as we reflect on the impact he made on the science and the friendship he so freely shared with us all.

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Preface to the Third Edition

Although a decade has passed since the publication of the second edition of *Immunotoxicology and Immunopharmacology,* the issues and research priorities faced by immunotoxicologists and immunopharmacologists remain the same: identification of agents that modify immune function, determination of mode or mechanism of action, and translation of laboratory or clinical data into scientifically sound prediction of risk or benefit to the exposed population. In keeping with the tradition established in the first two editions, this edition provides comprehensive reviews of the mechanisms underlying immunosuppression, allergy and hypersensitivity, and autoimmunity. Advances in basic immunology, cellular and molecular biology and genetics since publication of the last edition have increased our ability to detect and characterize events that follow manipulation of the immune system. Therapeutic modulation of the immune system has increased dramatically in the last ten years, resulting in the development of therapeutic agents that target specific cellular and humoral molecules. Technical progress in the basic sciences has likewise aided assay development, and increasingly sophisticated methods adapted from basic immunology and cell biology have enabled investigators to determine mechanisms of immunotoxicity at the level of signaling pathways and gene transcription.

In the third edition, mechanisms of environmentally induced immunosuppression, allergy, hypersensitivity, and autoimmunity have been updated to reflect progress made over the last decade. Similarly, trends in risk assessment and in model development to detect and characterize immunomodulation are addressed directly in chapters dedicated to regulatory issues, and indirectly in chapters focused on mechanisms of immunotoxicity. In some cases, expanded coverage is given to topics discussed in previous editions. For example, two chapters are dedicated to immunotherapeutic proteins, another to dietary supplements and foods with immunomodulatory properties, and another to the current and potential future uses of genomics and proteomics techniques to identify and characterize immunomodulators. A section on wildlife immunotoxicity was added to address immunotoxicity across a wide range of biological complexity, from invertebrates to marine mammals. New to this edition is a section dedicated to interactions between the immune and central nervous systems, and the consequences of altered nervous system function on immune homeostasis.

This book will be of interest to toxicologists, immunologists, clinicians, risk assessors, and others with an interest in accidental or deliberate immunomodulation. Although few of the chapters are written on an introductory level, background information and citations for review articles are included in most chapters that will provide a starting point for individuals seeking additional information.

Robert W. Luebke

Preface to the Second Edition

Although the philosophy and design of the second edition are consistent with the first, many changes have been made to reflect the metamorphosis of this area from a subdiscipline of toxicology to an independent area of research that can best be described as "Environmental Immunology." For example, chapters have been added that describe the role of immune mediators in liver, lung, and skin toxicity, in regulating drug- and chemical-metabolizing enzymes and in the immunosuppression produced by ultraviolet light, as well as immunotoxicology studies of non-mammalian systems. More emphasis has been placed upon the clinical consequences of immunotoxicity as well as on the interpretation of experimental data for predicting human health risk. A number of chapters from the first edition have been deleted, particularly those that provided descriptive overviews of the immune system, in order to limit the size of this edition while increasing the scope of immunotoxicology subjects.

Unlike the first edition, this book is divided into three major subsections, comprising immunosuppression, autoimmunity, and hypersensitivity. This division allows for a more comprehensive treatment of these important subjects with greater attention to test methods, theoretical considerations, and clinical significance. The section on immunosuppression begins with introductory chapters discussing consequences of immunodeficiency, human and animal test systems, and risk assessment. This is followed by chapters discussing various environmental agents, therapeutic drugs, biological agents, and drugs of abuse as well as immune-mediated toxicity that occur in specific organ systems. The second section is devoted to autoimmunity and includes discussions on the immunopathogenesis of autoimmunity as well as examples of chemical- and drug-induced autoimmune disease. The last section, which is devoted to hypersensitivity, has been greatly expanded from the first edition. This section begins with discussions on the clinical aspects of allergic contact dermatitis and respiratory hypersensitivity. This is followed by chapters describing mechanistic aspects of sensitization and the methods available for the toxicologic evaluation of chemical allergens.

This volume will be of interest to toxicologists, immunologists, clinicians, and scientists working in the area of environmental health. It should also be of interest to individuals involved in occupational health, safety assessment, and regulatory decisions. Although we assume that most readers have at least some understanding of immunology, we have attempted to prepare this book so that any individual interested in environmental sciences could follow it.

Michael I. Luster

Preface to the First Edition

Traditional methods for toxicological assessment have implicated the immune system as a frequent target organ of toxic insult following chronic or subchronic exposure to certain chemicals or therapeutic drugs (e.g., xenobiotics). Interaction of the immune system with these xenobiotics may result in three principal undesirable effects: (1) those determined by immune suppression; (2) those determined by immune dysregulation (e.g., autoimmunity); and (3) those determined by the response of immunologic defense mechanisms to the xenobiotic (e.g., hypersensitivity). The first section of this volume reviews the basic organization of the immune system and describes the cellular and humoral elements involved, the interactions and regulation of lymphoid cells, and their dysregulations that result in disease.

Toxicological manifestations in the immune system following xenobiotic exposure in experimental animals appear as alterations in lymphoid organ weights or histology: quantitative or qualitative changes in cellularity of lymphoid tissue, peripheral leukocytes, or bone marrow; impairment of cell functions; and increased susceptibility to infectious agents or tumors. Allergy and, to a lesser extent, autoimmunity have also been associated with exposure to xenobiotics in animals and man. Chapters are included in the second section which describe approaches and methodology for assessing chemical- or drug-induced immunosuppression or hypersensitivity.

Awareness of immunotoxicology was stimulated by a comprehensive review by Vos in 1977, in which he provided evidence that a broad spectrum of xenobiotics alter immune responses in laboratory animals and subsequently may affect the health of exposed individuals. Several additional reviews, as well as national and international scientific meetings, have reinforced these early observations. In several studies, alteration of immune function was accompanied by increased susceptibility to challenge with infectious agents or transplantable tumor cells, indicating the resulting immune dysfunction in altered host resistance. Clinical studies in humans exposed to xenobiotics have confirmed the parallelism with immune dysfunction observed in rodents. The latter sections in this volume describe studies with xenobiotics that resulted in immune modulation in rodents and man.

The sensitivity or utility of the immune system for detecting subclinical toxic injury has likewise been demonstrated. This may occur for one of several reasons: functionally immunocompetent cells are required for host resistance to opportunistic infectious agents or neoplasia; immunocompetent cells require continued proliferation and differentiation for self-renewal and are thus sensitive to agents that affect cell proliferation or differentiation; and finally, the immune system is a tightly regulated organization of lymphoid cells that are interdependent in function. These cells communicate through soluble mediators and cell-to-cell interactions. Any agent that alters this delicate

regulatory balance, or functionally affects a particular cell type, or alters proliferation or differentiation can lead to an immune alteration. One section of this volume is devoted to possible mechanisms by which xenobiotics may perturb lymphoid cells.

This volume should be of interest to toxicologists, immunologists, cell biologists, and clinicians who are studying mechanisms of xenobiotic-induced diseases. It should also be of interest to scientists faced with the challenge of the safety assessment of immunotherapeutics, biological responses modifiers, recombinant DNA products, drugs under development, and other consumer products. This volume should better prepare toxicologists for the challenges of the 21st century.

Jack H. Dean

Acknowledgments

The editors of the third edition thank the Target Organ Toxicity Series editors for their continued recognition of the need for an updated volume on immunotoxicology and immunopharmacology. We greatly appreciate the time, effort, and expertise of our colleagues who contributed chapters to the book, the patience of our colleagues at work, and of our families at home, who complained very little about the time spent editing this book.

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Part I

Immunotoxicology and Hazard Identification

1 Immunotoxicology: Thirty Years and Counting

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INTRODUCTION

The science of immunotoxicology arguably began in the early 1970s, following the recognition of increased sensitivity to infection following exposure of test species, including guinea pigs [1], mice, [2, 3] rats [4], ducks [5], hamsters and monkeys [6] to various xenobiotics. Reduced resistance to infectious disease was a well documented consequence of primary and acquired immunodeficiencies, but a novel outcome of xenobiotic exposure, leading some to characterize xenobiotic-induced immunosuppression as "chemical AIDS." Although the comparison was scientifically inappropriate, "immunotoxicity" was often thought of as synonymous with "immunosuppression" during the formative years of the discipline, although hypersensitivity, allergy, and autoimmunity were recognized as potential exposure outcomes. The first review in the field of immunotoxicology was published by Vos [7], followed in 1978 by the first symposium organized specifically to address this topic at the Gordon Research Conference on Drug Safety. The number of investigators and laboratories conducting immunotoxicology research increased significantly in the United States and Europe during the late 1970s and early 1980s. As research expanded during this period, many of the assays, methodologies, and approaches that are currently used to identify potential immunotoxicants were developed.

In 1984, the first international meeting of immunotoxicologists was organized by the Commission of the European Communities and the International Programme on Chemical Safety/World Health Organization in Luxembourg. This meeting, entitled "Immunotoxicology: The Immune System as a Target for Toxic Damage," summarized the state of the science and defined immunotoxicology as undesired direct or indirect effects of xenobiotics on the immune system causing suppression, an immune response to the chemical or its metabolites, or alteration of host antigens by the chemical or its metabolites [8]. Approximately 80 scientists from around the world, from the fields of immunology, pharmacology, pathology, and toxicology, discussed approaches for immunotoxicity assessment in rodents and discussed several compounds recently shown to cause immunotoxicity.

Immunotoxicology has matured over the intervening three decades, gaining recognition as a subspecialty of toxicology, and the interests of immunotoxicologist have broadened to focus on modulation, rather than only suppression, of the immune system by chemical and physical agents. Several areas of investigation including allergic contact dermatitis, respiratory hypersensitivity, and air pollutant toxicology, which originated independently, were merged into immunotoxicology as it was recognized that all involved perturbations of the immune system. In this chapter we will briefly explore the multiple paths that the field's progression has taken over time. This treatment is meant as a survey only, since adequate treatment of each topic requires more than a few paragraphs and many of the topics are discussed elsewhere in this volume or in recent reviews. Where appropriate, the reader will be directed to resources for more intensive coverage. Likewise, it is important to note that this survey will not take a strictly chronological approach since progress in all aspects of immunotoxicology has not been linear.

ORIGINS AND PROGRESS IN IMMUNOTOXICITY TESTING

The Tier-Testing Approach: Setting the Course for Modern Immunotoxicology

The majority of early publications that can be reasonably identified as comprising "immunotoxicology" reported altered resistance to infection in animals exposed to various environmental or industrial chemicals. Authors logically concluded that xenobiotic exposure suppressed immune function since the immune system is ultimately responsible for this resistance to infection. Subsequent studies demonstrated that suppression of various cellular and functional endpoints accompanied or preceded increased sensitivity to infection, and that administration of known immunosuppressants likewise decreased host resistance. The human health implications of these studies, that chemical exposure reduced resistance to infection, drove the initial focus of many immunotoxicologists on functional suppression, and provided the theoretical and practical underpinnings of immunotoxicity testing.

Although the experimental methods adopted by immunotoxicologists to evaluate immune function were those common to immunology laboratories, experimental designs were often ad hoc. This lack of standardization often made it difficult to compare chemical-specific results obtained in different labs and lead Dean and colleagues [9] to propose a "tier testing" paradigm. This approach was based, according to the authors, on the need for assays to be "relevant to the human experience and adaptable to certain practical considerations such as cost, reproducibility of data, ease of performance and application to routine toxicology studies." Using these criteria, a tiered approach was developed with differential priorities: screening assays to detect immunologic effects (Tier I) and a comprehensive suite of assays to provide an in depth assessment of immune function and host resistance endpoints (Tier II). A battery of assays from the screening tier was subsequently assembled into a hypothetical and practical test battery to screen for immunological effects of a chemical with potential immunosuppressive properties. This approach was tested with encouraging results using the known immunosuppressant, cyclophosphamide [10], and the testing paradigm was then further refined [11,12].

From these conceptual and early proof-of-concept studies, the tier-testing approach made a significant practical leap when the approach was employed by the National Toxicology Program in an inter-laboratory validation study between NIEHS (Research Triangle Park, NC), Virginia Commonwealth University (Richmond, VA), Chemical Industry Institute of Toxicology (Research Triangle Park, NC) and IIT Research Institute (Chicago, IL); each laboratory evaluated the same chemicals, using the same set of assays [13]. In this effort, both descriptive and mechanistic assays were employed including hematology, selected organ weights (spleen, thymus), and histology of lymphoid organs. Functional tests in this tier include T-dependent IgM antibody formation, natural killer cell function, and lymphocyte mitogenesis. (Mitogen-driven lymphocyte proliferation has poor predictive power and has been replaced by lymphocyte phenotyping in current tier testing protocols [14]). The results of this exercise, as well as follow-on studies to determine the biological significance of the findings, resulted in a series of watershed

publications [13–15]. The results and concepts developed in these early efforts provided the basis for moving immunotoxicology assessment forward, and has been extensively reviewed [16–19].

Use of Tier-Testing for Industrial and Environmental Chemicals

The earliest defined immunotoxicology test guidelines were developed to assess pesticides, since these chemicals have significant potential for large-scale human exposure. In 1996, the Office of Prevention, Pesticides and Toxic Substances (OPPTS) of the U.S. Environmental Protection Agency (EPA) published *Biochemicals Test Guidelines: OPPTS 880.3550 Immunotoxicity* [20], which described the study design for evaluating immunotoxicity in biochemical pest control agents. The panel of tests included in this guideline was taken directly from the National Toxicology Program's tier-testing approach and includes routine toxicology tests, as well as functional evaluation of humoral and cell-mediated immune function. The document describes the actual testing procedures to be employed, but little guidance was provided for interpretation of test results. Thus, a second document was published concurrently entitled *Biochemicals Test Guidelines: OPPTS 880.3800 Immune Response* [21]. This companion guideline provides a rationale for evaluating pesticides for immunotoxicity, more detailed explanations of testing strategies, and additional details on mechanistic assessments, including host resistance assays and bone marrow function.

Whereas immunotoxicity evaluation encompassed by the 880 series of guidelines would be expected to detect suppression of innate, cellular or humoral immunity, the number of required tests would greatly increase the financial and resource costs of testing. In 1998, the Agency published *Health Effects Test Guidelines: OPPTS 870.7800 Immunotoxicity* [22], describing immunotoxicology testing for EPA-regulated, non-biochemical agents that fall under the regulatory requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136 et seq.) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601). The testing approach mandated by 870.7800 reflects the more limited, case-by-case approach currently favored. Most notably, the functional assessment is limited to T-dependent antibody response (TDAR), natural killer (NK) cell function, and quantitation of T- and B-cells. The current (2006) version of the 7800 Immunotoxicity Guidelines calls for testing in mice and rats, unless data are available to show that absorption, distribution, metabolism and excretion are the same in both species. Although mandated for FIFRA and TSCA compounds, the guidelines call for exposure via the expected route of human exposure (oral, dermal or inhalation), and are applicable to a range of industrial and environmental chemicals. The U.S. EPA's Office of Air and Radiation, for example, requires that these guidelines be followed when air toxics are subjected to testing for immunotoxic potential.

Adaptations of the Tier-Testing Approach

Chemicals that do not fall under the testing requirements for pesticides may have immunotoxic potential. However, submitting all industrial chemicals for immunotoxicity

tier testing is impractical due to the expense involved and the numbers of animals that required. For this reason, microscopic examination of immune system organs has been used as a predictor of immunotoxicity, and as a trigger for functional testing. This concept was first explored by Shuurman colleagues [23], although it gained momentum from then until 2000, at which time the idea was developed in greater detail [24, 25]. Although the use of extended histopathology assessment as a routine immunotoxicology test was first widely adopted in Europe (due primarily to the inception of the regulatory document *Note for Guidance on Repeated Dose Toxicity (CPMP/SWP/1042/99)* [26], the approach has gradually gained support in the United States [27–29] and was incorporated in the ICH S8 immunotoxicology guidance (discussed below), in which histopathology plays an important role [30]. A recent study demonstrated that while the antibody response to sheep erythrocytes correctly identified 90% of known immunotoxicants in a dataset of compounds tested by the U.S. National Toxicology Program, extended histopathology correctly identified 80% of known immunotoxicants when minimal or mild histologic change in any tissue (spleen, thymus or lymph node) examined was accepted as evidence of immunotoxicity. However, mild change in any tissue also identified known negative compounds and tissues from vehicle control groups as immunotoxicants, whereas limiting calls to chemicals that caused moderate to marked tissue changes resulted in poor predictive performance, indicating that the criteria used to classify chemicals as immunotoxic must be carefully set to avoid high false positive and false negative rates [27, 28].

Seminal immunotoxicity experiments were conducted in rats [4], although the mouse became the preferred model, at least in the United States, because this species was commonly used by immunologists and reagents and inbred stains were readily available. However, the rat has traditionally been used in industrial chemical toxicity studies, and investigators worked to adapted testing methods [31] and performed comparative studies in mice and rats [32, 33], ultimately validating the use of rat as an alternative for immunotoxicity testing [34, 35]. This was followed closely by the publication of a collaborative study by the International Collaborative Immunotoxicology Study (ICICIS) workgroup on the use of the rat in immunotoxicology [36], which arrived at the same conclusion.

One other noteworthy development in the evolution of the tier-testing approach is the increasing use of sophisticated statistical analyses to evaluate the predictive value of data generated by these studies. Concordance analysis of NTP datasets provided the first insight into which tests were the most accurate in identifying immunotoxicants, and predicting changes in host resistance [15,16]. Others have used statistical methods to model various aspects of immunotoxicity testing and data interpretation. For example, immunotoxicity data for an individual compound are typically derived from several sets of animals, yet multivariate analysis is typically applied to datasets in which all endpoints are evaluated in all animals. However, Keil and colleagues [37] modeled the effects of obtaining data from different sets of mice and found that the purported disruption of the correlation matrices, critical to multivariate analysis, did not occur, indicating that not all variables must be derived from the same animal. This group also used multiple and logistic regression analysis to evaluate the relative contribution made by

individual effector mechanisms on host resistance endpoints and reported that moderate functional changes induced by an immunotoxicant predict altered resistance to bacterial or tumor cell challenge, although predictive endpoints were not necessarily those that immunologic dogma would suggest [38]. Shkedy and colleagues [39] reported success in fitting a nonlinear model to individual animal antibody responses to KLH to derive maximum likelihood estimates, which were then analyzed for treatment effects or using nonlinear mixed models to account for individual animal variability in antibody titer. Modeling efforts as described above may shape future testing methods by providing additional insight into modes and mechanisms of immunotoxicity, and the functional or observational endpoints that best predict changes in immune function.

The Emergence of Regulatory Guidance

As methods to evaluate immunotoxicity became more established and evolved to the stage of standardization, these techniques became a potentially useful tool to evaluate specialized toxicity to the immune system from a regulatory standpoint. We have previously examined how the U.S. EPA was responsible for some of the first such testing guidelines; however, the road to acceptance of such guidance for pharmaceutical development in both the United States and Europe (and, to a less obvious degree, in Japan and the rest of Asia) has been much less straightforward. Calls for regulatory guidance began in the early 1990s [40–43], leading to publication of the first codified regulations for immunotoxicology in 2000. Current regulatory guidelines for immunotoxicity hazard identification are discussed in chapter 2 of this book.

Europe: Note for Guidance on Repeated Dose Toxicity

In Europe, safety testing for pharmaceuticals is regulated by the Committee for Proprietary Medicinal Products or CPMP. In October of 2000, CPMP published *Note for Guidance on Repeated Dose Toxicity (CPMP/SWP/1042/99)* [24]; although the primary purpose of this particular document was to describe an overall approach to safety testing of pharmaceuticals, it was important as the first guidance document mandating specific immunotoxicology screening for pharmaceuticals. An appendix in the guidance document describes a staged evaluation, emphasizing that information gained in standard toxicology evaluation can be useful as a primary indicator for immunotoxicity. Functional tests may be incorporated to gain additional information, first as an initial screen and then progressing to extended studies as necessary. The choice of assays to be used includes combinations of functional tests known to be predictive of immunotoxicity, as described by Luster and colleagues [14,15].

As the first published requirement for immunotoxicology evaluation of drugs, *CPMP/SWP/1042/99* predictably was met with a combination of resistance and confusion. Much of this was allayed in a Drug Information Associated-sponsored workshop held in Noordwijk, The Netherlands in November of 2001. At this meeting, the intent of the guideline was clarified; a summary of this workshop, as well as an update, has been published [44, 45].

United States: Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs

In the United States, ensuring the safety of pharmaceuticals is the responsibility of the Food and Drug Administration Center for Drug Evaluation and Research (FDA/CDER). In October of 2002, CDER released a long-awaited document entitled *Guidance for Industry: Immunotoxicology Evaluation of Investigational New Drugs* [46]. This document is arguably the most comprehensive of any published guidance, and includes detailed descriptions of immune system-related adverse drug effects, including immunosuppression, immunogenicity, hypersensitivity, autoimmunity, and unintended immunostimulation. The document also includes suggested approaches and methodologies to evaluate each type of adverse immune effects. Like the CPMP document (described above), the FDA/CDER guidance advocates the use of information derived from standard repeat-dose toxicity studies to provide early evidence of immunotoxicity, with subsequent evaluations to be rationally designed to use a minimum of animals and resources while deriving the maximum amount of information. Subsequent to the publication of the FDA/CDER document, the primary author of the guidance published a manuscript describing the implications of the guidance [47].

ICH S8: Immunotoxicology Studies for Human Pharmaceuticals

The requirement for immunotoxicity testing in the CPMP guidelines, and reliance on clinical data to trigger testing in the FDA guidelines resulted in differing opinions on the utility of routine testing [48, 49]. Recognizing the need to globally standardize these regulations, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) initiated the process of compiling this document. The guidance "provides recommendations on nonclinical testing approaches to identify compounds that have the potential to be immunotoxic and guidance on a weight-of-evidence decision making approach for immunotoxicity testing." Similar to previous documents, the S8 guidance will apply to unintended immunosuppression and immunoenhancement, but will not address allergenicity or drug-specific autoimmunity [50–53].

BIOLOGICALS

Biologicals (i.e., therapeutics derived by biotechnology) present a unique challenge for immunotoxicity assessment for two primary reasons. First, many of these agents (such as cytokines, growth factors, etc.) are intended to modulate the immune response therapeutically, making it difficult to differentiate between efficacy and toxicity. Second, because many of these agents are proteinaceous, their introduction into a host can result in an immune response directed against the molecule itself; this can lead to alterations in pharmacodynamics or other adverse reactions. A detailed discussion of therapeutic biological molecules is presented in chapter 8 of this volume. One approach to testing protein immunomodulators was addressed by the International Conference on Harmonisation via the publication of *Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals*

S6 [53]. This document includes sections on immunogenicity as well as a brief section on immunotoxicity evaluation. Notably, the use of a standard tier approach was rejected in favor of case-by-case screening, followed by mechanistic studies as necessary.

VACCINES

In the past, vaccines have received only slight notice from toxicologists, possibly from the naïve notion that the nature of these medicines limited their toxic potential. We are increasingly recognizing this to be untrue, and thus the appropriate regulatory agencies have formulated guidance documents governing safety testing of these intentional immunomodulators.

For example, European regulation of vaccines is described in the CPMP's *Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines* [54]. Therein, immunotoxicology should be considered during toxicology testing, and vaccines should be evaluated for their immunological effect on toxicity (e.g., antibody complex formation, release of cytokines, induction of hypersensitivity reactions, and association with autoimmunity). Each vaccine is to be evaluated on a case-by-case basis.

Responsibility for safety of vaccines in the United States belongs to FDA/CBER. One of the primary documents describing vaccine studies is *Guidance for Industry for the Evaluation of Combination Vaccines for Preventable Diseases: Production, Testing and Clinical Studies* [55]. Animal immunogenicity is covered in detail in the document, although immunotoxicity is not specified as an area of concern. Another document, *Considerations for Reproductive Toxicity Studies for Preventive Vaccines for Infectious Disease Indications* [56], acknowledges the potential immunological reactions resulting from the vaccination process to exert unintended consequences. Specific guidance for actually performing such evaluations is not covered by any of these documents, but should be determined on a case-by-case basis depending on the regulatory circumstances [57,58].

THE LLNA: A CONCERTED EFFORT TO VALIDATE METHODOLOGY

While most published immunotoxicity testing guidelines are structured to detect immunosuppressants, hypersensitivity reactions are far more common. None of the assays included in standard tier-type protocols are appropriate for assessing the sensitizing potential of chemicals, and thus a specialized assay was required. Early testing strategies relied on tests in guinea pigs (see chapter 31), supplanted in 1989 with the murine local lymph node assay (LLNA) [59]. Over the course of the subsequent decade, Kimber and his collaborators amassed an impressive collection of studies demonstrating the utility of this assay for identifying contact sensitizers. In particular, inter-laboratory collaborations [60] demonstrated that the assay was sensitive, reproducible, and (most importantly) sufficiently robust to apply in a large-scale validation study. Therefore, The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) sponsored just such as study using the LLNA, which became the first assay

to be validated using their stringent criteria. The details of this process are too complex to review here, but the results have been published and are instructive reading [61–63]. Following validation, the LLNA became the standard assay for evaluating the sensitizing potential of chemicals and drugs. Detailed explanations of this assay and its use are covered in the OECD 429 guideline, *Skin Sensitisation: Local Lymph Node Assay* [64] and the U.S. EPA document *OPPTS 870.2600 Skin Sensitization* [65].

INTERPRETING LABORATORY ANIMAL DATA IN TERMS OF HUMAN RISK

While it is well established that immunosuppression can lead to an increased incidence or severity to certain infectious and neoplastic diseases, interpreting data from experimental immunotoxicology studies, or even epidemiological studies, for quantitative risk assessment purposes is problematic. This is particularly true when the immunological effects, as might be expected from inadvertent exposures in large populations, are minimal-to-moderate in nature, and values obtained for various immunological endpoints fall within a range considered to be normal for the population. Furthermore, detecting significant changes in rates of infection with common human pathogens in exposed populations is difficult against a background of infection in groups of individuals with no known exposure to immunotoxicants. Thus, the relationship between altered immune function and increased sensitivity or susceptibility to the types of infection likely to occur in individuals without primary or acquired severe immunosuppression has been the most difficult to establish. However, it is critical that a firm scientific basis for interpreting the outcome of immune function and host resistance studies in laboratory animals be established if results of Tier I and II data are going to be used to predict possible human effects as part of the risk assessment process. The infection risk posed by mild to moderate immunosuppression in humans, and interpretation of immunotoxicity data for human risk assessment, are discussed in chapter 3 of this volume.

ENVIRONMENTAL AND WILDLIFE IMMUNOTOXICOLOGY

Perhaps due to phylogenetic chauvinism, but as likely for more practical reasons, the evaluation of immunotoxicity has largely been confined to laboratory rodents, with the implicit (and often explicit) understanding that these mammalian species can serve as reliable surrogates for humans. This traditional approach may be somewhat myopic in that evaluation of species from chronically polluted sites may provide insight into the effects of chronic low level exposure to toxicants that may also affect humans. A variety of environmental pollutants have been evaluated for immunotoxic effects in non-laboratory species, including marine mammals, particularly seals [66, 67], birds [68], fish [69], and even invertebrates [70]. Although the level of immune system complexity is far different in invertebrates and mammals, many aspects of innate resistance to infection are phylogenetically conserved, and have been studied in detail. Assays developed by comparative immunologists and wildlife immunotoxicologists have been employed to

evaluate immune function in free-living species chronically exposed to environmental contaminants, and in laboratory-reared species under controlled conditions. Adverse effects observed in wildlife species often parallel those obtained when analogous endpoints are evaluated in traditional laboratory species. Thus, wildlife species may act as sentinel species for potential human effects [71] while simultaneously providing insight into the potential immunotoxicologic risk posed by contaminated sites to indigenous species. The three chapters in Section VI of this volume describe immune function and immunotoxicity in wildlife species, including invertebrates, selected vertebrates and marine mammals.

DEVELOPMENTAL, PERINATAL, AND REPRODUCTIVE IMMUNOTOXICOLOGY

For much of its history, immunotoxicology has used young adult rodents as the primary experimental species; this is logical, since the need to control as many variables as possible would suggest that a stable (i.e., mature) immune system would respond most reproducibly to outside influences such as toxic exposure. However, it has long been recognized that organogenesis and maturation represent periods of increased sensitivity and susceptibility to toxicants, and among the first immunotoxicity studies to be published evaluated the effects of gestational/neonatal xenobiotic exposure on the immune system [72,73]. As the evidence for increased sensitivity of the developing immune system mounted over the years, it was suggested that immunotoxicity studies should be included in standard reproductive toxicity screening studies [74], and that evaluation of immunotoxicity exclusively in adult animals may not predict effects in the developing organism [75,76].

In recognition of the increased vulnerability of the developing organism, both the U.S. EPA Food Quality Protection Act [77] and the U.S. EPA Safe Drinking Water Act [78] mandate that infants and children warrant special consideration in the risk assessment process. Immune system ontogeny and the sensitivity of the developing immune system to xenobiotics are discussed in detail in chapter 20 of this volume.

As was the case with tier testing, developmental immunotoxicology has been driven by expert workshops to reach consensus on the most important issues; three workshops were held in 2001 [79–81], and another in 2003 [82]. These workshops contributed to the development of a proposed testing framework to detect developmental immunotoxicity, which is described in detail in chapter 21.

FUTURE TRENDS IN IMMUNOTOXICOLOGY

Unintended Consequences of Therapeutic Immunomodulation

As noted above, the primary focus of immunotoxicology has been on suppression; many of the early techniques grew out of basic immunology research, in which the function of various components of the immune response was determined by selective manipu-

lation of these components, particularly in defining functional parameters critical to resistance to infection or neoplastic disease. However, the consequences of some forms of immunostimulation, including therapeutic manipulation of various components of the immune system, may be less obvious, but nonetheless adverse. Unfortunately, traditional testing paradigms are inadequate to determine these consequences; developing effective testing strategies is a major challenge of future immunotoxicologists since modalities for enhancing the immune system are increasing.

The recent rapid development of immunostimulatory therapeutics likewise has outpaced our understanding of the potential immunotoxicity associated with these drugs. One example is the unmethylated oligonucleotides (e.g., CpG ODN) that are being developed as Toll-like receptor (TLR) agonists for a variety of therapeutic applications. Although these molecules hold great promise, they have been associated with a variety of adverse reactions [83–87], and it is clear that novel testing approaches and assays will be necessary to understanding these reactions as development of these drugs progresses.

The adaptive immune response to most infectious agents is typically robust and includes a memory component that provides long-lasting protection against the specific agent. For most relatively innocuous agents that humans and animals are exposed to, this is sufficient to protect us. For the particularly dangerous organisms or their toxic products, vaccines (discussed below) are administered to provide protection without the risk of actual exposure. For most organisms and under most circumstances, this is sufficient. However, conventional adaptive responses may not offer adequate protection against biological warfare and bioterrorism agents, emerging biological threats such as methicillin-resistant *Staphylococcus aureus* or drug-resistant tuberculosis, or man-made organisms with yet undefined but potentially dangerous characteristics. As our understanding of the interaction between the innate and adaptive immune system improves, so does the potential to therapeutically manipulate the innate defenses to provide short-term, nonspecific protection. In this scenario, a therapeutic agent or combination of agents would be administered in advance (or immediately following) exposure to these threats [88,89]. Such agents include TLR agonists and other related pattern-recognition receptors [90] and molecules [91]. Application of knowledge gained from recent molecular and genetic immunology research has stimulated the development of additional classes of therapeutics that target very specific aspects of the immune response and may prove useful in the treatment of immunodeficiency and autoimmunity. Some of these agents have been subjected to clinical trials, and the efficacy and toxicity of these new therapeutic agents are discussed in Section II of this volume; protein-based immune response modifiers are presented in chapter 8 and immunostimulating biological molecules presented in chapter 9.

Finally, a particularly interesting ongoing challenge will be to understand the potential for "do-it-yourself" immune stimulation to have unintended consequences. There are now many herbal supplements, "functional foods" and other over-the-counter products that promise to boost the immune response and most are considered to be safe for use by the general public. Although there is limited published evidence of adverse immune system effects of these materials, some have been associated with autoimmunity [95,96]. See chapter 11 for a detailed discussion of the beneficial and potential adverse effects of nutraceuticals and functional foods.

Use of Transgenic Animal Models

The technology for specifically engineering mutations in the immune system of laboratory animals will increasingly give investigators the ability to evaluate perturbation of the immune response. The promise of this technology for immunotoxicology was first described by Lovik [97], and a number of recent uses of this technology for investigational immunotoxicology have been described [98].

In Vitro Immunotoxicology

Current public opinion and ethical considerations have stimulated efforts to reduce the number of animals used to test the toxicity of chemicals, drugs and personal care products. However, only limited effort has gone into developing *in vitro* or *in silico* methods to detect immune dysfunction. This may be at least partially attributable to the sheer complexity of the immune response, although there has been sufficient progress to warrant continued investigation along these lines. The exclusive use of *in vitro* assays may always have limited utility as a replacement for functional assays [99, 100], although the European Centre for the Validation of Alternative Methods (ECVAM) has sponsored at least two workshops of international experts to devise testing strategies based on functional assays [101, 102]. Rather, future directions of *in vitro* immunotoxicology will almost certainly take advantage of proteomics/genomics technologies, as has already been explored with the so-called CellChip [103, 104] and adaptations of cell-based high throughput screening for biological activity as used by the pharmaceutical industry. At some point in the distant future, *in silico* methods might replace animal testing in certain cases [105].

Application of GenomicsTtechniques as Tools for Hypothesis Generation and Mechanism of Action Studies

Evaluation of xenobiotic-induced changes in gene expression as a potential method to identify and classify potential toxicants has been pursued by industry and regulatory agencies worldwide as a means to screen and prioritize chemicals for functional evaluation. The U.S. EPA recently released a white paper discussing the potential uses of genomic data for regulatory purposes and risk assessment at the agency [106], and in recent years laboratories have begun to investigate the use of toxicogenomics to detect and characterize chemical modulation of the immune response. Current goals of toxicogenomics, which would also be important in immunotoxicology, include hazard identification by comparing microarray results with analyses of SAR or animal bioassays, or risk characterization by coupling genomic data with exposure assessment or cross-species comparisons. 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 efforts suggest that biological pathways can be identified consistently across platforms but direct gene comparisons are challenging, and that genomic data alone are insufficient and should be tied to a phenotypic anchor. A workshop was held in 2005 at the Environmental Protection Agency in Research Triangle Park, North Carolina, to address the potential of genomics techniques as an alternative or adjunct to traditional screening methods for immunotoxicity. The use of genomics techniques as a screening tool 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. Workshop participants concluded that the use of genomics holds promise as a means to identify potential immunosuppressive compounds and to generate hypotheses on potential modes and mechanisms of immunotoxicity [107]. The current and future uses of genomics and proteomics techniques by immunotoxicologists are discussed in chapter 6.

CONCLUSION

In this brief survey we have tried to convey a sense of the dynamic nature of immunotoxicology, a discipline that continues to evolve and incorporate new concepts and techniques while remaining true to its core premise: to evaluate the effect of chemicals and other agents on the structure and function of the immune system. We have explored some of the main inflection points along this evolution including the establishment of a structured testing approach (the tier), the establishment of regulatory guidelines that transformed immunotoxicology from a basic science only to a powerful tool to assess the safety of new drugs and other products, the refinement of approaches to the point when true standardization and validation could occur, and a glimpse into the future of the discipline. Immunotoxicology will no doubt continue to change, but doubtless the basic structure will remain solid for the next 30 years and beyond.

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2 Immunotoxicity Hazard Identification and Testing Guidelines

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INTRODUCTION

Regulatory approaches to immunotoxicology have evolved along with the science and therefore in most cases have been promulgated relatively recently. However, the origins of some regulatory test methods, such as those to identify potential for test article to produce allergic contact dermatitis, evolved with the science of immunology itself. From a regulatory perspective, the merging of immune-based hypersensitivity issues with what has traditionally been considered immunotoxicology has mirrored the scientific understanding that adverse effects on immune function are likely multidimensional rather than linear.[1] That is, xenobiotics that induce one type of adverse immune effect can modulate immune function in other often less predictable ways. It is not the purpose of this chapter to deal with these issues, but understanding the evolution of regulatory immunotoxicology should take these concepts into consideration.

In order to understand the use and intent of the various immunotoxicology regulatory guidelines and guidance documents, the difference between two concepts familiar to toxicologists should be emphasized. *Hazard identification* refers to a method which is essentially qualitative: that is, it is designed to detect the ability of a test article to produce a certain (in the context of toxicology) adverse effect, without reference to exposure issues. *Risk assessment*, on the other hand, takes into consideration method, dose, and duration of exposure, condition(s) of the exposed population, and concurrent

environmental and physiologic issues [2]. Another aspect of risk assessment is estimation of *incidence* or *prevalence* of adverse effects associated with exposure to test article, as well as potential *severity* of adverse effects.

IMMUNE-MEDIATED HYPERSENSITIVITY

Allergic Contact Dermatitis

The first published testing guidelines in immunotoxicology appear to have been for allergic contact dermatitis (ACD). Historically, this has been a significant issue with consumer products, pharmaceuticals, and in occupational health. ACD results from the reaction of a chemical with skin proteins to form allergenic protein-hapten complexes. The biology of ACD was studied extensively by Landsteiner and Jacobs in the 1930s and represents some of the most important fundamental work in immunology [3, 4]. In 1944, John Draize and colleagues utilized Lansteiner and Jacobs' work to develop the first method for testing the potential for a chemical to induce ACD [5]. Not to be confused with the test Draize developed for ocular irritancy, this method is still accepted by regulatory agencies (although it is no longer used to any significant extent). There are two important points in considering the Draize test for ACD: guinea pigs are the animals of choice, and the end-point is a clinical sign, rather than a surrogate or mechanistic marker [6].

Many other methods have been developed, primarily using guinea pigs, but the following have generally been accepted for regulatory purposes: the guinea pig maximization test, the split adjuvant technique, the optimization test, the Freund's complete adjuvant test, the Buehler test, and the open epicutaneous test [7]. The performance of the tests vary in detail and claimed sensitivity, but all share the basic idea of the original Draize test: induction by exposing the skin to test article (by various methods), followed by challenge administration. The end-point is erythema and edema, and is scored subjectively. Basically, the tests mimic human exposure and clinical signs, and differ only in the methods used to administer the test article. The most important point to remember with all of these methods is that the experimental endpoint is essentially subjective. There are numerous regulatory documents that either mandate or allow the use of these assays for environmental chemicals, cosmetics, consumer products, foods, and drugs [8–16].

In recent years, assays have been developed in mice that represent significant advances in testing for ACD. In the 1980s, Shayne Gad and his associates developed the mouse ear swelling test (MEST) [17]. In this method, induction is achieved by topical application of test article. Sensitization is assessed by applying the test article to the ear and swelling, generally measured using a micrometer, is the experimental endpoint. The importance of this test is that the endpoint is quantitative, rather than subjective. Although the MEST has not been used extensively, it is accepted as a regulatory test method by some authorities [16].

The most important advance in ACD testing has been the development of the murine local lymph node assay (LLNA). Although the general concept originated in the 1960s,

the method as we now know it was developed by Kimber and his associates beginning in the 1980s [18, 19]. The test is relatively simple: mice are treated with test article by application to the ear on three consecutive days. Two days after the final application, the mice are injected with radiolabeled thymidine and the lymph nodes draining the ears are collected five hours later. The nodes are processed into single cell suspensions and thymidine incorporation determined by β-scintillation counting. A three-fold increase in thymidine incorporation compared to vehicle control is considered to be a positive finding. This is a quantitative, mechanistically based assay which has undergone rigorous evaluation. Initially, the assay was accepted by regulatory authorities as a *screening* assay: if a positive result was obtained, this was accepted and no further testing was necessary. A negative finding, however, required confirmatory testing using one of the standard guinea pig assays for ACD.

The LLNA was one of the first test methods evaluated by the United States Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) [20–22]. This committee, made up of representatives from various regulatory and scientific organizations in the United States, was established by Congress to expedite the evaluation and acceptance of alternative test methods, primarily to reduce, refine, or replace animal studies. The LLNA was considered by ICCVAM for a number of reasons: it did not require the use of adjuvants, it did not rely on producing skin lesions, and it was an objective, quantitative assay. After extensive assessment, the LLNA was accepted by ICCVAM as a valid method to replace standard guinea pig assays for identifying test articles that have the potential to produce ACD. It is noteworthy that the LLNA was one of the first alternative methods accepted as validated by ICCVAM, and the studies used to support acceptance of the LLNA essentially set the standard for other candidate methods. The LLNA is now accepted as a stand-alone alternative to guinea pig ACD tests by most regulatory agencies [14, 15, 23].

Photoallergy

There are a number of compounds which can be photoactivated to form haptens capable of eliciting an immune-based hypersensitivity reaction, primarily a type IV skin reaction. Photoallergy usually presents as a form of ACD [24]. The most important difference between ACD and photoallergy is that the former is associated with direct dermal exposure to a chemical whereas photoallergic reactions are frequently due to disposition in the skin following systemic (often oral) exposure [25]. Photoallergy represents a regulatory conundrum: although it is known that compounds susceptible to photoactivation to form haptens absorb light in the ultraviolet-visible (UV-VIS) spectrum, this characteristic does not appear to be a reasonable criteria to trigger testing for what appears to be a relatively rare phenomenon. Regulatory agencies have published guidances that use the following general paradigm: if the test article absorbs in the UV-VIS spectrum and pharmacokinetic studies demonstrate appreciable accumulation in the skin, nonclinical phototoxicity testing should be conducted. If the test article is found to be phototoxic, other types of studies have been either suggested or required (e.g. photoirritation, photomutagenesis, photocarcinogenicity) [26–28]. However, methods for determining

photoallergenicity potential have not been widely accepted by regulatory agencies and are not routinely expected to be conducted. The usefulness of such determinations has been questioned, and clinical studies are generally considered more useful in drug development. At this time, photoallergenicity testing should not be considered a standard nonclinical safety determination.

Respiratory Hypersensitivity

From a public health perspective, asthma is probably the most important issue for immunotoxicologists. According to the US Centers for Disease Control and Prevention (CDC), asthma is the most common long-term disease of children in the United States. In 2001, the CDC estimated that 20.3 million Americans had asthma, and 12 million had an asthma attack in the previous year [29]. According to the World Health Organization (WHO), between 100 and 150 million people around the globe—roughly the equivalent of the population of the Russian Federation—suffer from asthma and this number is rising [30]. World-wide, deaths from this condition have reached over 180,000 annually. The principal impact that immunotoxicologists have had on the study of asthma has been research providing an understanding of the role of xenobiotic-induced pulmonary immune dysfunction. This research has provided support for various environmental policies designed to reduce air pollution.

It is important to remember that respiratory sensitization and asthma are related, but not identical, pathologies [31]. Asthma is a specific syndrome which appears to have genetic as well as environmental causes and there are numerous potential triggers which have been identified by immunotoxicologists [32]. However, asthma is not the same disease as other respiratory hypersensitivity syndromes (sometimes referred to as "chemical asthma," etc.) [33, 34]. Various regulatory guidance documents have sought to deal with the latter disease entities to ensure that xenobiotics are assessed appropriately for their ability to induce immune-based pulmonary hypersensitivity reactions [35–37].

There is no animal model currently available which has been evaluated sufficiently to form the basis of regulatory guidance on testing for xenobiotic-induced respiratory hypersensitivity [38, 39]. There are a number of methods, however, that can be used for hazard identification in investigative toxicology. That is, there are methods which have proven to be useful for screening purposes, but which have not been validated for regulatory purposes. For example, the methods developed by Karol and colleagues [40] and the related tiered method of Sarlo and Clark [41] have proven to be useful, but may be limited to identifying potent sensitizers. Some of the experimental endpoints, such as specific antibody production and assessment of lung function by plethysmography, can be incorporated into standard repeat dose inhalation toxicology studies. Unfortunately, there appears to be negligible effort in this area of research, at least for the purposes of regulatory validation.

Another approach builds on the notion, widely accepted among immunotoxicologists (and probably true), that respiratory sensitizers (at least small molecular-weight chemicals) represent a subset of dermal sensitizers [42]. Thus, assays for ACD, such as

guinea pig tests and the LLNA, could serve as reasonable screening methods. There are adaptations that have been proposed using dermal induction and respiratory challenge [43]. Methods that are more mechanistically-based may prove to be valuable. For example, there are adaptations of the LLNA in which serum IgE levels and specific cytokine patterns are determined since dermal sensitizers that induce Th2-type responses are more likely to be respiratory hazards [44]. Clearly, more work is needed in this area.

Anaphylaxis

It is ironic that possibly the first animal model of relevance to immunotoxicology was reported by Portier and Richet in 1902 [45]: in an attempt to induce tolerance to a sea anemone toxin, they accidentally produced a shock reaction in dogs. Since this was not the protective effect they had hoped to produce (*phylaxis* for protection in Greek), they named the reaction *anaphylaxis* [46]. The irony, of course, is that this serious reaction, mediated by IgE in humans, has proven to be notoriously difficult to predict based on animal studies. This is no trivial issue, since anaphylaxis is a serious, life-threatening reaction associated with exposure to drugs, foods, cosmetic ingredients, and other exogenous substances [47].

Probably the most widely used animal models for hazard identification of anaphylaxis were developed in the 1950s by Zoltan Ovary [48].There are three general forms of these tests: the passive cutaneous anaphylaxis assay (PCA), the active cutaneous anaphylaxis assay (ACA), and the active systemic anaphylaxis assay (ASA) [15]. All have similar study designs, with different challenge methods and experimental endpoints. Induction is usually attempted by immunization with and without adjuvant. In the PCA, serum is obtained, injected into the skin of recipient animals, followed by intravenous administration of the test article mixed with a dye (usually Evans blue). A blue response at the site of injection is taken as an indicator of specific IgE antibody in the donor serum (due to histamine release which produces vascular leakage of the dye into the skin) [49]. Although usually conducted in guinea pigs, there are many variations. One important issue that Ovary and subsequent investigators have attempted to solve by experimental manipulation has been the issue of whether IgE was in fact being detected [50, 51]. Using the nomenclature of the time, IgE is a “homocytotropic” antibody: that is, it will only have biologic activity when injected into the same (or closely related) species. IgG is “heterocytotropic”: that is, it has biologic activity in unrelated species [52]. Thus, immunization would be attempted in one species, such as the guinea pig, and challenge would be performed in both guinea pigs and a second species, such as rabbits. A positive reaction in guinea pigs and a negative reaction in rabbits was taken as evidence that the elicited antibody was IgE. There are, of course, many problems with this concept, not the least of which being that in guinea pigs IgG_1 is reaginic (that is, capable of inducing anaphylaxis), and although these animals also produce IgE, the role of this immunoglobulin type in the PCA reaction appears minor at best [53, 54].

The PCA and ASA assays are performed in similar fashion, except that instead of using dye leakage as an indicator in the challenge phase, these studies attempt to produce skin lesions and systemic anaphylaxis, respectively, as experimental endpoints [55].

These assays were valuable in the early study of allergy and anaphylaxis. However, the immune response, especially the induction phase of allergy (antibody production), to low weight compounds is genetically restricted. Therefore, when the heterogeneous genetic background of humans is considered, the use of these methods is limited [56].

Recent advances have attempted to build on a more sophisticated understanding of anaphylaxis biology. For example, modifications of the LLNA have been described which are similar to those used to identify potential respiratory sensitizers: the test articles being proteins and the objective of the studies to identify food allergens [57]. Oral exposure studies (using Brown Norway rats) have been developed for the same purpose. Unfortunately, anaphylaxis remains a challenging issue for small molecular weight compounds and in particular for drug development [58]. Occasionally IgE responses have been observed in nonhuman primate studies with candidate biologic drugs, but both the predictive value and the dependability of these responses are questionable.

A further complication in hazard identification of anaphylaxis is that some compounds can induce what is usually referred to as an "anaphylactoid reaction." This is a type of reaction Descotes has termed "pseudo-allergy": it appears to be anaphylaxis, but does not involve compound-specific IgE [59]. This has been a particular problem with certain types of drugs: iodine- and gadolinium-containing radiocontrast media, antisense oligonucliotides, and fluoroquinolone antibiotics in particular. The basis of the reaction is degranulation of basophils/mast cells with release of vasoactive compounds such as histamine and serotonin. There are at least three known mechanisms: direct destabilization of mast cells (probably related to high osmolar and ionic strength associated especially with radiocontrast media), activation of the alternate complement pathway and production of anaphylactic products (associated with antisense oligonucleotides), and dysregulation of arachidonic acid metabolism [60]. Anaphylactoid reactions can be, for the most part, reliably modeled in animals, at least for the purpose of hazard identification [61].

Systemic Hypersensitivity

In the recently concluded writing of the guidance on immunotoxicity studies for human pharmaceuticals, the Expert Working Group (EWG) of the International Conference on Harmonization of Technical Requirements for the Registration of Human Pharmaceuticals (ICH) faced a difficult issue. In considering the comments submitted in response to publication of the Step 2 draft guidance, it was clear that many thought the document was incomplete in that it dealt only with the general issue of unintended immunosuppression and did not address obviously important topics such as drug allergy and drug-induced autoimmune reactions. There were two reasons that the EWG had not included discussion of these topics in the guidance: there was very little in the way of guidance that could be provided on the subjects, and where useful test methods could be discussed (almost exclusively concerning ACD), there was no appreciable disagreement between the regional parties, thus no need to negotiate. However, in considering these comments from experts in immunotoxicology, it was clear that an important point was being made. Upregulation of immune responses resulting in adverse effects is just as important (if

not more important) as unintended downregulation. Thus, the EWG included unintended immune enhancement as a topic of concern in drug development [62].

What, exactly, is meant by the term "unintended immune enhancement"? Immunotoxicologists have, to a certain extent, adopted the mind-set that the parameters examined for immunotoxic effects tend to be of concern when altered in, for lack of a better term, a downward direction. That is, we look at changes in immune organ weights for signs of cellular depletion, atrophy, and necrosis. However, it has increasingly been recognized that changes in either direction are important clues, and should be evaluated accordingly. This has not always been standard practice: increased lymph node, thymus, or spleen weights in standard toxicology studies rarely trigger follow-on investigation. Yet these effects are often biologically significant, and should be investigated. Although not stated explicitly in the ICH Harmonised Tripartite Guideline "Immunotoxicity Studies for Human Pharmaceuticals" (designated ICH S8) [62], this was, at least in part, the reason for inclusion of unintended immune enhancement. Signs of inflammation, for example, could be a clue that a variety of immune-related adverse effects could have occurred. One simple explanation is that the test article is an immunosuppressant and the inflammatory reaction is due to uncontrolled infection [16], Another interpretation, however, is that inflammation could be indicative of the various immunopathies that are included under the general term "systemic hypersensitivity."

Systemic hypersensitivity refers to many immune-mediated adverse reactions: types II and III immunopathies (vasculitis, pneumonitis, nephropathy, etc.), skin reactions (such as morbilliform rash following oral drug administration, IgE-mediated anaphylaxis and urticaria, toxic epidermal necrolysis/ Stevens-Johnson syndrome), and autoimmune reactions such as drug-induced lupus being among the most important [63]. Systemic hypersensitivity should not be confused with "adverse immunostimulation", although the concepts overlap somewhat. Adverse immunostimulation refers to various effects associated with activation of immune effector functions, such as cytokine release syndrome [15]. The important difference between systemic hypersensitivity and adverse immunostimulation is that the former is antigen-mediated, whereas the latter is not an antigen-specific reaction.

Unfortunately, there are few methods which can be considered sufficiently evaluated to offer specific regulatory guidance on any of these adverse reactions. As will be discussed in later chapters, there are some experimental techniques which could be useful in certain situations, however. For example, the popliteal lymph node assay (PLNA) and its many modifications could be used to assess the autoimmunogenic potential of test article if signs consistent with this possibility are observed [64]. Immunohistochemical analysis of inflamed tissues could demonstrate immune complex deposition or other signs consistent with immunopathy [65]. Assays for drug-specific antibodies are usually difficult to develop and may give misleading results even if available, but in some situations such determinations might be useful [66]. Incorporation of autoantibody screening into standard repeat-dose nonclinical toxicology studies has not been a standard practice, but is now being performed fairly often in evaluating biologic drugs (especially since the recently identified problem of serious adverse effects such as aplastic anemia associated with recombinant erythropoietin) [67]. The important issue to consider here is that changes in immune system parameters should be taken seriously and evaluated appropriately.

UNINTENDED IMMUNOSUPPRESSION

In 1978, a conference was hosted by the FDA entitled "*Inadvertant Modification of the Immune Response: The Effects of Foods, Drugs, and Environmental Contaminants.*" One presentation given by Nelson Irey of the Armed Forces Institute of Pathology, is of particular interest [68]. In his presentation, Irey made the following observations based on adverse event reporting to the FDA: of 3900 adverse drug reactions (ADR), 486 (12%) appeared to be related to the immune system. These adverse events included tumors in transplant patients, opportunistic infections (especially in patients undergoing cancer radiation and chemotherapy), and hypersensitivity reactions (such as anaphylaxis and vasculitis). This percentage (around 10%) of adverse drug reactions having an apparent immune basis has remained a remarkably stable number over the years [66]. It is instructive to consider the details of the various studies of ADRs: where immunosuppression appears to be the basis of the adverse effect, almost invariably this was related to the pharmacodynamics of the drug or characteristics of the patient population. Hypersensitivity reactions, on the other hand, were much less predictable and even in cases such as penicillin-associated anaphylaxis, there were no reliable clues that such adverse events could happen in an individual patient [69], Basically, this remains the situation today.

Almost concurrently, with the publication of Vos [70], the science of immunotoxicology began as a distinct specialty. Prior to this important publication, immunology and toxicology had developed independently for the most part. In addition, it is important to remember that most of the early concern with immunotoxicity involved environmental and occupational exposure [71]. Thus, the earliest guidelines on immunotoxicology were developed by regulatory agencies concerned with inadvertent or unintended exposure to chemicals, many of which demonstrated important toxicities unrelated to the immune system [72]. For environmental and occupational exposure (and including such issues as food additives and cosmetic ingredients), directed immunotoxicity testing of xenobiotics as a standard practice appears justified. However, in the context of drug development, this seems less needed. It is beyond the scope of this chapter to engage in an extensive discussion of this point: suffice it to say that there are two critical differences between drugs and other products. First, the risk/benefit assessment is quite different, and secondly, humans are intentionally exposed to drugs, thus an assessment of risk can be made that would not normally be the case with environmental exposure [73, 74].

Thus, the general regulatory approaches to drugs and other chemicals differ with respect to immunotoxicity testing. For environmental chemicals and food additives, a modification of the tiered approach developed by Luster and his colleagues has been adopted by regulatory agencies [75–81]. According to published guidelines, the first tier consists of the antibody response to a T cell dependent antigen (typically sheep red blood cells), although both gross and histopathologic appearance of lymphoid tissues may suggest immune system toxicity. Follow-on studies are required if decreases in antigen-specific antibody production are detected. According to ICH S8, a weight-of-evidence approach is taken to determine the need for immune function studies [62]. The following factors should be considered in evaluating drugs for potential immunotoxicity: findings in standard nonclinical toxicology studies, the pharmacological

properties of the drug, the intended patient population, structural similarities to known immunomodulators, the disposition of the drug (that is, where the drug differentially distributes to immune tissues), and clinical signs suggestive of immunotoxicity (such as increased infections). Signs in standard nonclinical toxicity studies that could trigger functional immune testing include: hematological changes, immune system anatomical effects, changes in serum immunoglobulins, increased incidence of infections, and evidence of carcinogenicity in the absence of other plausible causes. If, upon evaluation of these various factors, it appears that a drug has immunomodulatory effects or other causes for concern exist (such as intended patient population or clinical findings), the potential effect of test article on immune function should be evaluated.

It is important to note that although there seems to be an important difference between regulatory approaches taken for environmental chemicals and drugs, in fact these are relatively minimal. In practice, if no signs consistent with immunotoxicity are observed in toxicologic testing of a pesticide, at this time it is likely that a company could seek product registration without conducting an immune function assay (although as a practical matter most companies would perform these tests). It is also important to remember that for environmental chemicals, animal data would be relied upon to do risk assessment as well as hazard identification. This is not usually the case with pharmaceuticals: instead, the risk assessment is based on data obtained in clinical trials [82, 83]. For example, product labels for all marketed herapeutic immunosuppresssants used to prevent rejection of transplanted organs contain a boxed warning that use of these drugs can cause lymphomas and other cancers. However, rodent carcinogenicity studies have, for the most part, failed to predict this risk [84].

Except for issues such as risk assessment and factors that would indicate the need to determine the potential effect of test article on immune function, the various guidelines are fairly consistent in what tests are recommended. ICH S8 contains a table suggesting the parameters that should be assessed in nonclinical toxicology studies: these parameters do not differ significantly from other guidelines (see Table 2.1). The

TABLE 2.1
Table of Parameters to Evaluate in Nonclinical Toxicology Studies (from ICH S8)

Parameter	Specific Component
Hematology	Total and absolute differential leukocyte counts
Clinical Chemistry	Globulin levels[1] and A/G ratios
Gross pathology	Lymphoid organs / tissues
Organ weights	Thymus, spleen (optional: lymph nodes)
Histology	Thymus, spleen, draining lymph node and at least one additional lymph node, bone marrow,[2] Peyer's patc,h[3] BALT,[4] NALT[4]

[1]Unexplained alterations in globulin levels could call for measurement of immunoglobulins.
[2]Unexplained alterations in peripheral blood cell lines or histopathologic findings might suggest that cytologic evaluation of the bone marrow would be appropriate.
[3]Oral administration only.
[4]For inhalation or nasal route only. BALT: bronchus-associated lymphoid tissues. NALT: nasal-associated lymphoid tissues.

default test for effect of test article on immune function is the T-cell dependent antibody response (TDAR, of which the plaque assay is a type).

Follow-on studies are also recommended as needed. These include determination of potential test article effects on blood or tissue immunophenotypes (by flow cytometry or immunohistochemistry), natural killer cell, macrophage, or neutrophil function, host resistance to infection or tumors, and cell-mediated immunity. The important issue in all of these guidelines is this: do not ignore signs of immunotoxicity, and assess these findings when observed.

CONCLUSIONS

Immunotoxicology has emerged as one of the most important specialty areas of toxicology. Adverse effects of xenobiotics on immune function result in significant morbidity and mortality (e.g., asthma). Unfortunately, guidelines are written as static documents in a changing scientific environment. There are several areas that have not been adequately addressed, to a great extent because the scientific basis for writing a useful regulatory document is not mature. For example, there is considerable interest in the issue of developmental immunotoxicology, but at this time the science isn't where it probably needs to be in order to propose a guideline or guidance document [85, 86]. Technologies such as genomics and proteomics hold great promise [87, 88]. Structure-activity databases are already being used to predict such adverse effects as ACD [89]. Changes in science should be kept in mind when reading and interpreting regulatory documents.

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3 Interpreting Immunotoxicology Data for Risk Assessment

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INTRODUCTION

While profound immunosuppression can lead to an increased incidence of infectious or neoplastic diseases, interpreting data from experimental immunotoxicology studies or epidemiological studies for quantitative risk assessment purposes can be problematic. This is because inadvertent exposures to immunotoxic agents may often be expressed as a mild-to-moderate change, reflected, for example, by a 15 to 25% decrement in an immune parameter compared to control values. To help address the clinical consequences of mild-to-moderate immunosuppression, we examined available experimental, clinical and epidemiological studies that examined the association between suppression of immune function and infectious disease, independent of the etiology of suppression.

In addition, we discuss the most likely clinical consequences of mild-to moderate immunosuppression and potential confounders as well as non-immune factors that may modify these disease outcomes. Cases of profound immunosuppression, such as primary immunodeficiency diseases or HIV/AIDS, are not discussed, as these represent extreme examples of immunosuppression where neither the specific clinical diseases nor the eventual outcomes have much in common with events that occur in individuals with mild-to-moderate immunosuppression.

DISEASES ASSOCIATED WITH IMMUNOSUPPRESSION

While both infectious and neoplastic diseases are associated with immunodeficiency, infectious disease incidence is more apparent and usually the focus of epidemiological studies as it represents the most rapid consequence of immunosuppression. Identifying the quantitative relationships between altered immune responses and frequency or severity of infectious diseases in epidemiological studies is challenging, as many factors contribute.[3] This is summarized schematically in Figure 3.1, where the appearance, progression, and outcome of infectious disease is viewed as an interrelationship between the virulence of the organism, infectious dose (number of organisms required to produce illness), the integrity of the host's anatomical and functional barriers, and the overall immunocompetence of an individual. The latter, in turn, is affected by genetics as well as age, gender, use of certain medications, drug or alcohol abuse, smoking his-

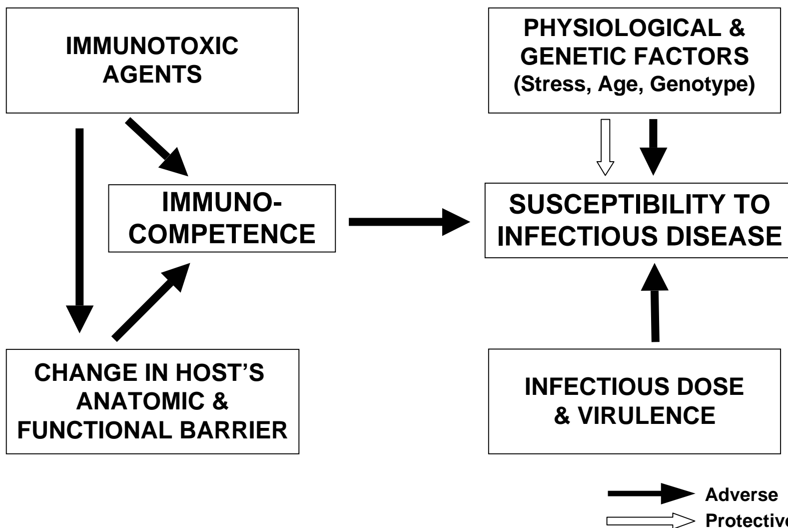

FIGURE 3.1 Schematic showing factors which influence infectious disease susceptibility.

tory, stress and nutritional status. These factors probably account for the considerable variability and large ranges reported in "normal" individuals.

The nature of the immunodeficiency can influence the type of microorganism responsible for disease progression. For example, extracellular pathogens, such as *Streptococcus pneumoniae* and *Haemophilus influenza* only multiply outside host cells, producing disease when they resist phagocytosis. Facultative intracellular pathogens (e.g., *Mycobacterium tuberculosis*) are generally phagocytized but resist intracellular killing. Thus, infections with extracellular or facultative intracellular organisms will be more frequent in individuals where impaired phagocytic mechanisms exist, such as neutropenia, or when humoral (i.e., antibody) deficiencies are present. Obligate intracellular pathogens, which include viruses, can not multiply unless they are within a host cell and are more commonly observed in individuals with defects in cellular (T cell) immunity.

Microbial agents associated with immunodeficiency disorders can also be classified as community acquired (common), opportunistic or latent pathogens. Community acquired pathogens, such as influenza, occur in the general population at frequencies associated with their infectious nature (i.e., virulence and ease of transmission). They occur in all age groups, but can be more severe in the very young or very old because of age-related immunodeficiency. Although influenza is responsible for more morbidity and mortality than any other infectious agent in recorded history, the low individual rates of common infections in the general population (only 1 or 2 episodes in an individual per year), combined with underreporting, make it difficult to detect changes in infection rates. Infections with opportunistic pathogens are less common in the general population, being more typical in individuals with profound immunosuppression such as HIV/AIDS. These microorganisms are commonly encountered in food, water, dust, or soil and include certain protozoans, such as *Toxoplasma gondii*, and the fungi *Candida albicans*, and *Pneumocystis carinii*. Latent infections, which are common but usually not clinically expressed in individuals with normal functioning immune systems, include cytomegalovirus (CMV), herpes simplex virus (HSV), and Epstein-Barr virus (EBV), all members of the herpes virus family. When the immune response is compromised, viral replication can ensue and cause disease such as cold sores. In some instances severe complications occur including CNS and ocular involvement or neonatal transmission with the development of serious postnatal infections.

Immunodeficiency has been associated with an increased incidence of viral-induced cancers, which tend to be more immunogenic than those that are chemically-induced. Cancers related to immunosuppression include leukemia and cancers of the skin (seen in transplant patients[4]) as well as Kaposi's sarcoma and EBV-associated B cell lymphomas (observed in HIV/AIDS patients).

IMMUNODEFICIENCY AND RELATIONSHIP TO INFECTIOUS DISEASE

Environmental Chemicals

Although a large number of human studies have evaluated immune system endpoints in occupationally and environmentally exposed cohorts, immune function, and infectious

outcomes generally have not been reported for the same cohort. Some studies, however, have described associations between chemical exposure, altered immune system endpoints and frequency of infections, thus providing some evidence of adverse effects associated with mild-to-moderate immunosuppression. Some of the more complete immunotoxicology studies have focused on persistent organochlorine compounds, formerly found in pesticides and industrial chemicals (e.g., polychlorinated biphenyls (PCBs)), in children following prenatal or postnatal exposure (via maternal diet and breast milk). Studies of accidentally exposed populations in Japan (Yusho) and China (Yu-Cheng) suggested an association of PCBs, their thermal breakdown products (quaterphenyls) and polychlorinated dibenzofurans, with immune abnormalities and increased infections. Children born to exposed mothers between 1978 and 1987 in the Yu-Cheng study group had lower levels of serum IgA and IgM and a higher frequency of respiratory infections and otitis media compared to matched, unexposed controls.[5–7] Similar results have been observed in the Yusho study population.[6] Recurrent respiratory infections and elevated blood levels of pentachlorophenol (PCP) were associated negatively with lymphocyte counts, CD4:CD8 ratios, and absolute counts of CD3+, CD4+, CD16+, CD25+, DR+, CD8+56+ and CD19+ cells.[8]

The association between PCBs and increased frequency of otitis media in children has also been described in other populations. A study of 343 children in the United States (Michigan), while not showing a general association between organochlorine levels and prevalence of infections, revealed a positive association between burdens of PCBs and DDE (the primary metabolite of DDT) or PCBs and hexachlorobenzene with otitis media.[9] In a study of Inuit infants in Artic Quebec, Canada,[10] the relative risk of recurrent episodes (at least three per year) of otitis media was higher in breast fed infants in the second and third highest tertiles of organochlorine exposure, compared to the lowest. At 3 months of age, breast fed infants with higher exposure levels had lower numbers of white blood cells and lymphocytes, and lower serum IgA levels at ages 7 and 12 months compared to bottle fed infants. In Dutch preschool children,[11] PCB levels in breast milk (non-ortho and coplanar PCBs) were also associated with increased recurrent otitis media and other symptoms of respiratory infection. In this sample, the body burden of PCBs at age 42 months was associated with a higher prevalence of recurrent otitis media and chicken pox. PCB body burden was not associated with differences in lymphocyte markers outside the normal range for age-matched children, although levels in breast milk and cord blood were positively correlated with lymphocyte counts and various T-cell subsets. Recent PCB exposure was found associated with decreased vaccination responses.[12]

The immunotoxicity of pesticides following human exposure has been reviewed previously.[12-15] One of the more comprehensive community-based studies examined a large ($n = 1600$) and well-defined population living near a priority Superfund site close to Aberdeen, North Carolina that contained organochlorine pesticides, volatile organic compounds, and metals. Compared to a neighboring community, residents of Aberdeen, ages 18 to 40, were found to have a higher incidence of herpes zoster (reactivated herpes infection causing shingles).[16] In a sub-study of 302 individuals, those living in Aberdeen had significantly higher age-adjusted levels of plasma DDE than those living in neighboring communities. Furthermore, higher levels of plasma DDE were related to lower lymphocyte responses to mitogens, and higher absolute lymphocyte counts

and serum IgA levels.[17,18] In a separate analysis, residents living nearer to the Aberdeen Superfund site had both a lower lymphocyte response to mitogen stimulation and a greater likelihood of having a lower percentage of CD16+ (NK) cells (<8%, the lower limit of the normal reference range).[19] The association seen with reactivated herpes infection is plausible in light of these changes, given that NK cells play an important role in the generation of cytotoxic T-cells required to help control viral infections.[20] In a study of highly exposed workers by the same authors, plasma DDE concentrations > 6 μg/ml were associated with decreased serum IgG levels.[17]

Chronic Stress

Chronic psychological factors (i.e., stressors), such as separation and divorce, care-giving for Alzheimer's patients or bereavement, produce low-to-moderate degrees of immunosuppression and increased incidences of infectious disease. In one of the few examples of human challenge studies, 394 healthy individuals were assessed for psychological stress and subsequently administered nasal droplets containing respiratory syncytial virus (RSV) or coronavirus.[21] The rate of respiratory infections ($p < 0.005$) and clinical colds ($p < 0.02$), as determined by virus-specific antibody levels and viral isolation, increased in a dose-responsive manner with increasing degrees of psychological stress. Although usually conducted in small cohorts, immune testing in chronically stressed individuals has also provided insights into the relationship between mild-to-moderate immunosuppression and disease.[22–23] In chronic stress populations showing an increased rate of infections, total circulating T cell numbers can be reduced to as much as 20% below mean control values, while the number of circulating B cells remain unaffected. Furthermore, CD4:CD8 ratios can be reduced as much as 40% and NK cell activity by 10 to 25% below mean control values. However, these changes can still be within the range of reported normal values. Associations have also been observed between chronic stress and reactivation of latent viruses, such as CMV, HSV-1 or EBV, as measured either by clinical disease or elevations in viral-specific antibody titers and this may be one of the more sensitive effects of mild-to-moderate immunosuppression.[24–29] (A comprehensive review of the association between stress, altered immune function and resistance to infectious disease, is presented by Yang and Glaser in chapter 29.)

Hematopoietic Stem Cell Transplantation (HSCT)

HSCT, which came into general practice in the 1980s, is employed in the treatment of certain hematological malignancies, aplastic anemia, and inborn genetic errors of cells originating from hematopoietic stem cells. Following cell grafting, immunodeficiency can persist for well over a year due to pre-grafting radiation treatment.[30] Thus, prospective studies can help identify quantitative relationships between immune function and disease as the immune system recovers. The incidence of infections can exceed 80% during the first 2 years post-engraftment with 50% of the patients having three or more infections. Opportunistic infections predominate, with fungi being the most common agent to cause disease, followed by bacteria and viruses.[30–31] Incidence data for upper-

respiratory infections is generally unavailable for these patients as these infections are seldom monitored in allogeneic bone marrow recipients. Although infections that occur in the first month following transplant are most likely due to neutropenia, later infections appear to be due to deficiencies in CD4+ T cells and B cells.

In a prospective study involving 108 transplant patients that were followed between days 100 and 365 post-engraftment, decreases in B, CD4+ and CD8+ T lymphocytes and total mononuclear cells were associated with an increase in infectious disease incidence ($p < 0.05$).[32] A smaller but more detailed study[33] evaluated 29 patients for 180 days preceding the one-year post-transplant exam using an infectious score that incorporated both frequency and severity. These studies showed a highly significant inverse correlation, ($p = 0.005$, in univariate analysis), between activated CD4+ T cell counts and total infectious score, but not with CD8+ T cell numbers, B cell numbers, serum immunoglobulin levels or delayed hypersensitivity responses (Figure 3.2). By comparing the efficacy of allogeneic marrow transplantation to blood stem cell transplantation,[32] it was demonstrated that a 1.7-fold lower rate of infections in blood stem cell transplants corresponded to approximately 4-fold higher CD45RA^{high} CD4+ (naive) T cell counts and 2-fold higher CD45RA^{low} CD4+ (memory/effector) T cell counts. In studies[34] that monitored immune cell recovery following bone marrow cell transplantation, the incidence of infection also was inversely correlated with CD4+ cell counts. However, only opportunistic infections were monitored and were almost exclusively present in patients considered severely immunosuppressed, (i.e., with CD4+ T cell counts of < 200 cells/mm^3).

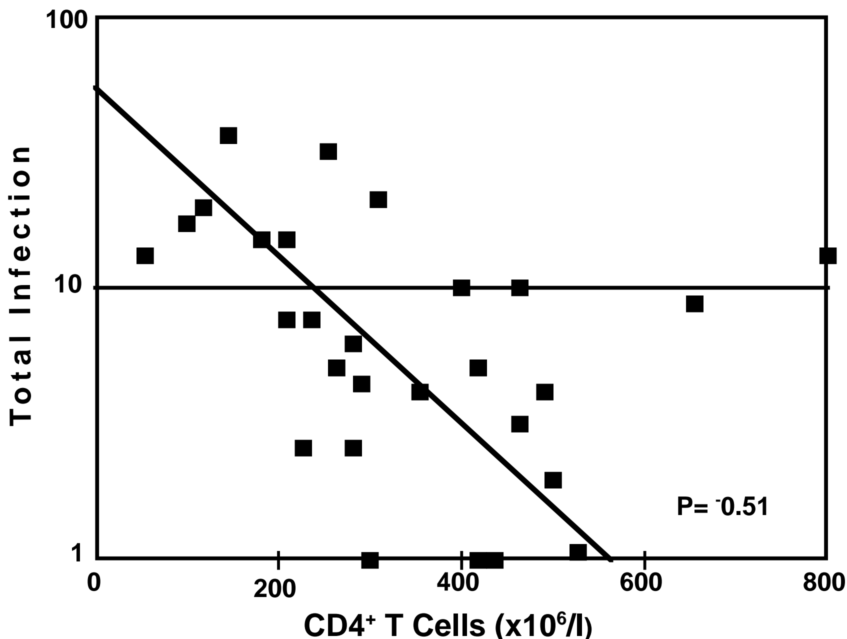

FIGURE 3.2 Twenty-nine patients were followed for 180 days preceding the one year post-transplant exam for CD4+ T cell counts and total infection score, which includes frequency and severity.[33]

Organ Transplant Patients

Studies in renal organ transplant patients have provided some insight into the long-term consequences of mild-to-moderate immunosuppression. While immunosuppressive therapies have greatly improved over the past 40 years, transplant patients are still predisposed to considerably higher rates of malignancies and infections than the general population. Infection rates range between 65 and 70% during the first 6 months post-transplantation, with CMV representing anywhere from 18 to 67% of the reported infections.[35] Increased skin cancers have also been noted in patients on long-term immunosuppressive therapy. For example, the risk of developing skin tumors following renal transplantation is 10% after 10 years and 40% after 20 years, while the incidence of squamous and basal cell carcinomas is 10- and 250-fold higher, respectively, than in the general population.[36] In examining 478 renal transplant patients, it was shown that the risk of lymphomas during the first 6 months post-transplantation increased proportionally with the intensity of immunosuppressive therapy.[37] In renal transplant patients, reduced IgG_1 subclass levels and CD4 T cell counts were the best predictors for infections (frequency of infections increased from 9% in patients with normal values to 38% with lower values).[38] Studies from a small cohort reported a reduction ($p < 0.04$) in the number of serious viral infections that occurred in transplant patients when the level of CD3+ lymphocytes were maintained above 500 cells/mm^3.[39]

PUBLIC DATABASES

In studies by the authors that are currently underway (Parks et al., submitted), we analyzed the relationship between commonly measured immune parameters (i.e., white blood cell counts and differentials) and self-reported history of cold sores (herpes labialis) over a 12-month period using data collected from the National Health and Nutrition Examination Survey.[40] Analyses were adjusted for HSV-1 antibodies to control for history of initial exposure to the virus and models were run to examine potential confounding or effect modification by other factors that might impact HSV-1 reactivation (e.g., smoking, history of upper respiratory infections). The study findings suggested that, even within the normal ranges, decreases in the peripheral neutrophil and increases in lymphocyte numbers are related to susceptibility to HSV-1 reactivation. Currently, a more comprehensive database, including immunophenotypes and self-reported coldsores, provided by the Multicohort AIDS centers,[41] is being evaluated in an attempt to confirm these findings.

EXPERIMENTAL ANIMAL STUDIES

Immunotoxicology data most often available for use in risk assessment is derived from experimental animal studies. Although animal models provide an opportunity to establish more reliable exposure estimates and conduct more informative tests than human studies, the level of accuracy that can be achieved using such data in extrapolating to humans is often a matter of debate. In immunotoxicology testing, a set of tests usually referred to

as "host resistance assays" have evolved in which groups of experimental rodents are challenged with either an infectious agent or transplantable tumor, at a challenge level sufficient to produce either a low incidence or minimal infectivity in the control group. As the endpoints in these tests have evolved from relatively nonspecific protocols (e.g., animal morbidity and mortality), to continuous measures, such as number or size of tumor foci, viral titers or bacterial cell counts, the sensitivity of these models has increased, although they are still limited by the number of animals that can be realistically devoted to a study (Table 3.1). While there have been considerable efforts to validate specific immunological endpoints, such as antibody responses,[42] histopathology,[43–46] quantitation of cell surface markers by flow cytometry,[47–49] or cytokine production,[50–51] there have been only two programs that have evaluated the sensitivity and predictive value of individual measures of immune outcomes with host resistance tests. These included studies conducted under the auspices of the U.S. National Toxicology Program (NTP)[52] and a smaller effort undertaken at the National Institute of Public Health and the Environment in the Netherlands which focused on the rat and was based on the OECD #407 guideline.[53–54] In both programs, host resistance tests were usually considered in a second or third testing level (i.e., tier) of evaluation, and were only performed when there were indications of alterations in a previous tier. However, data obtained from these programs indicated that host resistance assays for the most part was highly correlated with immune tests but were unlikely to detect subtle immunosuppression due to relative differences in sensitivity of the test models.

While it is rare for a single component of the immune system to be solely responsible for resistance to a specific infectious agent, certain immune measures correlate with the outcome of a host resistance assay. For example, reduced NK cell activity correlated with increased susceptibility to challenge with PYB6 sarcoma cells, B16F10 melanoma cells and murine CMV.[52;55–56] Suppression of cell-mediated immunity, complement deficiency and depressed macrophage and neutrophil function have been associated with decreased resistance to challenge with L*isteria monocytogenes*.[55;57–58] Clearance of

TABLE 3.1
Commonly Employed Experimental Disease Resistance Models.*

Challenge Agent	Endpoint Measured
Listeria monocytogenes	Liver CFU§, Spleen CFU, Morbidity
Streptococcus pneumoniae	Morbidity
Plasmodium yoelli	Parasitemia
Influenza Virus	Morbidity, Viral titer/tissue burden
Cytomegalovirus	Morbidity, Viral titer/tissue burden
Trichinella spiralis	Muscle larvae and adult parasite numbers
PYB6 Sarcoma	Tumor Incidence (subcutaneous)
B16F10 Melanoma	Tumor Burden (Lung nodules)

*For details[59,65–67]
§Each bacterial colony growing on artificial culture medium is assumed to arise from a single organism; colony forming unit (CFU) values therefore reflect the number of viable organisms recovered.

parasitic infections, such as *Plasmodium yoelii* and *Trichinella spiralis*, which involve both cellular and humoral components, are associated with depression of both arms of the immune system.[59–60] In studies conducted by the NTP, concordance between individual immune tests were compared to host resistance tests and found to range from relatively good (e.g., antibody plaque forming cell assay; 73%; NK cell activity 73%; and delayed type hypersensitivity response; 82%) to poor (e.g., lymphoproliferative response to LPS; < 50%).[45;52]

Deletion or "functional blocking" of specific immune components in experimental animals has also been used to elucidate the relative contributions of immune components to disease resistance.[61] This has been achieved via targeted gene disruption, resulting in animals deficient in a specific cell population or soluble mediator which contributes to host defense (e.g., CD4+ T cell knockouts), treatment of normal animals with selective toxic agents (e.g., the use of gadolinium chloride to block macrophage function) or administration of neutralizing antibodies against critical cell-specific surface receptors. Studies in which NK cells were depleted with antibodies to asialo GM1 demonstrated that at low levels of tumor challenge, a reduction of approximately 50% or more in NK cell activity was required before significant effects on resistance to NK-sensitive tumors were observed.[62] These studies also demonstrated that the level of suppression needed to alter host resistance was related to the challenge level of the tumor cells. In contrast, studies that have used monoclonal antibodies to effectively deplete CD4+ and CD8+ T lymphocytes from the peripheral blood have found little evidence of altered resistance to challenge with PYB6 sarcoma cells, a model considered to be dependent on cell-mediated immunity.[63] This may be due to the overlapping function of individual immune components or reflect the lack of effects of cell populations in the secondary lymphoid organs. Recently it was suggested that monitoring several immunological parameters concurrently provides information that might not be evident from studies using single tests.[64] Using dexamethasone, these authors demonstrated that, contrary to what might be expected based on the compound's suppressive effects on several immune parameters, relatively high levels of dexamethasone were required to decrease resistance to *Listeria monocytogenes*. At doses that suppressed many immune parameters, an increase in neutrophil numbers and nitrite production by peritoneal macrophages was observed, suggesting compensation for decrements in other immune parameters so that overall resistance to the pathogen was not compromised.

CONCLUSIONS

In contrast to profound immunosuppression, such as that which occurs in patients with HIV/AIDS or primary immunodeficiency diseases, exposure to immunotoxic chemicals or drugs is believed to be more likely to cause mild-to-moderate levels of immunosuppression (e.g., a 20% decrease in white blood cell counts). This review attempts to address, both qualitatively and quantitatively, the potential adverse health effects of moderate levels of immunosuppression. The following general conclusions can be surmised.

Considerable data is available suggesting that mild-to-moderate immunosuppression can lead to an increase in infectious disease. The types of infections that occur tend to result from either common pathogens (e.g., causing upper respiratory tract infections) or latent viruses (e.g., herpes cold sores), rather than opportunistic organisms such as *Pnuemocyctis carinii*. These are usually not life-threatening, except in certain susceptible populations, such as the elderly. Opportunistic infections, in contrast, are more prevalent in individuals where severe forms of immunosuppression are present, such as primary immunodeficiency diseases or HIV/AIDS.

Infectious disease studies are normally conducted over a short time, providing only snapshots and do not provide information on the consequence of chronic immunosuppression. The adverse health effects of chronic, low level immunosuppression have been addressed to some extent in transplant patients, primarily kidney transplants, who demonstrate an increase frequency of certain immunogenic tumors.

The major gap in clarifying the shape of the dose-response curve (i.e., between immune response and disease) is a lack of large scale epidemiological studies in populations with mild-to moderate immunodeficiency that have been monitored simultaneously for immune system parameters and clinical disease. Attempts in conducting such studies will be complicated by many non-immune factors which can affect infectious disease incidences.

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4 Mechanisms of Immunotoxicity

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INTRODUCTION

Over the last three decades, there has been considerable interest in the immune system as a potential target organ of toxicity following exposure to drugs, chemicals, or environmental pollutants, collectively referred to as xenobiotics. As a result, immunotoxicology, which focuses on the immune system as a "target organ," has emerged to detect, quantify, and interpret the adverse interactions of xenobiotics with the immune system. Immunotoxicology is relatively new, with the first review of the field published by Vos in 1977 [1] and regulatory guidance for immunotoxicology testing occurring within the last decade.

The immune system is a very complex and regulated organ system that involves the cooperation and interaction of a number of different cell types, cell products, tissues, and organs. The immune system consists of fixed primary (i.e., thymus and bone marrow) and secondary (i.e., spleen, lymph nodes, and gut-associated) lymphoid tissue, and various circulating immunocompetent cells. This unique organization may contribute to the immune system's vulnerability as a target organ for xenobiotics. For example, cells of the immune system undergo continual proliferation and differentiation for self-renewal to maintain immunocompetence and thus can be affected by xenobiotics that alter this cellular balance. In addition, data indicate that immune responses can

be regulated by other organ systems such as the nervous and endocrine systems [2–4]. Although the immune system is not typically thought of as a primary organ involved in the absorption or metabolism of xenobiotics, parts of the immune system (e.g., gut-associated lymphoid tissue) receive significant initial exposure after ingestion and immune cells have been reported to metabolically activate xenobiotics [5], and thus along with the liver, may play an important role in mediating a particular metabolites effect on immunocompetence.

Since immunocompetence is dependent on a series of complex, time-dependent cellular and cell product interactions, there are numerous potential target sites within the immune system that a xenobiotic may directly or indirectly (i.e., through interactions with other organ systems) affect and thus alter the regulatory function of the immune system, and ultimately immunocompetence (Figure 4.1). The interaction of xenobiotics with the immune system may result in several immunotoxic alterations: immunosuppression leading to alterations in host defense mechanisms against pathogens or neoplasia; uncontrolled proliferation (leukemias and lymphomas); or dysregulation of the immune response (i.e., allergy/hypersensitivity reactions; autoimmune reactions) [6]. The objective of this chapter is to describe the direct and indirect mechanisms that may contribute to the immune system as a target organ for xenobiotics. Importantly, many xenobiotics may affect the immune system by both direct and indirect mechanisms depending on the dose. Due to space limitations, only a select few xenobiotics will be discussed to illustrate prototype agents exhibiting different mechanisms of immunosuppression. A more comprehensive review of the immunotoxicity of various xenobiotics can be obtained from several excellent reviews [7,8]. In addition, the role of the immune system in producing immune-mediated disease (i.e., hypersensitivity, allergy, or autoimmunity) will be briefly discussed. Further detail regarding hypersensitivity and autoimmunity is provided in Section IX of this book.

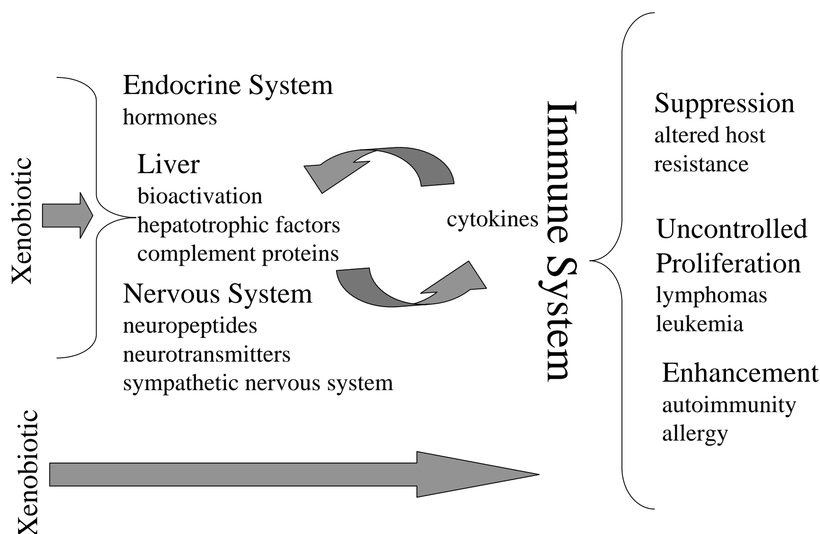

FIGURE 4.1 Scenarios by which chemicals, drugs and other xenobiotics may lead to alterations in immune function.

IMMUNOSUPPRESSION

There are two basic mechanisms by which xenobiotics may induce suppression of the immune system: (1) by direct action of the xenobiotic upon the lymphoid organs or cells involved in the immune response and (2) by indirect action of the xenobiotic on other organ or physiological systems, such as neuroendocrine interactions, metabolic activation of xenobiotics to toxic metabolites, or hepatic modulation, which then impact the immune response. Xenobiotics that suppress the immune system may display lympholytic, antiproliferative, or immunomodulatory effects (e.g., reduced cell number or organ weights; decreased production or release of cytokines; alterations in expression of surface receptors; alterations in immune cell communication), or a combination of these effects. A unique characteristic of the immune system is the ability of immunocompetent cells to be removed from the host, separated into various cell populations using a number of different techniques [9], and functionally evaluated *in vitro*. This allows for the conduct of a number of *in vitro* or *ex vivo* experimental approaches (e.g., cell stimulation and secretion, phagocytosis, separation-reconstitution studies utilizing the *in vitro* stimulation of specific antibody production) to identify the primary cell type(s) targeted by a xenobiotic and to help distinguish between xenobiotic-induced direct or indirect effects on the immune system. Immunotoxic compounds that act indirectly on immune cells will not have an effect on an *in vitro*-generated immune response. Lymphocyte subpopulations can also be enumerated via flow cytometry using fluorescently labeled monoclonal antibodies specific for cell surface markers [10], which permits the identification of the immune cell subtypes that are targeted by a xenobiotic. In addition to the evaluation of cell surface markers, flow cytometry can be used to further assess mechanisms as they relate to immunotoxicity [11].

Direct Effects of Xenobiotics

Xenobiotics may directly affect immune function (humoral, cell-mediated, innate, or host resistance); the size, composition (e.g., alterations in the numbers, or differentiation and maturation of B- or T-lymphocytes), or architecture of lymphoid organs; hematological parameters; cytokine production and/or release; the expression of receptors or ligands on the surface of immune cells; and receptor mediated signal transduction [8]. An example of direct acting xenobiotics is the cannabinoids. Cannabinoids have been reported to decrease the cellular and humoral immune responses, NK activity, and host resistance [12]. Cannabinoids are believed to affect the immune system through their interaction with a pertussis toxin-sensitive Gi-coupled cannabinoid receptor on lymphoid cells. Cannabinoid receptor transcripts have been reported in human tonsils, macrophages, spleen, and lymphocytes. [12]. Following binding, cannabinoids inhibit adenylate cyclase and thus prevent an increase in the intracellular cAMP that is associated with lymphocyte stimulation. A thorough discussion of cannabinoid-induced immunotoxicity is presented in chapter 32 of this volume.

Immunotoxicity of inorganic metals (e.g., lead, arsenic, chromium) may also occur via direct effects on the immune system or by inhibiting immunoregulation, which

may then result in immunosuppression and decreased host resistance, autoimmunity, or hypersensitivity [13–15]. Detailed discussions of lead and arsenic immunotoxicity are presented in chapters 13 and 17 of this book, respectively. Interestingly, exposure to high concentrations of metals in general results in immunosuppression, whereas, at lower exposures, immunoenhancement often occurs [1,16].

Indirect Effects of Xenobiotics

Neuroendocrine Modulation

In evaluating currently available data on endocrine active compounds (EACs), the Risk Assessment Forum of the EPA reported that the principal health effects following exposure to EACs included carcinogenesis, reproductive toxicity, neurotoxicity, and immunotoxicity [17]. A highly complex bidirectional interrelationship between the immune and neuroendocrine systems has been reported [2–4] and is discussed in detail in chapter 30 of this book. Nerve fibers containing neuropeptides and neurotransmitters are observed in various lymphoid tissues where they directly contact immune cells such as lymphocytes [2]. Conversely, products of the immune system (i.e., cytokines) have been reported to affect neuroendocrine functions [18], while various hormone receptors have been found on immune cells and a number of hormones have been reported to enhance (e.g., growth hormone, thyroid stimulating hormone, and prolactin), attenuate (e.g., gonadal steroids and endogenous opioids), or suppress (e.g., glucocorticoids and adrenocorticotropin) responses of the immune system [19]. Immune cells have also been reported to produce various peptide and protein hormones such as growth hormone, prolactin, luteinizing hormone, thyrotropin-stimulating hormone, and adrenocorticotropin [20]. Immune cell function is altered following the exogenous addition of neurotransmitters, neuropeptides, or cytokines *in vitro*. Thus, if a xenobiotic found in the environment alters the production or release of neurotransmitters and neuropeptides, it may also alter the function of the immune system [2].These interactions are discussed in detail in chapter 30. There is increasing concern that many environmentally persistent xenobiotics such as insecticides, herbicides, fungicides, as well as several industrial chemicals (e.g., polybrominated biphenyl, sytrenes, lead, mercury, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD]), may mimic or antagonize endogenous hormones and adversely affect not only the endocrine and reproductive systems but also the immune system [17,21]. Laboratory studies with EACs suggest that the reproductive and endocrine systems, and not the immune system, are the primary target organs of toxicity in young adult rats [22–24]. Further studies, however, are needed to evaluate the effects of low-dose chronic exposures to EACs on humoral, cell-mediated, and innate immunity as well as the developing immune system. Administration of xenobiotics, particularly at high doses that induce significant alterations in body weights, may also lead to a stress-induced, nonspecific immunotoxic response due to increased levels of circulating adrenal glucocorticosteroids (e.g., corticosterone), which have potent immunosuppressive activity [25].

Hepatic Modulation

The liver is a common target organ for injury resulting from exposure to various xenobiotics and has been reported to modulate immune responses through the release of a number of factors, such as acute-phase reactive proteins, L-arginase, or low density lipoproteins [26]. Following hepatic injury induced by xenobiotics (e.g., carbon tetrachloride, phenobarbitol, ciprofibrate, or α-hexachlorocyclohexane), a regenerative process is initiated by the release of a number of hepatotrophic mediators into the serum [26]. Such factors include complete hepatic mitogens [i.e., transforming growth factor-α, epidermal growth factor, hepatocyte growth factor/hepatopoietin A (HGF), hepatopoietin B, and acidic fibroblast growth factor], a number of co-mitogenic hepatotrophic factors, and transforming growth factor-β1 (TGF- β1) [26]. In addition, other factors such as α-fetoprotein or prostaglandin E2 are released but do not cause hepatocyte proliferation [27, 8]. A number of these factors have been reported to be immunomodulatory. For example, CCl_4-induced immunosuppression has been reported to be secondary to hepatotoxicity through the release of serum-borne hepatotrophic factors such as TGF-β1 [26]. CCl_4-induced serum factors were also found to increase the functional activity and number of B-cells in the spleen, while Con A-activated spleen cell supernatants from CCL_4-treated mice produced larger amounts of IL-2 than untreated spleen cells [26]. α-Fetoprotein also suppresses T-cell-dependent immune responses. Conversely, HGF may indirectly cause an increase in the number and activity of B cells, while other hepatotrophic factors have been reported to induce the proliferation of immune cells [26]. Thus, certain liver-derived factors released following liver damage can have both enhancing and inhibitory influences on immunocompetence depending on when the immune response is initiated relative to the hepatic damage [26].

Metabolic Activation

Xenobiotics may exert an indirect action on the immune system by being metabolically activated into toxic metabolites. An example of a xenobiotic requiring bioactivation is cyclophosphamide (CYP), the prototypical member of a class of drugs known as alkylating agents [29]. Upon entering the cell, the inactive, noncytotoxic drug is converted into phosphoramide mustard, a DNA alkylating agent that inhibits cell replication. CYP-induced immunosuppression involves a general reduction in lymphocytes as well as alterations in lymphocyte function [30]. CYP is often used as a positive control in immunotoxicology studies because it has been demonstrated to suppress both humoral and cell-mediated immune responses [31]. Benzene exposure results in bone marrow toxicity, and alters hematopoietic cell profiles and immune function [32], but data indicate that benzene-induced effects are due to reactive quinone intermediates that result from benzene metabolism [33,34]. Similarly, aflatoxin B1, a mycotoxin derived from fungi, requires metabolic activation in order to produce immunotoxic effects [35; see chapter 18 for a detailed discussion].

Data suggest that extrahepatic tissue (i.e., the immune system) may also play an important role in the *in situ* biotransformation of xenobiotics, such as polycyclic aromatic

hydrocarbons (PAHs) [5]. The macrophage was identified as the immune cell subtype capable of metabolizing the PAH benzo(a)pyrene (B(a)P) within the murine spleen [36]. Consistent with this finding are several studies demonstrating that the major cell type affected following exposure to B(a)P is the macrophage [5]. Macrophages have also been reported to metabolize certain drugs by a cytochrome P-450-dependent mechanism. Aryl hydrocarbon hydroxylase (AHH) has also been reported to be present and inducible in monocytes, while human peripheral blood lymphocytes and lymphocytes from a number of species have been shown to possess cytochrome P-450 [5].

Although the metabolic capacities of extrahepatic tissue, including cells of the immune system, are substantially lower than the liver, metabolic activation of xenobiotics in potential target tissues, such as the spleen or other immune organs, may play a critical role in determining immunotoxicity. The metabolic activation of xenobiotics in immune tissues may result in the formation of reactive electrophilic metabolites, which may then bind to cellular nucleophilic target sites such as DNA, RNA, and proteins that are important in mediating an immune response or maintaining cellular homeostasis [5].

DEVELOPMENTAL CHANGES THAT IMPAIR IMMUNE FUNCTION

The immune system has been identified as possibly having heightened susceptibility during maturation [37, 38, see also chapter 21 this volume]. Data suggest that some xenobiotics may cause effects that are more persistent or severe than those observed in adults (qualitative differences), or may induce immunotoxicity at lower doses (quantitative differences). The chronology of immune ontogeny and the development of normal immune capacities are important in understanding the susceptibility of the immune system during development. The development of the immune system during the gestation and neonatal periods is a dynamic process involving proliferation and differentiation of immune cells as compared to that of adults. Although the effects of xenobiotics on developing offspring have been reported, our ability to detect changes before maturation of the immune system is hampered by a lack of validated assays. Therefore, most of these studies have exposed animals (predominantly mice) *in utero* to xenobiotics and evaluated potential developmental immunotoxicity based on an assessment of the immune status of adult animals [39, 40]. A number of recent publications have reported on the most appropriate methods to assess developmental immunotoxicity [41–43], and this topic is discussed in detail in chapter 20 of this volume. These reports suggest the rat as the species of choice for screening for developmental immunotoxicology (DIT) potential and that an exposure design encompassing all critical windows of immune system development, which can then be integrated into existing developmental toxicity protocols, be employed to evaluate the DIT potential of xenobiotics.

IMMUNE-MEDIATED DISEASE

If one considers the immune system as a continuum, dysregulation of immune function can also be expressed in the development of immune mediated diseases via enhanced

immune responsiveness. These immune mediated disorders are commonly thought of in terms of *allergy* and *autoimmunity*. The mechanisms for these immune responses result from heightened responsiveness or loss of tolerance, and it is generally accepted that pathogenesis requires a combination of genetic and environmental factors. While there is complexity resulting from the interaction of susceptibility factors (e.g., health status, socioeconomics, lifestyle), at least 25 loci have been associated with allergy (or asthma) susceptibility [44]. Likewise, forms of autoimmunity have been associated with major histocompatibility complex (MHC) restrictions; autoimmune hepatitis can be associated with HLA DR3 and DR4 for smooth muscle and antinuclear antibody, respectively, and HLA DR7 for microsomal antibody [45]. Polymorphisms that impact metabolism may be a less obvious consideration but also could play a role in autoimmune reactions as the availability and reactivity of such xenobiotics as procainamide and hydralazine (acetylation), and D-penicillamine and sulfonamides (poor sulfoxidizers), might be altered [46,47]. While the specific mechanism(s) for the development of immune-mediated diseases remain vague, general attributes of most xenobiotics linked to immune dysregulation include chemical reactivity, resulting in the formation of neo-antigens, and the production of (pro)inflammatory mediators. Autoimmunity and autoimmune diseases are discussed in detail in Section VII of this book.

Xenobiotic allergy is most frequently attributed to a hypersensitivity reaction towards a chemical-specific, haptenized molecule. Hypersensitivity towards xenobiotics generally presents itself either as contact dermatitis, occurring 24–72 hours after dermal exposure, or as immediate, systemic allergy (e.g., urticaria, asthma) occurring soon after exposure. While CD8+, cytotoxic T-cells and Th1 cells are critical to allergic dermatitis and eczema reactions, CD4+ Th2 cells and IgE are primarily associated with Type 1 allergic responses [48]. Haptenation of a low molecular weight (LMW) xenobiotic serves as the initial event in the mechanism of hypersensitivity. Allergenic LMW chemicals are generally reactive and can bind to endogenous proteins (e.g., serum albumin, kerotinocyte proteins) following penetration through dermal and epithelial barriers to form new antigens.

In some cases xenobiotics (e.g., sulphamethoxazole) can be considered as “prohaptens” which need to be metabolized before binding endogenous proteins [49]. Likewise, Cheung and colleagues [50] reported the bioactivation of cinnamic alcohol into the protein-reactive chemical cinnamaldehyde, a well accepted skin sensitizer. In the case of photoallergic contact dermatitis, a “photohapten” becomes activated via ultraviolet A irradiation and binds to proteins, including those on the surface of antigen presenting cells, leading to the sensitization of specific T-cells [51]. A preference for particular amino acids (e.g., cysteine) has also been reported for reactive haptens [52]. For example, ofloxacin (a halogenated quinolone) demonstrates a propensity for binding to lysine residues found within the sequence of MHC class II [53].

Details on the cellular immune responses occurring following the recognition of xenobiotic haptens as antigens by the immune system are described in chapters 33–35 of this volume. Ultimately, a certain combination of mediators is selectively activated and subsequently helps determine and differentiate the characteristic immune response (e.g., Th1 vs. Th2). For example, dermal sensitizing chemicals (e.g., oxazolone and dinitrochlorobenzene) elicit a higher proportion of Th1 cytokines such as IFNγ and

IL-2 [54],which helps drive immune reactions primarily mediated via CD8+ T-cells. Conversely, diisocyanates and anhydrides are classes of chemicals associated with Th2 allergic responses that depend on cytokines such as IL-4 and IL-10, and the prototypic mediator of Type 2 allergic reactions, IgE antibody. These responses should not be considered all or none, but rather degrees of Th1/Th2 balance. Much remains unknown regarding the characteristics of these specific responses. Using diisocyanates as an example, both human and animal data demonstrate diversity in immune responses where IgE and cytokine profiles are concerned [55–57]. In cases of toluene diisocyanate (TDI) induced occupational asthma, the involvement of cells such as neutrophils, eosinophils and CD8+ T-cells support the possibility of non-Th2 mechanisms [58,59]. Partially due to limitations in the identification of hapten conjugates, the accurate detection of chemical-specific IgE antibody via immunoassay methodology is frequently questioned. Understanding the specifics of haptenation has contributed to difficulties in clearly elucidating mechanisms for low molecular weight xenobiotic hypersensitivity and thus, predictive models for hazard characterization. There are many more reactions that play a role in the generation of sensitization responses but which extend beyond the scope of this chapter (e.g., tissue remodeling, neuronal intervention and mediator release) [60,61]. This brief review greatly oversimplifies the current knowledge associated with allergic immune responses towards xenobiotics.

On the whole, autoimmune reactions are more diverse than allergy and are reviewed in detail in Section VII of this volume. Thus, understanding the pathogenesis of autoimmunity is a bit of a misnomer and not a matter of defining a single series of cellular and molecular events. Despite this, the immunological reactions for any given autoimmune response are similar to those identified for allergic responses, and "autoallergy" can be synonymous with autoimmunity, especially in the context of pharmaceutical agents. Drug and chemical associated autoimmune diseases typically disappear once the xenobiotic is discontinued [62,63]. The primary effector mechanisms of autoimmune responses are described in chapter 26. As discussed above in the context of allergic responses, inflammation frequently plays a role in autoimmune reactions as the associated cytokines are important in sustaining immune responses, and anti-inflammatory therapy has been shown to be effective in the development of certain disorders [64, 65]. Oxidative stress has also been implicated in the induction of autoimmunity in cases of xenobiotics, partially due to the effectiveness of some antioxidants in the treatment of specific autoimmune disorders [66, 67].

There are several hypothesized mechanisms by which xenobiotics might induce autoimmune disorders. One is through neo-antigen formation via modification or adduct formation with endogenous cellular molecules. Auto-antigens can be intracellular or extracellular proteins, nucleic acids, or other macromolecules that are slightly modified or bound by xenobiotics. The formation of adducts by xenobiotics is essentially equivalent to haptenation, described above for allergenicity. The primary difference appears to involve the ultimate antigen, as allergenicity results in immune recognition of a hapten conjugate with no specificity for native protein, while autoimmunity typically results in immune recognition of the endogenous protein or macromolecule. For example, hydralazine, halothane, and tienilic acid are three agents that have cytochrome (CYP450) reactive metabolites and form CYP450 adducts that subsequently elicit anti-

CYP450 antibodies leading to autoimmune hepatitis [68, 69]. Metabolic activation can also occur within the monocyte/macrophage and has been reported for many of the same xenobiotics (e.g., hydralazine) suggesting multiple mechanisms for xenobiotic autoimmune potential [68].

Auto-antigens can also result from novel immune recognition of unaltered endogenous molecules. During the process of clonal deletion, some auto-reactive T-cells may not be eliminated if respective antigens are not available within the thymus [70]. There may also be xenobiotics (e.g., cyclosporin A [71] and procainamide (hydroxylamine) [72]), which interfere with T-cell selection. If an auto-reactive T-cell repertoire exists, immune stimulation may serve to expose the atypical repertoire. Some xenobiotics such as procainamide and hydralazine have the potential to inhibit DNA methylation, which has been shown to increase expression for many cytokines and surface molecules with relevant associations to autoimmune responses [73]. In addition, xenobiotics such as procainamide and chlorpromazine have been associated with non-bilayer phospholipid arrangements (NPA) with anionic lipids of cell membranes. NPA are very immunogenic and have been identified as a potential trigger for lupus-like disorders [74]. While the key event(s) that trigger expression of an autoreactive disorder are still unknown, the self-antigens (e.g., liver enzymes and glycoproteins on platelets) for many diseases have long been identified [75–77].

Some xenobiotics may have divergent mechanisms of autoimmune responses. For example, hydralazine demonstrates adduct reactivity as well as inhibition of DNA methylation [68,73], while procainamide inhibits DNA methylation, forms immunogenic NPA, and disrupts clonal selection in the thymus [68, 72, 74]. It is this complicated pattern of effects that makes assessment of autoimmune potential in the laboratory for new xenobiotics almost impossible. Animal models can sometimes be recreated to resemble human disease [74], and thus may be useful for therapy considerations, but are difficult to utilize for screening chemicals for hazard potential due to the diverse nature of autoimmunity mechanisms and physiological presentation. While evidence supports many different mechanisms for xenobiotic-induced autoimmune reactions, none have conclusively demonstrated the critical events necessary to lead to the development of autoimmune disease. Therefore, it is difficult to predict or identify xenobiotics that might possess the potential to elicit autoimmune disorders.

CONCLUSION AND FUTURE DIRECTIONS

Over the last 30 years, our understanding of the mechanisms of immunotoxicity at both the cellular and molecular levels has seen unparalleled growth. Such growth can be attributed to our increased understanding of immune regulation and the development of technologies such as molecular biology methods and transgenic and knockout animals [78]. As a result, complex immune responses can be dissected into individual components and the mechanism of action of immunotoxic xenobiotics better understood. The emergence of advanced analytical profiling techniques (i.e., genomics and proteomics) in combination with bioinformatics offers a relatively new and powerful tool to further elucidate the mechanisms of immunotoxicity. When using such profiling techniques to

identify mechanisms of immunotoxicity, however, it is important to understand how the protein or gene expression profile observed relates to the administered dose and the kinetics of the xenobiotic-induced response [79]. While knowledge regarding the mechanisms of immune responses has advanced our ability to recognize and identify hazards as they relate to human exposure (i.e., immunotoxicity), existing data gaps still prevent a full and accurate risk assessment of immunotoxicity potential for many xenobiotics (especially in the cases of autoimmunity and hypersensitivity).

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5 Animal and *In Vitro* Models of Immunotoxicity

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INTRODUCTION

The immune system has evolved to protect the host against invasive microorganisms such as bacteria and viruses and against malignant cells. Inherent to its function, the immune system is spread over primary and secondary lymphoid organs (i.e., bone marrow, thymus, spleen, and lymph nodes) and it is, particularly, present at sites that are exposed to the outside, such as the mucosa of lungs, intestine, and skin. All immune cells derive from pluripotent hematopoietic stem cells present, in adults, in the bone marrow. During the first steps of the differentiation process, myeloid and lymphoid stem cells emerge. Subsequent differentiation into lymphocytes of the T and B lineages occurs within the microenvironment of thymus and bone marrow, respectively.

Immune responses are the result of an effective collaboration between innate (natural and relatively nonspecific) and acquired (adaptive, and extremely specific) com-

ponents of the immune system. Innate immune cells include granulocytes, monocytes/macrophages, natural killer (NK) cells and antigen presenting cells (APC). Antigen recognition by APC results in upregulation of costimulatory help signals, including receptor bound molecules (e.g., CD54, CD83, CD86), as well as soluble factors like cytokines. To become activated, T-lymphocytes require costimulatory signals in addition to recognition of antigen determinants, in the context of the antigen presenting molecules of the major histocompatibility complex (MHC).

It is also more or less accepted that T-cells, in particular T-helper cells ($CD4^+$), may develop into either Th1 cells or Th2 cells. By doing so, T-helper cells orchestrate the ensuing immune response by the types of cytokines they produce. Th1 cells, by producing IL-12 and γ-IFN, stimulate macrophages and/or cytotoxic T-cells to kill and destroy infected or malignant cells, or to initiate a delayed type hypersensitivity (DTH) reaction; Th2 cells, by producing IL-4, IL-5, IL-10, IL-13, trigger B-cells to initiate antibody production.

Being an aggressive system, control mechanisms have also evolved including central tolerance mechanisms (i.e., T-cell selection in the thymus), the requirement of second or costimulatory signals (absence of costimulation results in anergy), and a range of regulatory mechanisms (i.e., regulatory dendritic cells, and T-cells).

At least two specific properties make the immune system vulnerable to chemical or physical insults: (1) the immune system develops rather late in life (thymus development lasts at least until puberty), and some bone marrow-dependent immune components are continuously renewed (i.e., granulocytes), and (2) each pathogen attack, as well as immune surveillance, demands a delicate control of the balance between activation, silencing, and regulation of immune reactivity.

Immunotoxicology studies the effects of xenobiotics on the immune system; an immunotoxic compound is defined as a compound that can alter one or more immune functions, resulting in an adverse effect for the host. In particular, two main immunotoxic effects can be identified:

1. Decreased immunocompetence (immunosuppression), which may result in repeated, more severe, or prolonged infections as well as the development of cancer.
2. Immunoenhancement, which, as adverse effect, may lead to immune-mediated diseases such as hypersensitivity reactions and autoimmune diseases. Hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately, causing inflammatory reactions and tissue damage. The two most frequent manifestation of chemical-induced allergy are contact hypersensitivity and respiratory sensitization, both of which can have a serious impact on quality of life and represent a common occupational health problem. Hypersensitivity reactions are often considered to be increased at such a rate to become a major health problem in relation to environmental chemical exposure.

Clinical data unequivocally demonstrate that immunotoxicity is associated with significant morbidity and even mortality [1]. Immunotoxicity is therefore of considerable importance to the toxicologist who has the responsibility of identifying and characterizing the immunotoxic potential of chemicals and estimating the risk they pose to

human health. Thus, it is hoped that immunotoxicity potential of every new molecular entity would be systematically and specifically evaluated.

ANIMAL MODELS

Assessment of immunotoxic effects requires distinct strategies thus different animal models and assays have been proposed to characterize immunosuppression and sensitization. Before various experimental models are discussed, some general points must be considered. Due to the redundancy of the immune system, alteration of a single endpoint maybe not constitute sufficient evidence of immunotoxicity, making global assessment of all preclinical findings necessary. In addition, alterations in immune functions, which may be tolerated well in normal healthy adults, may have more serious consequences for those who are chronically sick, malnourished, or whose immune system has yet to mature or is in decline. Genetic factors also play a major role in immune responses (i.e., genetic predisposition plays a particularly important role in hypersensitivity and autoimmunity) and, as wide interindividual variability is unavoidable, sufficient numbers of animals or the use of inbred or genetically modified animals needs to be considered when planning immunotoxicity studies. Finally, the inclusion of immune endpoints applicable to animals and man may also be important.

Much of the methods development and validation efforts in the past have been focused on evaluation of immunosuppression and contact or dermal sensitization. Currently available animal models and assays are not valid to assess the potential for systemic hypersensitivity and, at this time, reliable models to assess autoimmunity are not available.

The question of which species of experimental animal is most appropriate for immunotoxicity studies is still a matter of debate. Toxicity test should be performed with species that will respond to a test chemical in a toxicological manner similar to that anticipate in humans (i.e. equivalent metabolism and target organ). Rodents, however, still appear to be the most appropriate animal model for examining the immunotoxicity of non-species specific compounds. It is important to mention that many assays commonly employed to assess immune functions can be performed, with slightly different protocols, in different animal species, including humans (Table 5.1). The following are reported nonclinical testing approaches to identify potential immunotoxic agents.

MICE

Many of the early immunotoxicity studies were performed in mice and several immune assays have been developed and used during the years [2]. As these efforts progressed, determination of the robustness of such tests became an important issue. Statistical analysis of experimental data collected by the U.S. National Toxicology Program, encompassing over 50 compounds, was performed to determine the robustness of such tests and their ability to accurately identify immunotoxic compounds [3, 4]. Tests that showed the highest association with immunotoxicity included the splenic antibody plaque forming cell (PFC) response to a T-cell dependent antigen (78% concordance), cell surface marker analysis (83% concordance) and natural killer (NK) cell activity

TABLE 5.1
Assay Commonly Employed to Assess Immune Functions in Experimental Animals and Humans

Immune Assay	Mouse	Rat	Nonhuman Primate	Human
Surface markers	X	X	X	X
Haematology	X	X	X	X
Serum Igs	X	X	X	X
Lymphoid organ weights	X	X		
NK cells activity	X	X	X	X
Mitogen assay	X	X	X	X
PFC	X	X		
DHR	X	X		X
Host resistance	X	X		(vaccination)

(69% concordance). While a good correlation was detected between changes in the immune tests and altered host resistance (e.g., 70% for the antibody PFC assay, 73% for NK cell activity, and 82% for the DTH response), no single test was, however, identified as fully predictive for altered host resistance [3,4]. Other tests, including the lymphoproliferative response to lipopolysaccharide (a B-cell mitogen) and routine leukocyte counts, were poor indicators of altered host resistance with 50 and 43% concordance, respectively.

Rats

Due to the lower availability of rat-specific reagents, development of immunotoxicity tests in this species evolved at a much slower speed relative to mice. However, since the rat has traditionally been the experimental model of choice for toxicologic studies of chemicals, the development of immunologic assays in rats was necessary. The pioneering work was carried out at the Dutch National Institute of Public Health and the Environment (RIVM) [5,6]. These studies were instrumental in formulating the OECD Guideline # 407 repeated dose 28-day oral toxicity study in rodents for the testing of chemicals. Changes to the OECD guideline #407 have been proposed, including enhanced histopathology of the immune system and inclusion of a functional assay, preferably one that involves immunization with a foreign antigen.

Animal Models of Hypersensitivity

Because a wide variety of xenobiotics can cause contact hypersensitivity, regulatory agencies have requested evaluation of this potential during development, registration, or certification of new materials. Several standardized predictive tests in guinea pigs and some tests in mice use the response in the elicitation phase as an indication of immune reactivity to the chemical [7]. Although guinea pigs have been the animal of choice for

evaluating sensitization potential over the past 50 years, during the last 10 years new predictive assays in mice have been developed. Classical guinea pig tests are relatively costly and time consuming. All use subjective endpoints and data interpretation is prone to difficulties. Less subjective techniques, such as measurement of ear thickness [8] or lymphocytes proliferation [9] have been proposed to evaluate the allergic response in the mouse models, the later having been validated.

In contrast, development of a reliable method to determine the potential for substances (proteins or chemicals) to cause IgE-mediated hypersensitivity reactions, including respiratory and food allergies, has been a difficult task. Animal models of food allergy presently under development include the Brown Norway rat, and a number of mouse models, including oral (C3H/HeJ) and intraperitoneal administration of proteins (such as BALB/c) models [10, 11]. Non-murine animal models such as the atopic dog model and the neonatal pig model are also presently under development and have produced promising results [12]. The guinea pig has been used to evaluate the potential of inhaled chemicals to cause respiratory hypersensitivity by evaluating challenge-induced respiratory reactions in previously sensitized animals. These approaches are associated with a number of important drawbacks and recently attention has focused instead upon methods that characterize the nature of immune responses induced in the mouse, and to a lesser extent the rat. One strategy that has been explored in both mice and rats is based upon the observation that only chemical respiratory allergens are able to induce a time- and dose-dependent increase in the total serum concentration of IgE [13]. More recently an alternative method, known as cytokine fingerprinting, has been developed [14,15]. The method exploits the fact that chemical respiratory allergens selectively induce Th2-type responses and production of relatively high levels of the cytokines IL-4, IL-10 and IL-13 by activated lymph node cells. In contrast, contact allergens, associated with the development of selective Th1-type immune responses, induce high levels of interferon γ (IFN-γ) and interleukin 12 (IL-12), but only small amounts of IL-4 and IL-10. The method, however, has not yet been fully validated and studies are continuing.

IMMUNOTOXICITY: HAZARD IDENTIFICATION

Although a universal protocol for immunotoxicity screening does not exist, many regulatory agencies consider that the initial screen for potential immunosuppression should be done within standard toxicity studies. This approach reflects ethical considerations to reduce the number of animals used and, it is preferable to include immunological test measurements in existing studies rather than in separate experiments that only assess immunotoxicity. As part of a standard toxicity study, the following parameters should be evaluated for signs of immunotoxicity:

1. Changes in total and differential white blood cell counts
2. Alterations in immune organ weights and histology
3. Decreased basal plasma immunoglobulins
4. Increased incidence of infections
5. Evidence of carcinogenicity in long term studies

If there are signs of immunotoxicity, the decision to conduct additional immunotoxicity testing is determined on a case-by-case basis following a weight of evidence review of the data. The type of additional immunotoxicity testing that is appropriate will depend on the nature of the immunological changes observed. Although histology of the lymphoid organs has been claimed to be a reliable tool to predict immunosuppression [16], many immunotoxicologists believe that an immune function assay, especially a T-cell-dependent antibody response [3], should also be considered as an initial screen [17].

Immune Status: Hematology, Organ Weights, and Histopathology

A comprehensive investigation of potential chemical-induced immunotoxicity combines detailed histological examination of immune system tissues and selected quantitative and functional assays. Hematology endpoints include both total and differential white blood cells counts and total serum immunoglobulin levels. Both relative and absolute numbers of WBCs should be quantified. However, absolute numbers provide more biologically relevant information, as the use of percentages of cell types alone may mask cytopenia or excessive numbers of a cell type, which would lead to falsely high or low numbers of a particular cell [18]. Nevertheless, changes of at least 10–20% in blood leukocyte number are necessary before being considered biologically relevant. Total serum immunoglobulin (Ig) levels (IgG, IgM and IgA) can be easily quantified in rodents [19] and in nonhuman primates [20] using the enzyme-linked immunosorbent assay (ELISA). However, the determination of total serum Ig levels in experimental animals has not proven very useful, since pronounced effects on immune function are required before significant changes in total serum Ig levels can be observed.

With respect to pathology, histopathologic evaluation should be carried out on both primary and secondary lymphoid organs, which include the spleen, thymus, bone marrow, and draining and distal lymph nodes. It is, however, difficult to make firm conclusions regarding the degree of changes. Organ weight changes and hematology results must always be taken into account when evaluating histopathology. Changes in immune organ weights are relevant and significant changes are indicative of an immune effect. The concordance with immunotoxicity is 68% and 61% for thymus and spleen weights respectively [3, 4].

Although changes in WBC numbers or shifts in lymphocyte subsets observed in many studies may not be accompanied by changes in immune function [3, 4], immunophenotyping of lymphocytes in spleen or peripheral blood, using monoclonal antibodies directed to cell-surface markers and flow cytometric techniques, has become an important tool in immunotoxicology [18]. Such data can be useful in elucidating the mode of action of the chemical under investigation.

Immune Functionality: Measurement of B and T Lymphocytes, NK Cells, and Macrophage Functions

The functional capacity of the immune system should be established before concluding that a given compound is immunotoxic. Many functional assays have been developed

and validated [2–4]. Functional assays are indeed essential in the investigation and characterization of immunotoxic events. They can provide information on the mechanistic basis for xenobiotic-induced immunotoxicity. However, due to difficulties in interpreting the clinical relevance of small changes, functional assays are not, at present, included in routine toxicity tests.

In rodents and nonhuman primates, challenge with specific antigens has been highly predictive of effects on humoral immunity [3, 4, 20].

The response to an antigenic challenge involves the sequential and tightly orchestrated interactions of competent immune cells, including antigen-presenting cells, and activated T- and B-lymphocytes. Consequently, much information can be derived from challenging the host with foreign antigens. *In vivo* challenge with antigen will, however, require the use of a satellite group of animals.

In rodents, sheep red blood cells (SRBC) are routinely used for immunization. The antibody response is determined using the plaque forming cells assay (PFC) or by plasma SRBC-specific antibody titer [21, 22]. As an alternative to SRBC, other T-cell-dependent antigen may be used, including keyhole limpet hemocyanin (KLH), tetanus toxoid (TT), or pneumococcal antigen.

Cell-mediated immunity (CMI) has been studied in rodents and nonhuman primates mainly using three methods: the lymphocyte transformation assay (LT) in response to the T-cell mitogens phytohemaglutinin (PHA), concanavalin A (Con A), to specific antigens such as TT or by activating the T-cell receptor with anti-CD3 antibody; the mixed lymphocyte response (MLR) assay using allogenic cells; and the DTH response using dinitrochlorobenzene or oxazolone as the sensitizing agent. [20]. The LT and MLR assays, being *ex vivo-in vitro* assays, can be run concurrently, while the DTH assay requires *in vivo* challenge. Of these assays, the LT assay has provided useful information regarding the mechanism of action for some chemicals but its predictive value for immune functional impairment is low [3, 4]. The MLR assay, although a potentially useful assay for predicting chemical-induced adverse immune effects, has not been standardized and validated across laboratories and its concordance with host resistance assays or its predictive value for immune effects is presently not known.

A number of cells, including cytotoxic T lymphocytes, NK cells, and mononuclear phagocytic cells, are endowed with cytotoxic abilities and thus mediate important immunosurveillance mechanisms against neoplastic cells and viral infections. In immune-compromised hosts, a correlation has been observed between low NK cell activity and morbidity [22–25] or the incidence and severity of upper respiratory tract infections [24].

In addition to their cytotoxic properties, the monocyte/macrophage lineage of cells is important in antigen recognition, processing and presentation to T-lymphocytes. These cells are potential targets for chemical-induced immunotoxicity and assays to study functional aspects should be included in the experimental design. Various assays have been used in experimental animal models. Peripheral blood monocytes or peritoneal macrophages have been used as the source of phagocytic cells. SRBC or latex particles are commonly used to assess phagocytosis. Flow cytometric techniques are also available for quantifying the phagocytic response of cells [26]. However, none of these assays or techniques has undergone validation and their predictive value is presently unknown.

HOST RESISTANCE ASSAYS

Several host resistance assays have been developed for use in both mice and rats. These assays provide important clues to the clinical relevance of small changes in the frequency, distribution or functional activity of immunocompetent cells. Several models of infectivity have been developed [27]. These include infection with bacteria, viruses, and parasites, including *Listeria monocytogenes*, *Streptococcus pneumoniae*, *Influenza virus, Trichinella spiralis*, and *Plasmodium*. It should be noted that the immune defense mechanisms that are operational in combating infection are different for each of the infectivity models, thus the choice of the host resistance model should be made based on previously identified changes in immune function parameter [28]. It is, however, important to mention that examination of the susceptibility of animals to challenge with pathogens or tumor cells is expensive, requires large number of animals, special housing conditions and may be difficult to perform.

APPROACHES TO *IN VITRO* IMMUNOTOXICOLOGY

Although evaluation of immune function following *in vivo* exposure to a test material is the most relevant situation, it is increasingly desirable to limit the use of animals whenever possible. Moreover, there are certain situations (e.g., when a test material is anticipated to be dangerous to handle or is prohibitively expensive) when a totally *in vitro* system would be very advantageous. Finally, *in vitro* tests are particularly valuable in determining the molecular mechanism of action of immunotoxic compounds that directly affect immune cells. Many of the techniques that serve as the *in vitro* portion of *ex vivo* immunotoxicology testing can be used as stand-alone assessments in a totally *in vitro* test system [29].

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) in the United States and the European Centre for the Validation of Alternative Methods (ECVAM) in Europe have been established to coordinate issues related to the development, validation, acceptance, and national/international harmonization of toxicological test methods that offer significant advantages over conventional animal studies with respect to animal welfare considerations (i.e., refinement, reduction, and replacement alternatives). The validation of the *in vitro* test should be against information gained from humans, rather than the results from laboratory animal species. To date, the only new test method relevant for assessing immunotoxicity to be validated for regulatory purposes is the Local Lymph Node Assay (LLNA). In Europe, a whole blood assay has been prevalidated for use in *in vitro* immunotoxicology [30, 31]. In this assay, the compound under study is added to whole blood, and lymphocytes and monocytes are stimulated to produce interleukin-1β and interleukin-4, respectively, by the addition of lipopolysaccharide or staphylococcal enterotoxin. The whole blood assay system has been evaluated in a variety of systems and the results suggest the validity of this model for assessing immunotoxicity [32–35]. Advantages of the human whole blood culture system are presented in Table 5.2.

In general, compounds that are capable of damaging or destroying the bone marrow will often have a profoundly immunotoxic effect, since the marrow is the source of im-

TABLE 5.2
Advantages of the Human Blood Cell-Based in Vitro Test.

- Species differences between humans and animals are avoided.
- Human primary cells are employed in their physiological proportions and environment, avoiding preparation and cultivation artifacts.
- Culture techniques are extremely simple.
- *In vitro* testing is less expensive and time-consuming than *in vivo* testing.
- The same test can be employed *ex vivo* and *in vitro.*
- The number of compounds and concentrations tested can be increased.
- The amount of substance required is dramatically reduced, allowing testing at earlier stages of drug development.
- Effects on different blood cell populations can be tested in a single model.
- Changes of cellular immune response can be quantified, enabling potency testing.

mune system effector cell progenitors. Thus, if a compound is myelotoxic the material will be a *de facto* immunotoxicant. The methodology for evaluating myelotoxicity *in vitro* using bone marrow culture systems is well characterized [36].

Compounds that are not overtly myelotoxic may still selectively damage or destroy lymphocytes, which are the primary effectors and regulators of acquired immunity. This toxicity may result from the destruction of rapidly dividing cells by necrosis or apoptosis. A variety of methodologies are available for this purpose (e.g., colorimetric, flow cytometric assays). If the cells are viable (80% or greater), basic functionality could be determined by performing specific functional assays.

Measurement of Potential Effects on Natural Immunity

NK cells are involved in non-specific immunity. CD3- CD16+ CD56+ cells account for 7–41% of the lymphocytes in human peripheral blood (absolute counts vary between 130 and 1,000/mm^3 [37]. Both their numbers and their functional activity have been studied for a long time by toxicologists [38]. NK cells are enumerated based on surface markers (mainly CD56) and their cytotoxic function is usually assayed *in vitro* using (^{51}Cr) labeled target cells (K562 erythroleukemia cells as human NK targets or YAC-1 lymphoma cells as rodent targets), or flow-cytometric cytotoxicity assay. Other functional parameters, such as cytokine production, can be evaluated in purified NK cell preparations [39]. NK cell assays are exquisitely sensitive to modulation by toxic substances, but the significance is often questioned. Indeed, changes in NK activity are not often related to pathological conditions of the immune system and the predictive potential of such assays after *in vitro* exposure is the subject of intensive investigation [40–41].

The use of other immune cells such as mast can also be considered. At the moment, however, there is no strong evidence for a role of eosinophils directly activated by compounds.

Measurement of Potential Effects on Humoral Immunity

In animals, the T-cell-dependent antibody response is considered to be the "gold standard". However, there are currently no good systems for *in vitro* antibody production using human cells. Development of human *in vitro* systems will require optimization of antigen (preferably using antigen relevant to human exposure, such as TT), culture conditions, and assay endpoints. In addition, there is some concern whether a primary immune response can actually be induced in human PBL. One potential starting point would be an *in vitro* immunization culture system based on the Mishell-Dutton assay (this assay is not considered optimal for this use due to significant variability in results—and often a complete lack of success—between laboratories).

Measurement of Potential Effects on Cell-Mediated Immunity

The activation of specific immune responses involves the proliferation of lymphocytes. For T-cells, the stimulatory agent can be a combination of anti-CD3 and anti-CD28 or mitogens such PHA or ConA. Dysregulation of cell homeostasis may have severe adverse effects on immune functions, increasing susceptibility to infections and cancer, as well as favoring the development of autoimmune diseases.

The mitogen-stimulated proliferative response widely used in immunotoxicology and in clinical immunology, is an *in vitro* correlate of activation and proliferation of lymphocytes specifically sensitized by antigen *in vivo*. *In vitro* stimulation of lymphocyte proliferation is an easy assay. Both plant lectins (e.g., PHA, Con A, PWM, etc.) as well as LPS, purified protein derivative of tuberculin (PPD), anti-CD3 or anti-CD28 antibodies, can be used to stimulate T- or B-cell proliferation in whole blood. *In vitro* antigen-specific and mitogen non-specific activation of lymphocytes results in myriad biochemical events, including calcium influx, protein kinase C activation, and phospholipid synthesis, culminating in DNA synthesis and cell division [42,43]. Thus, xenobiotics interfering with signal transduction pathways are likely to alter mitogen-induced lymphocyte proliferation.

Measurement of Cytokine Production

The activation of any immune response is dependent upon the production and release of cytokines. Cytokines are released as one of the first steps in the immune response and quantitative alterations can be used as a measure of immunomodulation. Due to the highly pleiotropic and redundant nature of cytokines, in which a single function may be affected by multiple cytokines simultaneously, it is advisable to include the broadest panel of cytokines possible in any *in vitro* system evaluating this endpoint. A plethora of assay systems are available for measuring cytokines and their receptors including ELISA, flow cytometry, and molecular biology techniques such as PCR [44]. Depending on the stimulus, human blood cells release different cytokines, originating from several blood cell populations. Cultures of whole blood allow assessment of effects on monocytes or lymphocytes by employing selective stimuli. The model has been shown to reflect several aspects of immunotoxicity such as immunostimulation, priming and inhibitory effects.

Stimulation for 24 hours with LPS leads to the release of interleukin-1β, IL-6, IL-8, TNF-α and by prolonging the incubation period from 48 to 72 hours, the whole blood model can detect the release of other lymphokines [45], including IL-2, IL-4, IL-13 and IFN-γ. Skewing of the T-helper cell response to antigens can likewise be detected by evaluating the pattern of cytokine release, corresponding to a predominance of Th1 or Th2 cytokine production. The predictive value of these approaches is currently under investigation.

Cell Culture Models for Evaluating the Sensitizing Potential of Xenobiotics

Besides its barrier function, the skin has been recognized as an immunologically active tissue. Keratinocytes (KC) may convert nonspecific exogenous stimuli into the production of cytokines, adhesion molecules and chemotactic factors [46]. After keratinocytes, Langerhans cells (LC) comprise the second most prominent cell type in the skin (2 to 5% of the epidermal population), and represent the principal APC in the skin [47]. Due to their anatomical location and their significant role in the development of allergic contact dermatitis (ACD), the use of both these cell types to evaluate sensitizing potency *in vitro* is justifiable. In principle, a test system comprised of KC alone may not be useful in establishing allergenic potency as these cells lack antigen presenting capacity. However, in addition to chemical processing, LC activation requires the binding of cytokines produced by KC as a result of initial chemical exposure. The irritant capacity of allergens might present an additional risk factor so that irritant allergens may be stronger allergens than non-irritant ones [48]. In that case, the potency of chemicals to induce cutaneous sensitization may be assessed as a function of KC cytokine expression.

Starting from the *in vivo* observation that in mouse IL-1α expression by KC was selectively increased after *in vivo* application of contact sensitizers but not tolerogen or irritant [49], similar results were reproduced *in vitro* using the murine KC cell line HEL30 [50]. Similar results were also obtained by van Och and colleagues [51] and, furthermore, the authors observed that the ranking of potency was similar to the ranking established using the local lymph node assay. Similarly, using human KC it has been demonstrated that allergens but not irritants or tolerogens induced IL-12 [52, 53]. Trinitrobenzene sulphonic acid induced the expression of CD40 on KC, whereas the irritant sodium dodecyl sulphate did not [54]. Together these studies indicate the possibility of identifying contact sensitizers using murine or human keratinocytes.

On the other hand, DC form a sentinel network able to detect, capture, and process antigens including invading bacteria, viruses, products of tissue damage and haptens [55–57]. Upon antigen capture, DC undergo a maturation process leading to the upregulation of co-stimulatory molecules (CD86, CD80, CD40), MHC class II molecules and the CD83 protein [58]. Thereafter, DC migrate to the T-cell areas of lymphoid organs where they lose antigen-processing activity and become potent immunostimulatory cells. These maturing DC acquire the ability to migrate through expression of chemokines and chemokine receptors and downregulation of molecules such as E-cadherin.

Knowledge of DC physiology has progressed considerably because of the discovery of culture techniques, in the early 1990s, which support the *in vitro* generation of large numbers of DC from hematopoietic progenitors [59]. Two main protocols to generate

DC, from either monocytes or $CD34^+$ hematopoietic cell precursors (HPC), have been described. Generating DC from murine bone marrow $CD34^+$ HPC has been used as an alternative, but this procedure is time consuming and requires a significant number of animals. The establishment of human *in vitro* models of DC offered the possibility to demonstrate that haptens were able to directly activate cultured DC derived from peripheral blood monocytes or from $CD34^+$ HPC [55,60–63]. Several studies confirmed these observations by demonstrating the upregulation of maturation markers (CD83, CD80, CD86, CD40) on human DC [64–67]. Increased production of cytokines (IL-12p40, IL-8, TNF-α and IL-1β) has also been reported upon hapten stimulation [55,62,68], although significant differences have been noted between experimental systems.

Molecular Immunotoxicology

In vitro testing approach can be expanded beyond simple screening to more mechanistic evaluation. The characterization of specific interference with cell signaling induced by an immunotoxicant can lead to a better understanding of the molecular mechanism of action. The sciences of immunology and immunotoxicology have evolved to the point where the molecular mechanism(s) of action can be defined. These types of studies are demanding to assure a good understanding of the profile of immunotoxicity. Study designs must be "targeted" and should consider the primary cellular target and the specific functional immune parameter affected by the chemical. For example, some areas of investigation that would naturally follow from a demonstration of overall xenobiotic-induced cytokine modulation (as would be detected in the whole blood model) might include:

1. At what stage is cytokine production affected: transcription, transduction or release?
2. Is cytokine production skewed toward a discrete phenotype (e.g., Th1 vs. Th2)?
3. Are cytokines overproduced (i.e., immunoenhancement) or underproduced (i.e., immunosuppression) in response to a stimulus?
4. Does a chemical induce cytokine production in the absence of an obvious stimulus?

ADVANTAGE AND DISADVANTAGE OF ANIMAL AND *IN VITRO* MODELS OF IMMUNOTOXICITY

With the exception of whole-animal host resistance assays, the actual testing approach can be described as *ex vivo-in vitro* in that exposure of the immune system to potential immunotoxicants takes place *in vivo*, with subsequent immunological evaluation taking place *in vitro*. Although this approach obviates many uncertainties (effect of xenobiotics on primary or secondary lymphoid tissue, potential requirements for metabolism/biotransformation, etc.), the use of whole animals presents many secondary issues, such

as expense, ethical concerns, and eventual relevance to risk assessment for humans. *In vitro* methods using human derived immunocompetent cells should provide a first step toward establishing a practical and predictive *in vitro* immunotoxicology testing paradigm.

It is recommended that a flow chart/decision tree approach be used to evaluate whether or not a compound is immunotoxic (initial screening). Detection of compounds as potential immunotoxicants can then be followed up by more detailed *in vitro* mechanistic assays

The validation of an *in vitro* method to detect immunotoxicity depends on high quality *in vivo* data. It is essential that a sufficiently large number of positive and negative reference compounds, including both drugs and chemicals, be tested. To this end the establishment of a human database is strongly recommended. This should be accomplished by a coordinated effort from governmental agencies, medical institutions and industry.

In vitro exposure is most straightforward for direct immunotoxicants. However, materials that require biotransformation would require special culture systems (e.g., culture in the presence of S9). Furthermore, an additional limitation of *in vitro* methods would be the physicochemical characteristics of the test material, which may interfere with the *in vitro* system. Such characteristics may include the need for serum, effects of vehicle on cells (such as DMSO), and chemical binding to cells. *In vitro* systems do not take into account the interactions of the different components and it is difficult to reproduce *in vitro* the integrity of the immune system. Finally, *in vitro* systems do not account for potential neuro-immuno-endocrine interactions.

Much progress needs to be achieved before *in vitro* tests could indeed replace the use of animals in immunotoxicology. Furthermore, there is a need for research to develop *in vivo* as well as *in vitro* models to detect autoimmunity and immunostimulation.

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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	78
	GSTP1 Ile105Val	79
Alcohol and chemical-induced Hepatitis	TNFα -308, -238	80
	IL-1β +3953, -511	81
Cancer (benzidine-induced)	GSTP1 Ile105Val	82
Chemical-induced neurotoxicity	IL-1α -889	83
	TNFα -308	84
Chronic beryllium disease (CBD)	TNFα -308	85
	HLA-DPB1(Glu69)	44
Chronic obstructive pulmonary disease (COPD)	TNFα -308	86
	TGFβ codon 10	87
Coal workers' pneumoconiosis (CWP)	TNFα -308	88
Silicosis	IL-1RN +2018	89
	TNFα -238, -308	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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7 The Use of Multiparameter Flow Cytometry in Immunotoxicology and Immunopharmacology

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INTRODUCTION

The continual development and evolution of novel and powerful technologies has allowed scientists to answer increasingly complex questions about the basic mechanisms underlying cellular homeostasis for the benefit of human health. Our understanding of how cells proliferate, thrive, adapt, and die in response to numerous environmental

conditions are at the heart of the fields of pharmacology and toxicology. The study of fluorescently labeled cells allows for an extension of our understanding of the consequences of various compounds on both the life and death of a cell. As such, flow cytometry has become a valuable technique for the study of toxicological effects as they relate to human health.

Flow cytometry is literally the measurement of various properties/characteristics of single cells (particles) as they flow in single file past a beam of laser light. The light that is scattered by the cell or emitted as fluorescence is collected, filtered, and then converted to digital values that are stored on a computer for analysis (Figure 7.1; see color insert following page XXX). However, while flow cytometry examines cells at the single-cell level, one cell at a time, flow cytometric technology allows several different measurements to be made on thousands of individual cells within a very short period of time. Thus, with flow cytometry, distinct subpopulations of cells present within a mixed population of cells can be identified and characterized without physical purification.

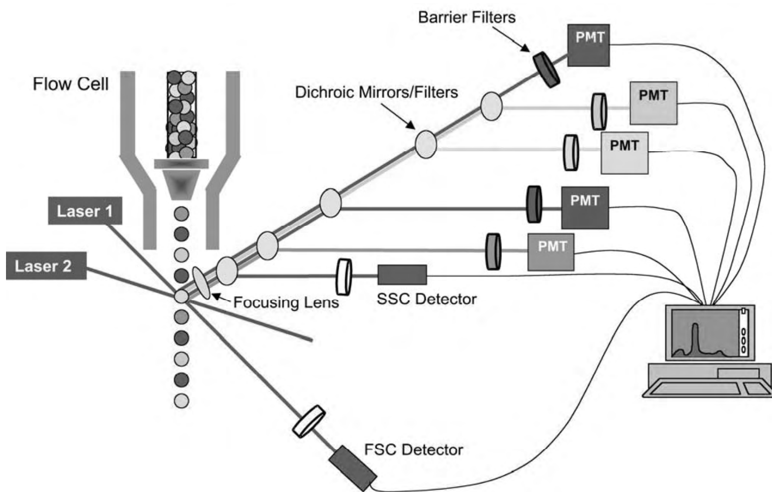

FIGURE 7.1 (See color insert following page 296) Representative diagram of the mechanics of a flow cytometer. Fluorescently-labeled cells are passed through one or more beams of light (lasers) which causes the fluorochrome to become excited and emit photons. A filter in front of the laser captures the forward scatter of light from each cell (a general measure of cell size). The spectra emitted from each cell as it passes through the light beam(s) is deflected through a series of filters that measure the 90° side scatter of light (a general measure of 'granularity' that can be used to distinguish cells with different internal structures) via a photodiode, or the light spectra of each analyte from the cell. Dichroic mirrors reflect light below specific wavelengths to photomultiplier tubes (PMT) and allow longer wavelengths to pass. Additional filters are typically used to target specific wavelengths. Filters may be short pass (allow shorter certain short wavelengths to pass while blocking longer wavelengths), long pass (allow long wavelengths to pass while blocking shorter ones), or band pass (allow a discrete range of wavelengths to pass while blocking others). PMTs collect the specific photons, amplify the signal and covert it to analog voltages. These analog signals are subsequently converted to a digital signal by an analog digital converter (ADC) and then sent to a computer for data storage and eventual analysis. Adapted from Dr. J. Paul Robinson, Purdue University Cytometry Laboratories.

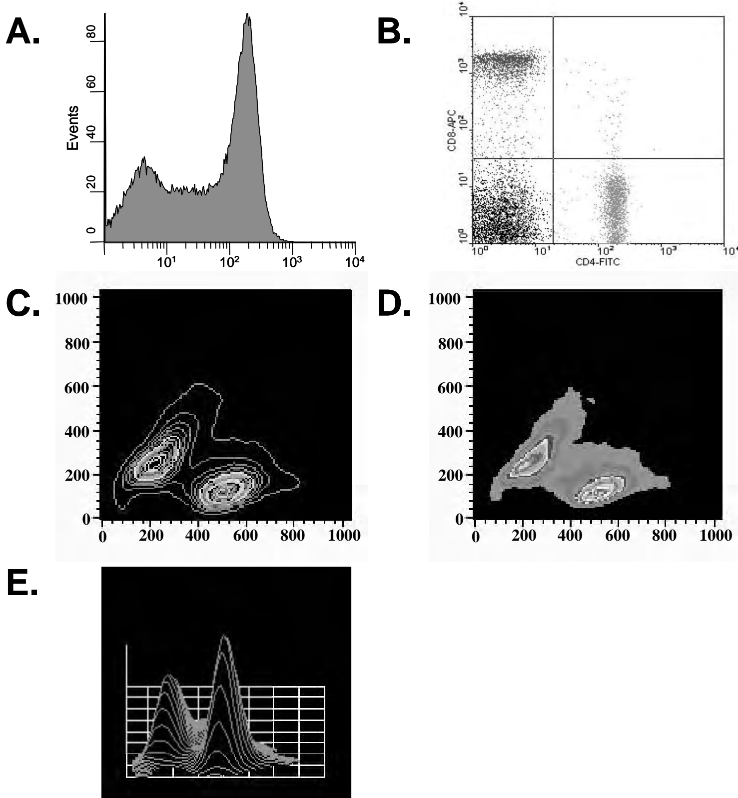

FIGURE 7.2 (See color insert) Representative graphical data displays. (A) Histograms evaluate the distribution of a single parameter over all the cells in a data file, the average relative intensity and the proportion of cells that fluoresce with higher energy (brighter) or lower energy (dimmer) than a specific value. Percentages of "positive cells" for a given parameter can be measured. (B) Dot plots are two parameter evaluations of a population. Each dot represents a value that corresponds to a dedicated channel and shading within each quadrant provides an evaluation of the relative intensity within each channel. Like histograms, percentages of "positive cells" for each parameter can be measured. These plots provide the added benefit of allowing the researcher to determine whether some cells possess dual labeling (double positives) or no labeling at all (double negatives) with either fluorochrome. Contour plots (C) and density plots (D) are similar in that the relative intensities of events in a single channel are represented by contour lines similar to those on a map. As the number of cells increases within a given area, the number of contour lines also increases. (E) The isometric, or 3D, plot is essentially a contour plot in three dimensions.

The digital data from the flow cytometer are collected in list mode files that contain a record of all of the fluorescent events associated with each individual cell in a sample. For any individual cell, the number of events collected is dependent on the number of fluorochromes utilized in the analysis that bind to the cell, which can be amplified by increasing the variety/number of lasers utilized to excite the fluorochrome. These raw data can then be analyzed on-line or off-line by software provided by the manufacturer of

the instrument or purchased from independent vendors (e.g., FlowJo, WinMDI, WinList, ExPO, CellQuest, FCS Express), and can be viewed graphically as a single parameter (histograms) or dual parameters (dot plots, contour plots, density plots, isometric plots), as shown in Figure 7.2. Typically a flow cytometer can display, at a minimum, five individual parameters (forward scatter, side scatter, and green, orange, and red fluorescence). When additional fluorochromes are used in an evaluation, a "gate," or region around a cluster of cells with specific characteristics (e.g., $CD4^+CD8^-$), can be set such that additional evaluations can be examined in that specific subpopulation.

Originally, most flow cytometers utilized single argon ion lasers and could detect 2 or 3 colors at a single time. However, with the advent of multiple lasers (gas and solid-state, low power air cooled, high power water-cooled, and new non-cooled) and advanced electronics, present-day flow cytometers can simultaneously detect up to 10 different fluorescent molecules, permitting the capture of an extensive array of information on a single cell. Flow cytometers are also finding use for fluorescence resonance energy transfer (FRET) analyses, to determine when and where two or more biomolecules, often proteins, interact within their physiological surroundings or one another. In addition to the expansion of laser variety, the choice of fluorochromes has been expanding as well. Fluorochromes may be conjugated to proteins such as antibodies to specific cellular targets, or may be dyes that become fluorescent as a function of intracellular enzymatic activity, pH, or ionic fluxes. Tandem dyes (multiple dyes conjugated together), allow a broader use of certain fluorochromes when the number of lasers is limited because the photon emission from the first dye provides the excitation for the second. When combined with multiple lasers, the number of evaluations that can be made simultaneously increases dramatically because it is possible to make multiple analyses on subsets of cells after gating. In addition to forward angle light scatter (FALS) and side light scatter, the analysis can range from the evaluation of 2 populations (1 fluorochrome) to 60 or more (6+ colors) in a single sample. When combined with cell surface marker identification information multiparameter flow cytometry becomes a powerful technology for assessing changes in membrane integrity and DNA, along with a wide variety of dynamic intracellular biochemical processes within individual cells or discrete populations. A representative list of fluorochromes/dyes and applications for these evaluations can be found in Table 7.1 and Table 7.2. The only limitation is the number of lasers and nonoverlapping emission spectra of the fluorochromes used. As a general rule, "bright" fluorochromes are used for unknown or low-density markers, while "dull" fluorochromes are used for known or high-density markers.

Flow cytometry methodologies provide important tools for identifying target cells of immunotoxicity. Because the immune system represents a heterogeneous mixture of cells participating in innate and adaptive immune responses, it is very useful to use surface phenotypes to identify target cells affected by immunotoxicants. Toxicity may be reflected in many ways, including alterations in intracellular biochemical pathways associated with cell proliferation, activation, or differentiation or the production bioeffector molecules. Flow cytometry allows one to rapidly examine literally tens of thousands (or more) of marker-defined cells at both the intracellular and extracellular biochemical levels (Table 7.1). The remainder of this chapter highlights multiparameter flow cytometric approaches to examining chemically-induced immunotoxicity and immune-mediated toxicity in cells *in vitro* or *ex vivo*.

TABLE 7.1
Representative Fluorochromes/Dyes and Applications for Functional Flow Cytometry[a]

Application	Fluorochrome/Dye	Excitation Wavelength (nm)	Emission Wavelength (nm)	Comments
Phenotyping	Alexa Fluor® 350	350	442	Blue
	Alexa Fluor® 405	405	440	Blue
	Alexa Fluor® 488	488	530	Green
	Alexa Fluor® 546	546	573	Orange
	Alexa Fluor® 647	647	667	Red
	Allophycocyanin (APC)	650	660	Red
	APC-Cy7	647	774	Infrared
	Cascade Blue®	405	440	Blue
	Cyanine (Cy) 3	550	565	Orange
	Fluorescein isothiocyanate (FITC)	488	515	Green
	Pacific Blue®	405	440	Blue
	PE-Cy5	488	670	Red
	PE-Cy7	488	767	Infrared
	Peridin-Chlorophyll (PerCP)	470	670	Red
	PerCP-Cy5.5	488	695	Far Red
	Phycoerythrin (PE)	488	578	Yellow
	PE- Texas Red®	488	615	Orange
	Texas Red®	596	615	Near Red
DNA analysis	Acridine Orange (AO)	460/502	650/526	RNA / DNA
	7-Aminoactinomycin D (7-AAD)	546	655	DNA (G-C-rich areas)
	Chromomycin A_3	458	600	DNA (chromo some analysis; G-C-rich areas)
	Diamino-2-phenylindole (DAPI)	359	461	DNA
	DRAQ5	647	670	DNA (viability)
	Ethidium Bromide	510	595	DNA / RNA
	Hoechst 33342	346	460	DNA (viability; A-T-rich areas)
	Hoechst 33258	346	460	DNA (chromo-some analysis)
	Propidium Iodide (PI)	536	617	
	SYTO/SYTOX	[b]	[b]	DNA / RNA Cyanine dye
	TO-PRO-3	642	661	DNA / RNA
Intracellular Ions	Indo-1	335	405	Calcium
	Fluo-3	488	530	Calcium
	FuraRed	488	660	Calcium
	Rhod2	488	570	Calcium
	Quin2	339	492	Calcium
	PBFI	334	504	Potassium
	SBFI	334	525	Sodium

(Continued)

TABLE 7.1
Continued

Application	Fluorochrome/Dye	Excitation Wavelength (nm)	Emission Wavelength (nm)	Comments
Reporters	Green Fluorescent Protein (GFP)	488	510	Gene silencing with siRNA
	Yellow Fluorescent Protein (YFP)	519	534	
	Cyan Fluorescent Protein (CFP)	434	477	
	DsRed	558	583	
Other	Laser Dye Styryl (LDS)-751	543	712	Nucleated cell detection; mitochondria
	MitoTracker Red	560	610	Mitochondrial membrane potential
	JC-1	498	525 monomers 590 aggregates	Mitochondrial membrane potential
	MitoSOX™ Red	510	580	Mitochondrial superoxide
	Rhodamine 123	515	525	Membrane potential
	bis-(1,3-dibutylbarbaturic acid)trimethine oxonol ($DiBAC_4(3)$)	493	516	Membrane potential
	3,3'-hexyloxacarbocyanine iodide ($DiOC_6(3)$)	482	516	Membrane potential
	FcOxyBURST® Green	495	524	Oxidative burst
	Monochlorobimane	380	461	Intracellular GSH
	SNARF-1	488	530–640	pH
	Fluorescein	495	519	pH
	Calcein	496	517	pH > 5
	carboxyfluorescein diacetate succinimidyl ester (CFSE)	488	515	cell proliferation
Apoptosis	YO-PRO-1		488	510

[a] The fluorochromes and dyes presented here are unconjugated. They may be conjugated to immunoglobulins (e.g., anti-CD3 or anti-caspase antibodies) or other proteins/peptides (e.g. Annexin-V).
[b] These dyes vary in their excitation and emission spectra, as well as their permeability and selectivity for nucleic acids.

IMMUNOPHENOTYPING

Flow cytometry is now commonly used in immunotoxicity studies to assess changes in relative frequency and number of lymphoid and myeloid cells in the spleen, lymph nodes, bone marrow and/or peripheral blood of rodents, and in the peripheral blood of humans. A list of selected cell surface markers useful in immunotoxicity studies is shown in Table 7.3. Notably, the majority of available reagents are specific for murine antigens with human reagent availability a close second. Reagents for rat, primate, and

TABLE 7.2
Applications of Multiparameter Flow Cytometry to the Evaluation of Immunopharmacologic and Immunotoxicologic Actions and Mechanisms

Cell size
Cytoplasmic Granularity
Phenotyping (Surface Antigens)
Cell Cycle Analysis and Kinetics
Apoptosis
Calcium Flux
Chromosome Analysis
Micronuclei Analysis
Intracellular pH
Intracellular Glutathione
Oxidative Burst
Cell Viability
Endocytosis / Phagocytosis
Active Transport
Cellular Proliferation
Intracellular Cytokine Production
Intracellular Signal Transduction
Intracellular Free Ions (e.g., Na, K, Ca)
Intracellular Enzymes
Stem Cell Isolation
Cellular Membrane Potential
Mitochondrial Membrane Potential
Gene Reporter Assay
Gene Silencing (siRNA)

dog are much more limited, an interesting conundrum as these are the primary species used for toxicity testing of drugs and chemicals.

While changes in cell phenotypes have proved useful in some settings to characterize the immunotoxicity of xenobiotics,[1] phenotypic analysis alone is often not a sensitive indicator of low dose immunotoxicity for many agents that alter immune function. Xenobiotics that exert selective toxicity on lymphoid and myeloid cells may be discovered through immunophenotypic analysis. However, most agents produce immunotoxicity at doses much lower than those required to produce cytotoxicity or interfere with primary lymphoid organ differentiation. Some of the most potent immunosuppressive chemicals that have been tested, such as cyclosporine A, do not alter immunophenotype at doses that are immunosuppressive. On the other hand, when phenotyping is linked to assessment of functional parameters of the cells, immunotoxic effects are more likely to be identified.

There are literally hundreds of markers that are currently available for the mouse and human than can be used to characterize lymphoid and myeloid cells and subsets in primary and secondary lymphoid organs. Many of the markers expressed in mammals are highly conserved across species and have been designated as genetic clusters of differentiation (CD). CDs can be identified with fluorescently labeled monoclonal antibodies. As presented previously, when combined with other fluorescent probes, important information on intracellular biochemistry and cell function can be obtained. Many of the biochemical markers used by immunotoxicologists are common to other

TABLE 7.3
Selected Markers for Immunophenotyping Peripheral Blood, Spleen Cells, Lymph Nodes, or Bone Marrow

Cell Population	Mouse	Rat	Dog	Primate
HSC Stem Cell	CD34 SCFR (CD117)			CD34
Lymphoid Stem Cell	IL-7R (CD127)			
Myeloid Stem Cell	GCSF-R or GM-CSFR (CD114, CD115, CD116)			
Common Leukocyte marker	CD45	CD45		
Dendritic cells	CD11c CD207 CD289 (TLR9)	CD11b/c		
Lymphocytes				
Pan-T cell marker	CD3 CD2 Thy 1.1/1.2 TCRβ CD28	CD3	CD3 CD5	CD3
Helper T cell	CD4	CD4	CD4	CD4/CD3
Cytotoxic T cell	CD8	CD8	CD8	CD8/CD3
Treg	FoxP3			
Activated T cells	CD25↑ (IL-2R) CD69↑ CCR7			
Pan-B cell marker	CD45R/B220 CD19 mIg (μ, δ, γ, α, ε heavy chain, or κ, λ light chain)	CD45RA	CD21	$CD20^+/CD3^-$
Activated B cells	MHC II ↑	MHC II ↑		MHC II ↑
Plasma cell	PC-1			
Monocytes/ Macrophage	CD14 MHC II ↑ CD11b (Mac-1) CD282 (TLR2)	MHC II ↑	MHC II	CD16 MHC II ↑
Natural killer cells	NK1.1 CD94 CD25↑ CD69↑ CD161c	CD161		$CD16^+/CD3^-$
NKT	CD3 / CD56	CD3 / CD161		

↑ = inducible

organs and tissues, such as those associated with intracellular signaling, altered Ca^{2+} homeostasis, cell proliferation and cycling, oxidative stress, and apoptosis. There are also many markers that are somewhat unique to the immune system involving cell-cell interactions, cytokine, and chemokine receptors, and lineage specific markers. The next section will illustrate a few markers from each category and will present specific applications in immunotoxicology.

FUNCTIONAL FLOW CYTOMETRY FOR EVALUATION OF IMMUNOTOXICITY AND MECHANISM OF ACTION

Cell Cycle and Proliferation Markers

One of the first applications developed for flow cytometry was cell cycle analysis.[2] There are numerous intercalating fluorescent DNA and RNA staining reagents that can be used to determine the amount of DNA in cells, an indicator of cell cycle stage and progression, as demonstrated in Figure 7.3. Nucleic acid dyes may be selective for DNA

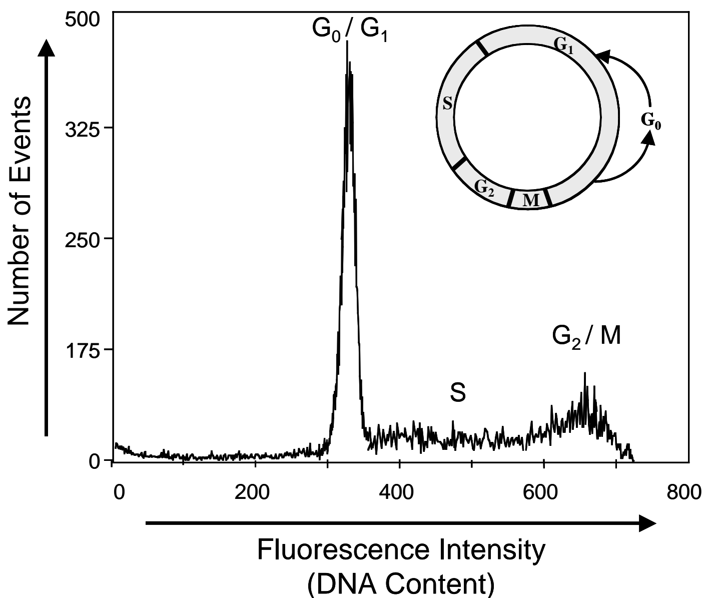

FIGURE 7.3 Flow cytometric analysis of cellular DNA content. Normally proliferating cells were fixed with ethanol, treated with RNAse, and stained with propidium iodide to label DNA. DNA content in the population was evaluated flow cytometrically to determine the percentage of cells in each phase of the cell cycle. During G_1, cells begin to synthesize RNA and protein and their DNA content is defined at 2N (diploid). S-phase begins when the cells commit to DNA synthesis and chromosomal replication. During this time, DNA content is between 2N and 4N. In the G_2 phase, cells complete DNA synthesis (4N; tetraploid), continue to grow and prepare to divide (mitosis, M). Upon completion of the process, each daughter cell contains the same genetic material as the original cell (2N) and roughly half of its G_2 level of cytoplasm. Cells may enter a quiescent (non-dividing) state where they exit from the cell cycle, but may be re-activated in response to an external stimulus.

or may stain both DNA and RNA (Table 7.2). Specificity can be critical, for example, when distinguishing red blood cells infected with malaria parasites from reticulocytes, or cells with abundant ribosomes from other nucleated cells. The most commonly used dyes include propidium iodide (PI), acridine orange, DAPI, and Ho-33342. Ho-33342 is unique in that it can be used to stain DNA in viable cells, whereas the other agents require membrane permeabilization. Bromodeoxyuridine (BrdU) is also used for cell cycle analysis, although this method is more involved and requires incorporation of BrdU into replicating DNA, followed by cell permeabilization and detection using fluorescent anti-BrdU (or secondary) antibodies.

A newer flow cytometric technique for assessing proliferation is evaluation of cell division history using dyes such as 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE). These fluorescent dyes are taken up by cells and cleaved by esterases, causing their retention in the cytoplasm. Upon cell division, the CFSE distributes equally to the daughter cells, resulting in a progressive 50% reduction in fluorescence intensity with each cell division. When CFSE-labeled cells are co-stained with antibodies to lymphocyte surface markers, one can directly compare the influence of chemical exposure on the proliferative capacity of different subsets of cells in the same sample. Another advantage of the CFSE method is that it can be coupled to the measurement of activation markers on specific cell subpopulations, providing additional insight into mechanisms of action of immunotoxicants. For example, in studies to understand the mechanisms of AhR activation on $CD4^+$ T cells, Funatake et al. used CFSE-labeled donor T cells to track their activation status after injection into an F1 host. Flow cytometric analysis revealed a dramatic change in activation phenotype of the donor CD4+ cells induced by TCDD exposure.[3] This was reflected in increased expression of CD25 and decreased expression of CD62L that occurred after the cells had undergone 3-4 cell divisions; yet there was no overall effect on cell division (Figure 7.4). These results contributed to the novel discovery that AhR activation in proliferating T cells promotes the generation of a regulatory T cell population.

Indicators of Cell Injury and Death

There are many fluorescent probes that can be used as indicators of cell injury and stress. An agent that disrupts the membrane of a cell will cause it to become permeable to dyes such as propidium iodide (PI) or 7-amino actinomycin D (7-AAD). Thus, these dyes are useful for simple tests of membrane integrity. There are also fluorescent vital dyes, such as diacetylfluorescein (FDA), carboxyfluorescein diacetate (CFDA), and calcein that are de-esterified intracellularly into fluorescent molecules that indicate cell viability. Several approaches to assessment of cell death pathways including the use of TUNEL reagents to detect DNA strand breaks,[4] annexin V as a marker for cell membrane phospholipids associated with apoptosis, and direct measurement of caspases and apoptotic signaling pathways (e.g., Fas, FasL) using fluorescent-tagged antibodies.[5,6] Oxidative stress can be measured in cells using 5-(and-6)-carboxy-2',7'- dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) for the measurement of intracellular H_2O_2, dihyrdoethidium (DHE) for intracellular super oxide anion, MitoSOX™ Red for mitochondrial superoxide, and

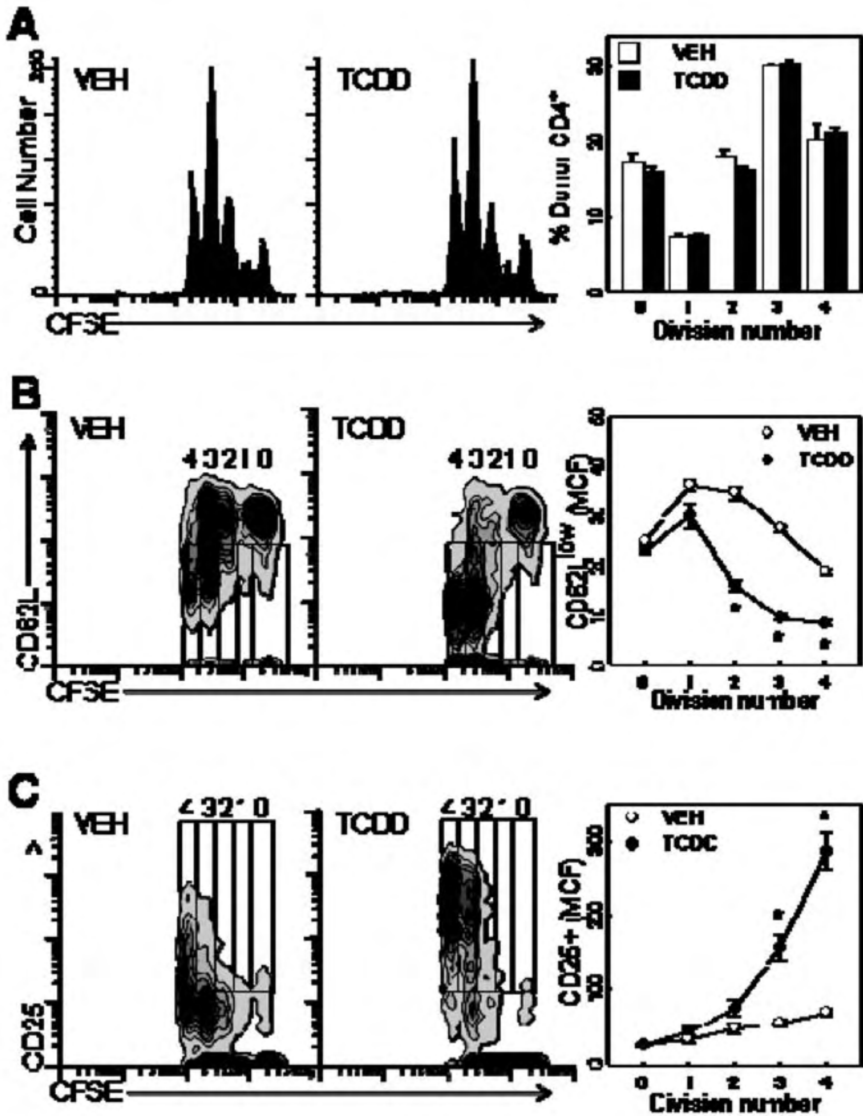

FIGURE 7.4 (A) Donor CD4+ T cell proliferation, as reflected by dilution of the fluorescence associated with CFSE, is not altered by TCDD exposure. Changes in expression of T cell activation markers (B) CD62L and (C) CD25, are induced by TCDD exposure and are dependent on cell division. Data represent donor CD4+ T cells responding to alloantigen in F1 hosts 48 hours after adoptive transfer. F1 host mice were treated with vehicle or TCDD one day prior to injection of CFSE-labeled donor T cells. Adapted from Funatake et al., 2005.

dihydrorhodamine for peroxnitrite. Intracellular glutathione can be detected by reaction with monoclorobimanes (MCB) and monobromobimanes (MBB).[7]

Another indicator of cell injury induced by genotoxic chemicals is the stabilization of p53 protein in cells. p53 is phosphorylated in response to DNA damage and strand break by the ATM and ATR pathways. Several investigators have utilized antibodies to p53 to examine increased levels of protein following chemical treatment of cells.[8,9]

Extracellular Markers of Cell Activation

Expression of many adhesion molecules and growth receptors is altered on leukocytes during cell activation and differentiation. Many of these molecules are lineage associated and can be used to identify subsets as well as altered expression following exposure to xenobiotics. Several examples of adhesion molecules that are up- or down-regulated in response to immune activation or toxicants are discussed below, including CD25 (IL-2R), CD62L (L-selectin), and CD69 (C-type lectin). Other markers of cell activation and proliferation that are being increasingly utilized include various cytokine and chemokine receptors, particularly CCR7 for T cells, and proliferating cell antigens such as Ki67 protein.[10]

Intracellular Functional and Biochemical Analyses

There are several flow cytometry assays that can be used to assess cell signaling in lymphoid cells. Intracellular Ca^{2+} assays have long been used by immunologists to assess lymphocyte activation and signaling through surface receptors.[11] Free intracellular Ca^{2+} is increased in B and T cells following antigen receptor activation or cross-linking of surface receptors via IP_3-dependent release from the endoplasmic reticulum. Thus, xenobiotics that interfere with Ca^{2+}-dependent signaling pathways would be expected to decrease the release of Ca^{2+} following antigen or mitogen receptor activation. Interestingly, several types of xenobiotics have been found to mobilize Ca^{2+} in the absence of receptor activation leading to inappropriate signaling, or in some cases Ca^{2+}-dependent cell death. Polycyclic aromatic hydrocarbons (PAHs) in particular exert complex effects on Ca^{2+} pathways in lymphoid and other cells. Certain PAH metabolites, such as benzo(a)pyrene 7,8-diol, 9,10-epoxide activate protein tyrosine kinases.[12] This is probably indirect due to inhibition of protein tyrosine phosphatase (PTPase), as various electrophiles and oxidants are known to inhibit PTPase. Certain redox-cycling PAHs have been shown to increase intracellular Ca^{2+} in lymphocytes after many hours of treatment. The mechanism is likely due to NADPH and ATP depletion in mitochondria leading to Ca^{2+} overload. Recently, it was shown that certain ortho quinones of benzo(a)pyrene (e.g., 7,8-BPQ) have a unique mechanism of elevating intracellular Ca^{2+} by activation of the ryanodine receptors (RyR) that control Ca^{2+} influx through membrane channels. As shown in Figure 7.5, 7,8-BPQ produces a rapid increase in intracellular Ca^{2+} in murine spleen cells. Two other BPQs (1,6-BPQ and 3,6-BPQ) did not increase Ca^{2+} at early time points, although they did after 18 hours due to overall oxidative stress.[13]

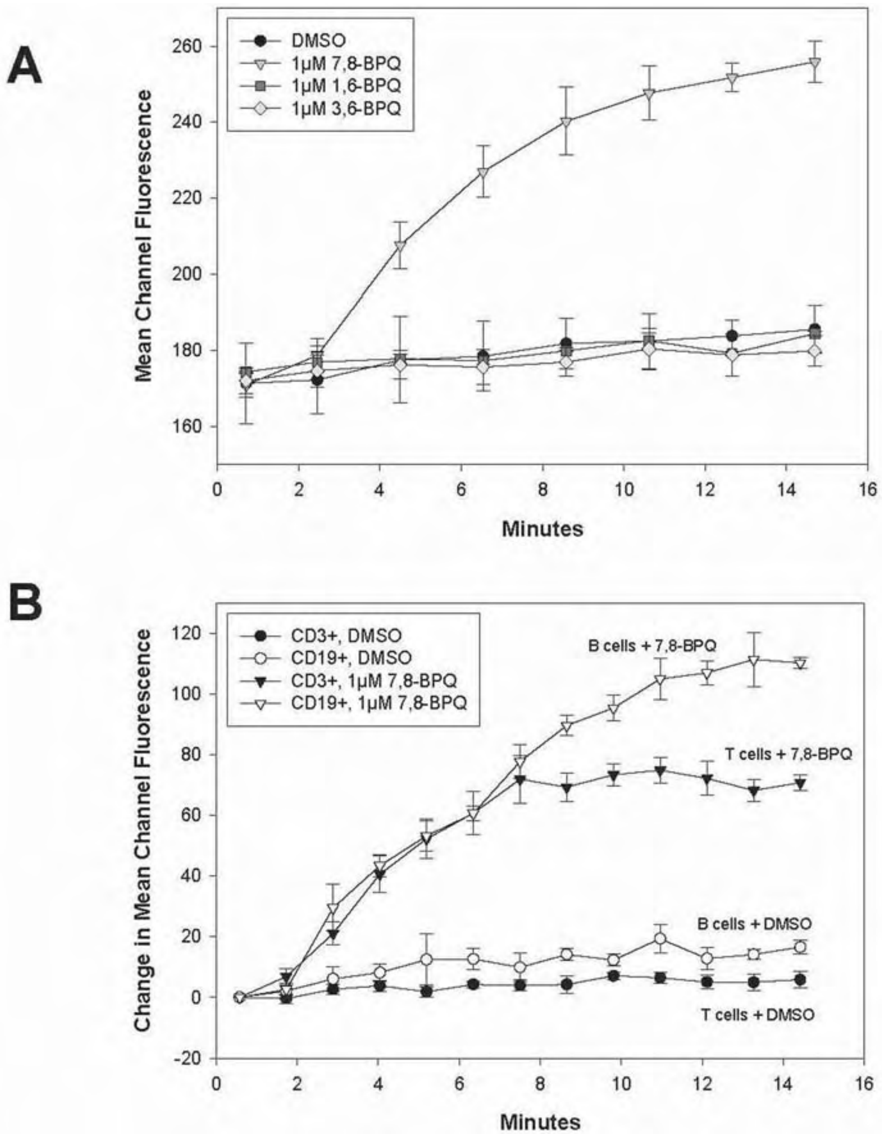

FIGURE 7.5 7,8-BPQ increases intracellular Ca^{2+} in murine spleen cells (A) and in both B and T cells (B). Single cell suspensions were prepared from murine spleens. Splenocytes were loaded with Fluo-3/AM dye for one hour and then treated with 7,8-BPQ, 1,6-BPQ, 3,6-BPQ, or DMSO (control). Surface-marker-defined T cells and B cells were treated with 7,8-BPQ or DMSO. Following treatment, the immediate intracellular Ca^{2+} response was continuously monitored for 15 minutes. Results are shown as the change in Mean Channel Fluorescence ± SEM. The numbers shown in this figure were the averages of triplicate determinants. Adapted from Gao et al., 2005.

Use of Antibodies to Evaluate Intracellular Markers

Due to improvements in cell permeabilization techniques over the past few years, it is now possible to use a variety of antibodies to study intracellular protein expression in different cell class subtypes. When combined with surface marker expression in lymphoid cells, these assays become extremely powerful for assessing the effects of xenobiotics on defined cell populations. Examples of assays that can be performed in lymphoid subsets include intracellular cytokine expression[14] and various cell signaling reagents ranging from general anti-phosphotyrosine reagents to specific phosphoproteins referred to Phosflow technology.[15] An example of Phosflow technology in surface marker-defined human peripheral blood leukocytes is shown in Figure 7.6. In this experiment human peripheral blood leukocytes (HPBL) were treated with H_2O_2 to induce

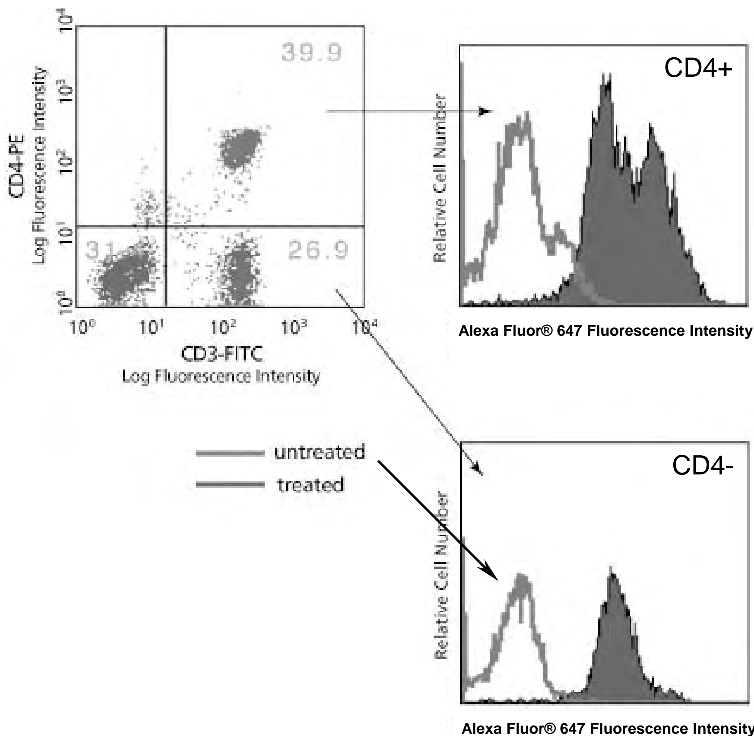

FIGURE 7.6 (See color insert) Detection of intracellular tyrosine phosphorylation (pY) of ZAP70 in CD4+ and CD4- human peripheral blood leukocytes (HPBL) following stimulation with H_2O_2. HPBL were either untreated or were treated with H_2O_2 to induce signaling of ZAP70/Syk pathways identified by phosphorylated tyrosine 318 (Y318) on ZAP70 or Y352 of Syk kinase. Cells were simultaneously stained with reagents for ZAP70(pY318)/Syk(pY352), CD3 (all T cells), and CD4 (T-helper cells). The $CD3^+CD4^+$ cells (T-helper) and the $CD3^+CD4^-$ (which constitute mainly $CD8^+$ cytotoxic T cells) were identified in the HPBL population. Cells were gated on the $CD3^+CD4^+$ or $CD3^+CD4^-$ populations (upper left dot plot), and the ZAP70/Syk signaling in these populations (upper and lower right histograms) was determined by fluorescence intensity differences in the treated (red, filled) and untreated (green, open) populations. Reproduced with permission from BD Biosciences.

signaling of ZAP70 pathways identified by tyrosine phosphorylation of tyrosine 318 (Y318) on ZAP70. The $CD3^+CD4^+$ cells and the $CD3^+CD4^-$ (which constitute mainly $CD8^+$ cells) were identified in the HPBL population. The results showed an increase in the amount of fluorescence from the ZAP70-PY319 in H_2O_2 treated cells. Both $CD4^+$ and $CD4^-$ cells responded to the treatment.

Measuring T Cell Activation In Vivo

$CD4^+$ T cells are the primary determinants of the type and robustness of an immune response, playing important roles as helper, effector and regulatory cells. Because of their extensive and essential functions, $CD4^+$ T cells are recognized as important targets for pharmaceutical drug development as well as key targets of immunotoxic chemicals. Until recently, the ability to measure changes in the responses of $CD4^+$ T cells to specific antigenic stimulation *in vivo* has been an elusive goal due to their low frequency of occurrence (estimated at around 1 in 10^6 cells). Even flow cytometry was not sensitive enough to detect the antigen-specific $CD4^+$ T cells *in vivo*, and methods for identifying T cells responding to an antigen required in vitro culture or analysis of a polyclonal T cell response.

T Cell Receptor (TCR) Transgenic Models

One novel approach to overcome the low frequency of antigen-specific T cells has been the development of T cell-receptor transgenic mice. The transgenic receptor is specific for an antigenic peptide presented in the context of an MHC molecule (Class II to activate $CD4^+$ cells or Class I to activate CD8 cells) and is expressed by all of the T cells in the transgenic mouse. To more closely mimic a natural physiological milieu, small but detectable numbers of the transgenic T cells are adoptively transferred into syngeneic recipients, and their response to antigen challenge is tracked by flow cytometry using an anti-clonotypic monoclonal antibody. Furthermore, the antigen-specific changes can be validated as those occurring in the transgenic T cells but not in the bystander T cells of the same recipient.

The DO11.10 TCR-transgenic model, specific for chicken ovalbumin (OVA) peptide 323-339 in the context of MHC Class II, has been widely used in studies of $CD4^+$ T cell responses,[16] including immunotoxicity studies.[17,18] DO11.10 T cells can be easily identified using the anti-TCR antibody KJ1-26 following their injection into syngeneic Balb/c mice. Figure 7.7 shows that immunization of the mice with OVA results in a significant increase in the percentage of transgenic $CD4^+$ $KJ1\text{-}26^+$ T cells in the spleen. When the spleen cells are co-stained for various markers of T cell activation, the expected increased expression of CD69, CD44, CD11a and decreased expression of CD62L is readily apparent in comparison to the $KJ1\text{-}26^-$ bystander $CD4^+$ T cells. The antigen-specific $KJ1\text{-}26^+$ T cells also show increased forward angle light scatter (FALS), a flow cytometric indicative of blastogenesis. In addition to lymphoid organs, activated transgenic $CD4^+$ $KJ1\text{-}26^+$ T cells are detectable in the blood on day 3 to 5 after primary antigen challenge.[18] Sampling peripheral blood mononuclear cells (PBMC) in

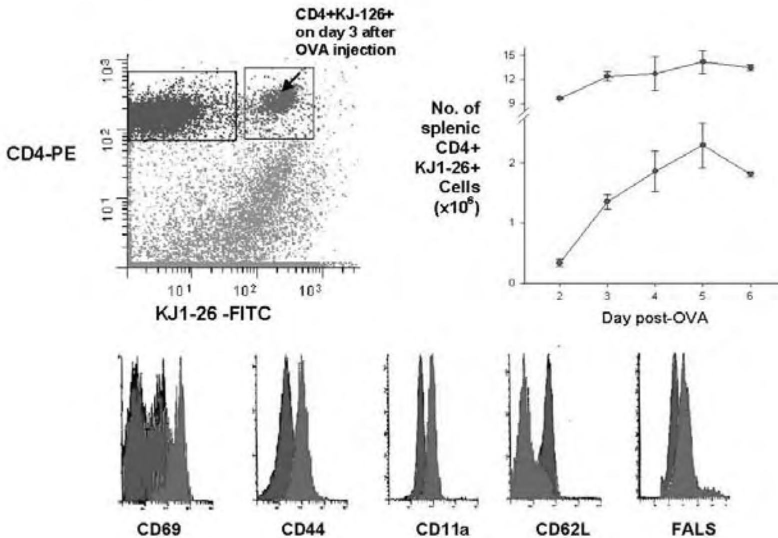

FIGURE 7.7 (See color insert) Adoptively transferred DO11.10 transgenic T cells can be identified by expression of CD4+ and KJ-126 in spleen cell suspension from Balb/c mice after ovalbumin (OVA) immunization. Balb/c mice were injected iv with DO11.10 spleen cells containing 3-5 x 10^6 $CD4^+KJ\text{-}126^+$ cells and immunized by intraperitoneal injection of 2 mg OVA emulsified in complete Freund's adjuvant 2 days later. OVA immunization increases the frequency of KJ+ T cells and alters the expression of various surface molecules consistent with T cell (Tc) activation.

rodent studies is not commonly done at the present time, but could be cost-effective in immunotoxicity studies for tracking T cell responses over time in individual animals. PBMC data from rodent studies could also be directly compared to data from primate and human studies that routinely use PBMC for analysis.

MHC-Peptide Tetramer Technology

Another innovation that utilizes flow cytometric analysis of antigen-specific T cells in vivo has been the development of soluble MHC-peptide tetramer reagents. Most of the current reagents are Class I tetramers designed for the study of antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTL). Until their development, CTL were measured by limiting dilution assays in vitro or by lytic activity after expansion of the CTL in vitro. The tetrameric reagents are linked to a fluorochrome, and their binding with high affinity to $CD8^+$ T cells bearing receptors specific for the peptide can be readily detected by flow cytometry. Remarkably, the use of tetramers revealed that the virus-specific primary and memory $CD8^+$ T cell responses to viral infections are more than tenfold greater than previously estimated using limiting dilution analysis.[19] Recently, tetramer technology was successfully utilized by Lawrence and Vorderstrasse in flow cytometric studies to determine the influence of TCDD exposure on the antigen-specific $CD8^+$ memory T cell response to influenza virus.[20] Class II tetramers are also being developed and, along with Class I tetramers, are now available from several commercial sources.

COUPLING FLOW CYTOMETRY WITH IMAGE ANALYSIS—ASSESSING THE ROLE OF MACROPHAGES IN TISSUE INJURY

Macrophages are critical cellular effectors of nonspecific host defense. In response to tissue damage, these phagocytic leukocytes, along with neutrophils or PMN, rapidly migrate to sites of injury initiating an inflammatory response. When appropriately orchestrated, this leads to destruction of invading pathogens, removal of tissue debris, and ultimately, to wound repair. However, when control mechanisms go awry, as observed following exposure to toxicants, the inflammatory response can become chronic, resulting in persistent swelling, pain, and eventually, to increased or prolonged tissue injury.[21] Techniques in flow cytometry/image analysis have proven to be particularly useful in assessing the contribution of macrophages and inflammatory mediators to tissue injury. Probes are commercially available to assess changes in macrophage structure, antigen expression and functional activity following toxicant exposure.

The first step in macrophage responsiveness to tissue damage involves cellular "activation," which occurs following exposure of the cells to inflammatory signals generated at sites of injury. The process of activation is associated with morphologic, functional and biochemical changes in the cells.[22] Thus, macrophages become enlarged and highly vacuolated, and exhibit greater membrane ruffling and an increased cytoplasmic to nuclear ratio. When activated macrophages are analyzed by flow cytometry, these physical and structural changes are reflected by alterations in their light scattering properties. Whereas forward angle light scatter, which is roughly proportional to the size of the cells, increases as macrophages become activated, right angle light scatter or side scatter, a measure of cell density, decreases.[23] By following changes in the light scattering properties of macrophages isolated over time from animals exposed to toxicants, it is also possible to identify new subpopulations of cells that appear at sites of injury. For example, using flow cytometry, Ahmad and colleagues identified a small relatively dense subpopulation of macrophages that accumulates in the liver during the pathogenesis of acute endotoxemia.[23] It was hypothesized that these cells were newly recruited macrophages responding to tissue injury. To investigate this possibility, the phenotype of the macrophages accumulating in the liver following hepatotoxicant administration can be analyzed using antibodies against F4/80, a marker present on mature resident macrophages,[24,25] and macrosialin (CD68), which binds to activated macrophages;[26,27] this was done using acetaminophen as a model hepatotoxicant. It has previously been demonstrated that hepatic necrosis induced by acetaminophen is accompanied by a marked accumulation of macrophages in the centrilobular regions of the liver, areas particular sensitivity to toxicity.[28,29] In control mice, F4/80 was expressed in large quantities by resident liver macrophages (Kupffer cells). Administration of acetaminophen to the mice resulted in a 75% decrease in F4/80 positive macrophages that was observed 24 to 48 hours after treatment.[30] In contrast, expression of CD68 increased two-fold, 24 to 72 hours after administration of acetaminophen and this was associated with inflammatory macrophages. Interestingly, the loss of the chemokine MCP-1, or its receptor CCR2, resulted in a decrease in the number of $CD68^+$ macrophages in the livers after acetaminophen administration, confirming that these cells are in fact derived from infiltrating cells. These results demonstrate the utility of immunofluorescence and flow cytometry/image analysis to identify activated macrophages in a tissue following toxicant exposure.

Macrophage activation is also characterized by increased functional responsiveness, including chemotaxis, phagocytosis and cytotoxicity, and these activities can also be investigated using techniques in flow cytometry/image analysis. The accumulation of macrophages at sites of injury is mediated by chemotactic factors such as bacterially-derived f-met-leu-phe, C5a, and various chemotactic cytokines or chemokines (ex., MCP-1, MCP-3, MIP-1 and Rantes), that orchestrate the inflammatory response. Studies have shown that expression of MCP-1 and its receptor, CCR-2, are upregulated in the liver following acetaminophen administration to mice.[30,31] This is directly correlated with increased numbers of inflammatory cells in the tissue. As indicated above, the fact that these cells do not accumulate in the livers of CCR-2 knockout mice demonstrates the important role of MCP-1 in inflammatory cell recruitment into the injured tissue. Once localized at sites of injury, locally generated inflammatory mediators activate macrophages to phagocytize and kill invading pathogens, and to rid the injured tissue of dead cells and debris. Phagocytosis by isolated macrophages can be easily quantified by flow cytometry using fluorescently-labeled bacteria, beads, apoptotic cells, or tumor cell targets.[32,33] Using flow cytometry, it is possible to quantify the percentage of phagocytic cells, as well as the number of particles phagocytized per macrophage.[33]

Activated macrophages also generate increased quantities of cytotoxic mediators, including reactive oxygen and nitrogen intermediates, which have been implicated in tissue injury in a number of model systems including endotoxin and acetaminophen-induced hepatotoxicity.[34–38] Biochemical probes such as 2',7'-dichlorofluorescin diacetate (DCFH-DA), dihydrorhodamine (DHR) 123, and dihyrdoethidium (DHE) are useful for assessing production of these mediators by activated macrophages and their role in tissue injury.[39,40] Using these probes it has previously been demonstrated that macrophages accumulating in the liver following hepatotoxicant treatment of animals release increased quantities of hydrogen peroxide and peroxynitrite.[41,42] Similar increases in production of reactive oxygen and nitrogen intermediates have been observed in the lung following exposure of animals to endotoxin.[43,44] An example of the type of data generated using DCFH-DA and DHR is shown in Figure 7.8 and Figure 7.9. In both the liver and the lung, increased production of reactive nitrogen intermediates is associated with increased expression of inducible nitric oxide synthase (iNOS). Antibodies to iNOS are commercially available and alterations in expression of this enzyme in activated cells can also be detected by flow cytometry/image analysis.[44,45] The observation that inhibiting macrophages with gadolinium chloride blocks excessive production of reactive oxygen and nitrogen intermediates, and prevents tissue injury, provides strong support for the idea that macrophages and oxidants are important in the pathogenesis of toxicity.[38,46]

Another characteristic of activated macrophages is increased production of inflammatory cytokines like tumor necrosis factor-α (TNFα) (Figure 7.10). Typically considered a proinflammatory cytokine, TNFα up-regulates expression of cell adhesion molecules, facilitating the recruitment of inflammatory cells to sites of injury.[47] TNFα also primes phagocytes to produce cytotoxic oxidants. More recent studies have suggested a new and potentially more important role for TNFα in the maintenance of homeostasis and in initiating tissue repair.[48] Findings that expression of TNFα in the liver is biphasic, increasing both early (within 3 hours) and again later (24 to 48 hours)

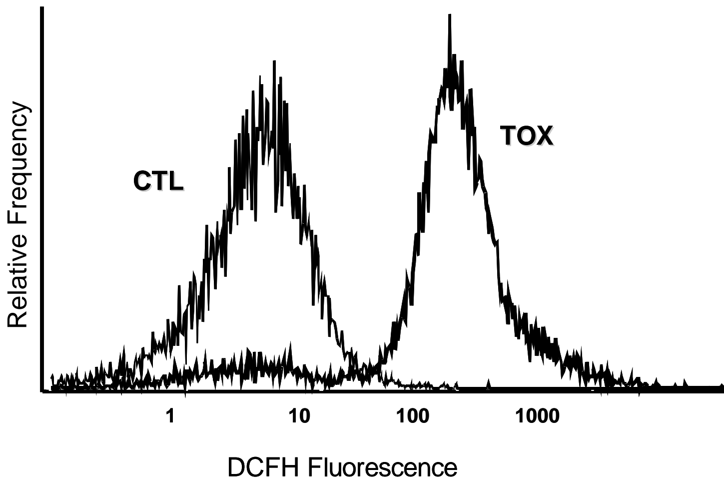

FIGURE 7.8 Production of reactive oxygen intermediates by activated macrophages. Cells isolated from the livers of control (CTL) or toxicant (TOX)-treated mice were incubated with phorbol myristate acetate for 15 minutes at 37°C, followed by the indicator dye, DCFH-DA. After 15 minutes, the cells were analyzed for green fluorescence by flow cytometry on a Coulter Cytomics FC500 flow cytometer (Beckman Coulter). For each analysis, at least 10,000 events were collected and analyzed using CXP software.

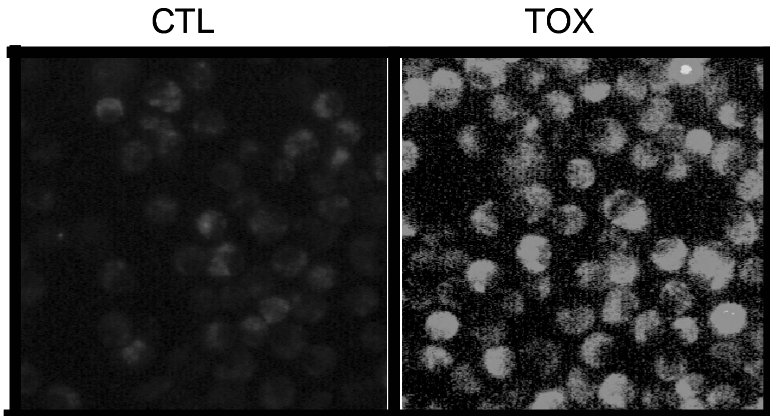

FIGURE 7.9 (See color insert) Peroxynitrite production by activated macrophages. Cells isolated from control (CTL) and toxicant (TOX)-treated animals were cultured overnight in the presence of IFNα + LPS. Phorbol myristate acetate was then added. Thirty minutes later, the cells were loaded with DHR 123. After 5 minutes incubation, the cells were rinsed and analyzed for fluorescence associated with peroxynitrite production by confocal microscopy.

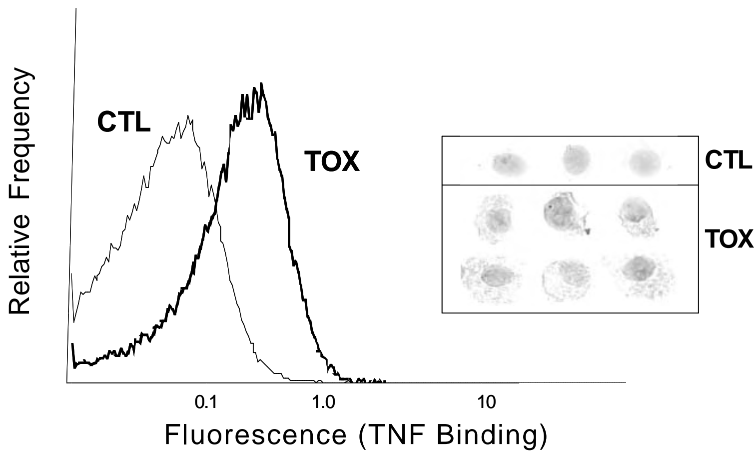

FIGURE 7.10 Activated macrophages express increased quantities of TNFα. Macrophages isolated from the lung of control (CTL) or toxicant (TOX)-treated mice were incubated with anti-TNFα antibody for 1 hourr at room temperature. Cells were then washed and incubated with FITC-conjugated anti-goat IgG. After 45 minutes, the cells were washed with PBS and analyzed by flow cytometry on a Coulter Cytomics FC500 flow cytometer. For each analysis, at least 10,000 events were collected and analyzed using CXP software. Inset: Macrophages were stained with anti-TNFα followed by peroxidase labeled secondary antibody. Cells were visualized microscopically after incubation with diaminobenzidine.

after acetaminophen administration to mice, are consistent with the pathologic and protective roles of this cytokine in hepatotoxicity.

Inflammatory cytokines such as TNFα exert their biological activity by binding to specific receptors on sensitive targets.[49] Thus, in addition to analyzing cytokine expression in tissues and isolated cells using specific antibodies, receptors for inflammatory mediators can also be evaluated as a measure of macrophage activation. For example, following administration of toxicants such as endotoxin to rats, expression of receptors for the bioactive lipid, platelet activating factor (PAF), is upregulated in macrophages.[50,51] This is associated with a marked increase in PAF-induced intracellular calcium mobilization, measured by the calcium sensitive fluorescent indicator dye Indo-1.[52] PAF also causes a rapid decrease in intracellular pH in macrophages that was quantified using the pH sensitive fluorescent dye SNAFL-calcein.[53] This decrease occurred more rapidly in macrophages from toxicant treated rats, when compared to controls.[54] These data suggest that biochemical responsiveness is also altered in macrophages activated following xenobiotic-induced injury.

A question arises as to the mechanisms that may be involved in regulating macrophage activation and excessive mediator production in tissues following xenobiotic-induced injury. Receptor binding has been linked to activation of a number of biochemical signaling pathways including tyrosine kinases, mitogen activated protein kinases, and phosphoinositide 3-kinase/Akt, ultimately leading to activation of nuclear transcription factors. Commercial antibodies to various proteins involved in these signaling pathways can be used, together with flow cytometry and/or fluorescence imaging to assess their

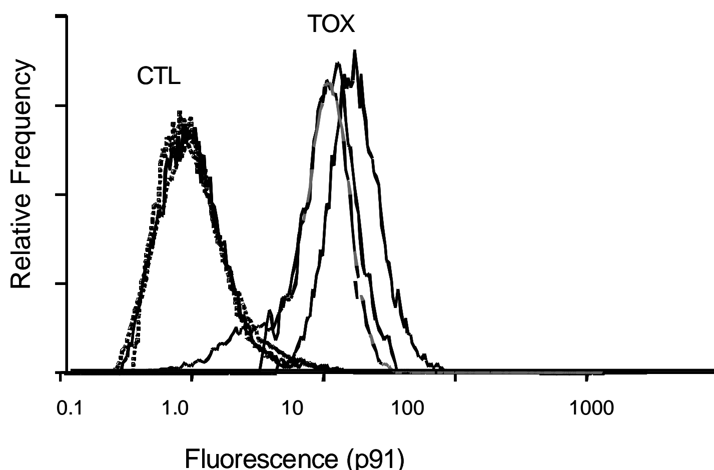

FIGURE 7.11 Increased expression of Stat-1 (p91) by activated macrophages. Cells isolated from the lung of control (CTL) or toxicant (TOX)-treated mice were incubated with anti-Stat-1 (p91) antibody for 1 hour at room temperature. Cells were then washed and incubated with FITC-conjugated anti-goat IgG. After 45 minutes, the cells were washed and analyzed by flow cytometry on a Coulter Cytomics FC500 flow cytometer. For each analysis, at least 10,000 events were collected and analyzed using CXP software. The responses of three representative samples of macrophages from control and toxicant-treated animals are shown.

role in macrophage activation. An example of using flow cytometry and a specific antibody to Stat-1 (p91), an important transcription factor protein mediating the action of interferon gamma[55] is shown in Figure 7.11. The fact that increased expression of Stat-1 is observed in macrophages from toxicant-treated animals suggests that this transcription factor may be important in regulating the functional activity of these cells.

In summary, flow cytometry is clearly useful in evaluating macrophages and their role in toxicity. A major advantage of this technology is the rapid and accurate identification of subpopulations of responding cells from within a mixed population. There is no doubt that the utility of flow cytometry will increase in the future as new fluorescence probes are developed that allow investigators to more clearly assess various macrophage characteristics and the response of these cells to xenobiotics.

CHALLENGES

In the future, it is likely that many labor-intensive functional immunological assays performed *in vivo* or *ex vivo* will be replaced by high throughput multiparameter cytometric-based assays. The challenges faced both today and in the future have been recently described.[56] For example, translational aspects of the nonclinical data to the clinical setting are difficult because despite much similarity, there are still species differences in some of the very basic aspects of the immune system. Additionally, reagents

are more widely available for murine and human immune markers, yet most industrial toxicology is performed in the rat, dog, and primate. As noted previously, reagents in the dog are very limited and have not been validated across laboratories. Often human reagents will cross react in primate; however, if binding is to different epitopes of the same protein, there may be variations in reagent performance, and the same clone and fluorochrome can vary in performance between commercial suppliers. Finally, there is the issue of computer validation for Good Laboratory Practice (GLP) Guidelines. Some manufacturers provide a validation protocol that is intended to comply with clinical laboratory requirements, but it is unclear if there is consistency across vendors. What is clear is that all instruments for GLP studies should be rigorously, routinely, and consistently calibrated and periodically verified against a validation protocol. Standard operating procedures (SOPs) for these activities, as well as cell prep, and data gathering and analysis are needed.

CONCLUSIONS AND FUTURE DIRECTIONS

Scientific progress has resulted in the continual development and evolution of useful and powerful techniques, such as multiparameter flow cytometry and image analysis, to answer questions of cellular maintenance and function (and the impact chemicals may exert) for the benefit of human health. Multiparameter flow cytometry provides researchers with a capacity to simultaneously examine multiple cellular characteristics on thousands of cells in a relatively short period of time.

The advances in the development of commercially available flow cytometers have, like other technologies, grown almost exponentially. In the past two decades or so, cytometers have gone from single laser instruments that needed a dedicated room to accommodate their size to multi-laser benchtop models, mobile units for "field studies," and the near future promises of almost briefcase-sized portable (carried) models that can be transported directly to the subject, patient, or test species. Moreover, like other technologies, the expense has dramatically decreased such that instead of being a technology confined to core facilities at major universities or single industrial facilities, nearly any lab that needs or desires an instrument can afford one. Data analysis software and access to experts or others working in the field has also eliminated the need for highly trained and dedicated personnel. A list of key resources available is located in Table 7.4.

In addition to flow cytometers, cytometric instrumentation has evolved to include Laser Scanning Cytometry (LSC). The LSC is a cross between a flow cytometer and an imaging cytometer. Data are equivalent to the flow cytometer but is slide-based. Endpoints include light scatter and fluorescence, but the instrument can also record position of individual cells on the slide so that cells of interest can be relocated and re-evaluated or photographed. LSC is valuable when cell morphology is important.

Finally, multiparameter flow cytometry has the potential to improve existing methodologies for the evaluation of immunotoxicological endpoints such as the natural killer cell (NK) assay and the local lymph node assay (LLNA) to evaluate innate immunity and chemical-induced allergic disease. Accepted methodologies typically utilize radio-

TABLE 7.4
Useful Flow Cytometry References and Web Sites

Practical Flow Cytometry, 4th Edition H.M Shapiro, Editor (John Wiley & Sons, Inc., New York), 2003.
Flow Cytometry: A Practical Approach M.G. Ormerod, Editor (IRL Press, Oxford), 1994.
Current Protocols in Cytometry J.P. Robinson et al., Editors (John Wiley & Sons, Inc., New York), 2006.
Flow Cytometry Protocols, 2nd Edition T.S. Hawley and R.G. Hawley, Editors (Humana Press, Totowa, NJ), 2004.
Introduction to Flow Cytometry J.V. Watson, Editor (Cambridge University Press), 1991.
Flow cytometry Data Analysis: Basic Concepts and Statistics J.V. Watson, Editor (Cambridge University Press), 1992.
Flow Cytometry: First Principles, 2nd Edition A.L. Given, Editor (Wiley-Liss, New York), 2001.
www.cyto.purdue.edu ListServe (Hosted by The Purdue University Cytometry Laboratory),
www.isac-net.org International Society for Analytical Cytology (ISAC).
http://flowcyt.salk.edu Salk University.
http://facs.scripps.edu/index/html Scripps Research Institute.
http://science.cancerresearchuk.org/sci/facs Cancer Research UK.

active materials for evaluation of cytotoxicity or proliferation. Investigators are actively pursuing techniques to evaluate these endpoints using multiparameter functional flow cytometry.[57–61] The development of new and improved instrumentation, reagents, and correlation of the information obtained with results in standard immune function tests and clinical evaluations will clearly continue to increase the demand for the powerful and rapidly advancing technology.

ACKNOWLEDGMENTS

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Part II

Immunopharmacology and Immunotoxicology of Therapeutics

8 Targeted Therapeutic Immune Response Modulators

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INTRODUCTION

A new era of interest in immunomodulatory drugs began with increased funding for immunology basic research that originated from two sources: (1) the proven effectiveness of life-saving treatments for degenerative diseases and hematologic cancers, and (2) the public health campaign, joining with patient advocacy groups, to develop new treatments for HIV/AIDS. A decade of focused research in immunology in the 1980s led to fundamental discoveries in the field of leukocyte biology, including the identification of key targets of immunomodulation (e.g., primary and secondary signals in antigen presentation) that fueled the development of new drugs and immunosuppressive treatments for solid organ graft rejection and bone marrow transplantation, as well as novel immunomodulatory treatments for autoimmune diseases.

While the clinical trials for the more traditional small molecule drugs tacrolimus (Prograf®) and mycophenolate mofetil (CellCept®) were nearing completion, new molecular/immunologic and fermentation process developments enabled the full-scale drug manufacturing of monkey or human chimeric monoclonal antibodies (mAbs), and the development of humanized mAbs. Taken together, the new research and more cost-effective fermentation production methods enabled clinical trials for a new field of compounds having low intrinsic (systemic) toxicity with targeted and potent effects on immunity. The agents approved in this area have revolutionized the available therapies for rheumatoid arthritis (RA; e.g., Enbrel®), Crohn's Disease (CD; e.g., Remicade®), psoriasis (e.g., Raptiva®), and multiple sclerosis (MS; e.g., Tysabri®). The mention of

these new biotherapeutics is not intended in any way to recommend them as safe or effective for any given patient; they are merely listed as examples in each of their disease categories for purposes of illustration. MAbs are a good representative example for discussion because a number have been approved since the first approval of a murine mAb drug, Orthoclone-OKT3. In the field of autoimmune diseases, Remicade® was the first chimeric mAb and Humira® was the first "human" mAb to be approved.

In contrast to protein therapeutics, effective small-molecule drug treatment of certain cancers and transplantation require sophisticated health care facilities and expert medical practitioners to oversee post-transplant treatments, patient compliance, and manage risks of chronic therapy in light of side effects. Approximately one in five persons worldwide will suffer at some point in their life from one of the over 80 known autoimmune diseases or disorders. This list of diseases is expanding every year as the role of immunity in the pathogenesis of diseases is further elucidated, such as in the case of delineating the immunologic basis of psoriasis in the 1990s. The public health and worldwide market impact of new drugs with a large therapeutic index is enormous. Distinct advantages of mAbs with large sections of humanized sequence include long serum half-lives and infrequent administration.

Another important tenet that reinforces drug development in this area is the discovery of shared immune-dysregulated mechanisms among a number of autoimmune diseases: a single drug may be effective across a number of diseased populations, such as Remicade® in CD and RA. Furthermore, although T-cell activation is regarded as central to the pathogenesis of a number of diseases, including RA, there is a growing body of evidence for the role of B cells in this disease process. B cells can function as antigen-presenting cells (APCs), activate T cells, secrete pro-inflammatory cytokines, and produce autoantibodies that can directly or indirectly damage tissue and maintain a memory response to autoantigens. Treatments have been designed that target B cells that are involved in the cellular processes that drive RA. Basic discoveries can point to synergistic mechanisms which may suggest appropriate combination biologic immunomodulatory treatments for diseases such as RA. If this synergism is approached with care and supported by extensive research, targeted therapies may be combined to modulate only the immune mechanisms critical in a specific disease, and in doing so might one day virtually arrest disease progression. The art in this field is to identify the disease-selective immunomodulated pathways and subtly readjust the dysregulated immune processes, without complete blockade of important processes involved in host defense. The knowledge of basic immunology and target populations is expanding rapidly. The efficacy of a number of recently approved protein therapeutics is spearheading the rapid growth of this field as we work out this delicate balancing act with controlled animal and human experiments.

This chapter serves to illustrate a number of growth areas, and raise the consideration that some of the hallmarks of desirable drugs in this area affect immunity in potent and sometimes unintended manners.

CYTOKINE THERAPIES

Cytokines are important mediators of the immune system and many therapies targeting them have been designed to either augment the activities of the immune system to destroy

tumors or infectious pathogens, or down regulate or suppress responses in inflammatory, hypersensitivity, or autoimmune diseases. Cytokines have also been exploited for their ability to stimulate growth and differentiation of hematopoietic cells and their progenitors to help reverse the myelosuppressive effects of chemotherapy.

Interleukin (IL)-12 is a key regulator of cell-mediated immunity that has therapeutic potential in cancer and infectious disease. IL-12, which is produced by macrophages, increases IFNγ production in natural killer (NK) and T cells, enhances the cytotoxicity of NK and T cells, and promotes T-helper cell Type 1 immune responses both *in vitro* and *in vivo*. In preclinical models, IL-12 enhances the protective immune response against bacteria,[1] intracellular pathogens,[2] and tumors[3] in a variety of murine models. Treatment with recombinant human (rh) IL-12 was generally well tolerated in nonhuman primates when administered for 2 weeks, with a transient decrease in circulating lymphocytes and reversible thrombocytopenia and anemia.[4] Similar findings were observed with murine IL-12 in mice.[5] The initial clinical trial with rhIL-12 was designed to evaluate the pharmacokinetic and pharmacodynamic properties when given as a single dose with a 13-day rest period, followed by 5 daily doses with a 16-day rest period. This trial established a safe dose that was then utilized in a larger Phase 2 trial. However, when patients were administered multiple doses, this previously safe dose resulted in severe multi-organ toxicity and two deaths.[6] In subsequent studies conducted in mice and primates to investigate the mechanism of the toxicities observed clinically, it was determined that a single-dose of IL-12 followed by a rest period protected the animals against the toxicity observed with daily dosing; this protection was associated with an attenuated IFNγ response. This study highlights the complexity of cytokine activity and the importance of evaluating their safety under the same treatment regimens that will be administered clinically.

Several pro-inflammatory cytokines, such as TNFα, IL-1, IL-6, are important in the initiation and maintenance of various autoimmune diseases, such as RA, CD, and psoriasis. Thus, targeted therapies, which have been developed to inhibit their activity, have resulted in clinical improvement of these patients. Currently, there are three TNFα inhibitors (etanercept, infliximab, and adalimumab) and one IL-1 receptor antagonist (anakinra) that have been approved for the treatment of at least one of these diseases. In addition, a number of other anti-cytokine therapies are in clinical development. The TNFα antagonists will be reviewed here.

Etanercept (Enbrel®) is a recombinant fusion protein consisting of extracellular domains of the human p75 tumor necrosis factor receptor (TNFR) linked to the Fc fragment of human immunoglobulin G1 (IgG1). Its major biological activity is due to the binding of TNF resulting in decreased levels of biologically active TNF.[7] Etanercept also binds to lymphotoxin, which may contribute to some of its activity. Although etanercept contains the Fc region of IgG1, it does not fix complement or lyse cells expressing membrane TNF in the presence of complement, and does not lead to the killing of TNF-producing cells. Etanercept has been approved for the treatment of a number of autoimmune diseases, including RA, juvenile RA, psoriatic arthritis, ankylosing spondylitis, and psoriasis. Infliximab (Remicade®) is a chimeric IgG1 anti-TNFα mAb that has been approved for the treatment of RA, CD, and ulcerative colitis. Infliximab binds with high affinity to both the soluble and transmembrane form of TNFα, thereby inhibiting its binding to the TNF receptor and neutralizing the biological activity of

TNF.[8] When infliximab binds to membrane bound TNF, it can also cause the *in vitro* destruction of immune cells by antibody-dependent cellular cytotoxicity.[9] Additionally, the binding of infliximab to membrane-bound TNF on activated T cells promotes apoptotic T-cell death.[10,11] Adalimumab (Humira®) is a fully human IgG1 mAb to TNF developed through phage display technology. Adalimumab has been approved for the treatment of RA and psoriatic arthritis. Similar to infliximab, adalimumab binds to both soluble and membrane bound TNF, blocking its interaction with TNF receptor and lysing cells expressing TNF on their surface.[12] Unlike etanercept, neither infliximab nor adalimumab binds lymphotoxin.

Although this class of agents has dramatic therapeutic effects in patients, there are a number of important safety concerns due to the role of TNF as a central mediator of inflammation and immunity. Inhibition of TNF has been associated with some rare, but serious adverse events, including granulomatous infections (particularly mycobacterium tuberculosis [TB]), demyelinating disorders, autoimmune disorders, hematologic/lymphoproliferative disorders, and congestive heart failure. In addition, infusion-related events and the development of neutralizing antibodies have been a concern for some patients. Whether some of these safety concerns are directly mediated by TNF inhibition or are a consequence of the disease itself and/or the co-morbid condition of the patient population and their concomitant use of medications has yet to be determined.

There is a vast amount of published literature from preclinical models that document the role of TNFα in the protective immune response against infectious agents. The administration of anti-TNF antibodies or soluble TNF receptor Ig fusion proteins reduces host defenses in various clinically relevant intracellular and extracellular infection models, including Listeria,[13] TB,[14] cutaneous leishmaniasis,[15] and streptococcal pneumonia,[16,17,18] leading to an increased incidence of infection-induced mortality. In models of latent TB in mice,19 these agents have resulted in the reactivation of TB and demonstrated the requirement of TNF for the formation and maintenance of granulomatous inflammation that is necessary to contain the intracellular organisms. The clinical experience with currently marketed TNFα inhibitor therapies also suggests a reduced defense against certain infections in humans, including TB, which has resulted in serious and sometimes fatal infections and sepsis and is reflected in the manufacturer's product labeling.[7,8,12] A quantitative assessment of risk of TB due to TNF-antagonist is difficult to ascertain as the cases to date have been predominantly reported through passive surveillance. The risk for TB in RA patients is associated with multiple other factors, including age, country of origin or current residence, previous exposure history to persons with TB, concomitant therapy with other immunomodulators, and disease activity. Patient screening has proven to be an effective means to reduce the incidence of this serious adverse effect.

The role of TNF in tumor development is unclear. Although TNF has tumor cytolytic activity in both *in vitro* and *in vivo* tumor models,[20] mice deficient in TNF do not have a propensity for the development of spontaneous tumors.[21,22,23] Animal carcinogenicity data are not available on the oncogenic potential of theses marketed TNF-blocking agents, as they are either not active in rodents and/or are immunogenic, precluding long-term rodent studies. In the double-blind and open-label clinical studies with the TNF-blocking agents, the overall incidence and types of malignancies observed were generally similar

to the overall general population, whereas the incidence of lymphoma increased.[24,25] However, the incidence of lymphoma was commensurate with the increased incidence expected in the RA patient population with severe disease.[26] Since RA patients have a chronically dysregulated immune system, this defect may lead to an increased risk for cancers, particularly those of the lymphoproliferative type.[27,28,29,30] Furthermore, the risk for cancers is likely further increased due to the use of concomitant immunosuppressive medications in the RA patient population.

In addition to concerns of infections and malignancies, there have been rare cases of autoimmunity and CNS demyelination reported in RA patients treated with different TNF-antagonists. There have also been reports of increases in the percentage of RA patients with positive anti-nuclear antibody and anti-double stranded-DNA serologies, putative markers of autoimmune disease, lupus-like syndromes, and autoimmune skin rashes.[31] In MS patients, neurologic and demyelinating events, including exacerbation of existing multiple sclerosis or optic neuritis, have been observed with the use of TNF antagonists.[7,32] The potential for an increase risk of congestive heart failure is also a concern due to a slightly elevated mortality rate in clinical trials with etanercept and infliximab for the treatment of congestive heart failure itself. Although a causal relationship has not been conclusively established, these adverse events may possibly be related to TNF biology and that patients with a history of these diseases should avoid its use.

SELECTIVE COSTIMULATORY MODULATORS

Full activation of T cells in an immune response requires the delivery of at least two signals from APCs.[33] The first signal, conveyed through the interaction of the T-cell receptor (TCR) with major histocompatibility complex molecules on the APC, provides antigen specificity. The second signal, mediated by costimulatory molecules on T cells and the interaction with their ligands on APC, serves to initiate, maintain, and regulate the T-cell response. The absence of a second signal after exposure of the T cell to antigen can result in an unresponsive or anergic state. There are numerous co-stimulatory molecules expressed on T cells, including CD28, CD154 (CD40 ligand), the CD28-like inducible T-cell co-stimulator precursor (ICOS), CD27, CD134 and CD137 (41BB). Of these, the CD28-CD80/CD86 pathway is one of the most prominent and well characterized. Resting T cells predominantly use the CD28 receptor on APCs (CD80/86) to initiate activation, and selective blockade of CD28 inhibits the initiation of T-cell activation. After TCR engagement, T cells rapidly upregulate CD154, ICOS, CD27, CD134, CD137 and several other receptors that amplify and sustain or regulate their activation. Within days of activation, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4, CD152) is expressed on the surface of T cells. CTLA-4 binds with greater avidity for CD80/CD86 than CD28. CTLA-4 ligation also transduces a negative signal to the T cell, thereby down regulating the immune response.[34,35,36,37] The interaction of CD28-CD80/86 molecules is also important in the homeostasis of regulatory CD4+, CD25+ T cells, which are essential for the maintenance of tolerance to tissue-specific antigens.[38,39,40] These molecules play an important role in the pathogenesis of infectious disease, autoimmune disease, graft rejection, and graft-versus-host disease. Costimula-

tory antagonists prevent or ameliorate these diseases in preclinical models. Several of these molecules are being or have been evaluated clinically and a few will be discussed below.

Abatacept (Orencia®) is a recombinant, soluble, fusion protein consisting of the extracellular domains of human CTLA-4 linked to a fragment of the Fc domains of human IgG1. It is the first in a new class of co-stimulation blocking drugs recently approved for the treatment of RA. The binding of abatacept to CD80/86 blocks CD28-mediated costimulation, inhibiting the activation of T cells and the secondary activation of macrophages and B cells. Thus, abatacept works early in the inflammatory cascade. CTLA-4 is the high avidity receptor for CD80 and CD86, having a higher affinity for CD80/86 than CD28. Thus, abatacept can effectively inhibit binding of CD80/86 to CD28. Although abatacept binds to Fc receptors, the human IgG1 region contains mutations in the hinge region that result in reduced complement fixation.[41,42]

In vitro, abatacept decreased antigen-specific T-cell proliferation and proinflammatory cytokine production by human naive T cells and partially inhibited antigen-specific circulating human memory T-cell proliferation and cytokine production, while preserving CD8+ T-cell cytolytic activity and the production of TNF-α from LPS-activated monocytes.[41,43] *In vivo*, abatacept decreased antigen-specific antibody production to T-cell-dependent antigens in rodents and nonhuman primates.[44,45] Abatacept was also efficacious in a number of animal models of T-cell-mediated disease, including solid-organ transplant rejection, RA, systemic lupus erythromatosus (SLE), and MS.[46,47,48 49,50,51]

Several studies have evaluated the effects of CD28 modulation on host responses to a number of pathogens, including lymphocytic choriomeningitis virus, vaccinia virus, influenza virus,[52] vesicular stomatitits virus, herpes simplex virus, murine leukemia virus, Listeria,[53,54] and Leishmania.[55,56] The results of these studies suggest that the protective *in vivo* immune responses to most pathogens are largely preserved in animals treated with abatacept. However, abatacept may increase the risk of clinical infection to some pathogens that require a CD4+ T-cell-mediated mechanism for clearance, such as herpes simplex virus.

The long-term safety of abatacept was thoroughly evaluated in preclinical studies since abatacept is pharmacologically active in a number of species and suppresses the antibody response to itself. Abatacept was clinically well tolerated for up to 6 months of treatment in mice or 1 year in monkeys, with no evidence of opportunistic infections or target-organ toxicity.[41] The most notable concern identified during preclinical testing was an increased incidence of malignant lymphomas and mammary gland tumors (in females) in the mouse carcinogenicity study. The increased incidence of lymphomas and mammary tumors observed in mice treated with abatacept was likely associated with a decreased control of murine leukemia virus and mouse mammary tumor virus, respectively, in the presence of long-term immunomodulation.[57] No mutagenic potential of abatacept and no chromosomal aberrations in human lymphocytes with abatacept were observed in a battery of *in vitro* genotoxicity studies. Although the preclinical mouse carcinogenicity data suggest that abatacept may increase the risk for development of virally induced or virus-associated tumors, a similar signal was not observed in monkeys over a 1-year treatment period. In a 1-year toxicity study in cynomolgus monkeys, abatacept was not associated with any significant drug-related toxicity, and

reversible pharmacologic effects consisted of minimal transient decreases in serum IgG and minimal to severe lymphoid depletion of germinal centers in the spleen and/or lymph nodes. No evidence of lymphomas or preneoplastic morphologic changes was observed, despite the presence in these animals lymphocryptovius (LCV), an oncogenic Epstein-Barr virus-like gamma herpes virus known to be associated with these findings in immunosuppressed monkeys.[52] The incidence of malignancies in clinical data appear to be within the expected range. However, only longer-term clinical data will resolve this issue.

Similar to what was observed preclinically, abatacept treatment was also well tolerated in psoriasis and RA patients; these patients had mild transient decreases in serum IgG and IgA, but no decrease in peripheral blood lymphocyte counts or overall serum Ig levels. Abatacept-treated patients did have an increase in certain bacterial (upper respiratory tract) and viral (herpes simplex virus) infections that were generally mild to moderate in severity, responded well to treatment, and had an outcome similar to placebo-treated patients. Mycobacterial, disseminated viral, and invasive fungal infections were rare.[41,58,59] These clinical findings are generally consistent with the results from the repeat-dose mouse and monkey studies and data obtained with host-resistance models: the absence of any clinical manifestations associated with bacterial, fungal, or viral infection. These data suggest that although abatacept selectively alters immune function, it does not profoundly suppress host immune responses against most pathogens in both animals and humans.

The blockade of CD154 on T cells by anti-CD154 antibodies disrupts its interaction with APCs expressing CD40 that prevents T-cell proliferation and the production of inflammatory cytokines and antibodies that may attack the body's own tissue.[60] CD154 is overexpressed in a number of autoimmune diseases and blocking its interaction with CD40 in various animal models of autoimmune disease appeared to ameliorate the disease condition.[61] In addition, antibodies against CD154 prevented transplant rejection in a primate model of kidney transplantation,[62,63] with some monkeys not rejecting their kidney for a significant period time even after antibody treatment was discontinued.

Toralizumab (IDEC 131) is a humanized mAb and hu5C8 is a chimeric mAb that bind to CD154 expressed on T cells. Both antibodies were in Phase 2 clinical development (toralizumab for MS and CD; hu5C8 for lupus, thrombocytopenic purpura, and kidney transplantation) when clinical trials were halted because of concerns for thromboembolism.[64] During Phase 2 clinical trials with hu5C8, two lupus patients suffered heart attacks, an obese bedridden transplant recipient died of a pulmonary embolism, and approximately 10% of the 100 patients taking hu5C8 experienced excessive clotting. Eventually, toralizumab re-entered clinical trials only to have a CD patient develop a blood clot; at least two other elderly patients also developed blood clots, but none of these findings could be definitively linked to the drug.[65]

It is still not clear what the precise cause of the excessive clotting with hu5C8 was or if toralizumab has the same liability. Although never proven, one theory is that in addition to binding to T cells, hu5C8 also activated platelets through Fc receptor interactions. In animal studies that compared hu5C8 with an aglycosyl form of the antibody with reduced binding to Fcγ and complement, it was demonstrated that while Fc activity was not necessary to suppress humoral immune responses or delay disease progression

in a mouse model of lupus, it was critical in the prevention of allograft rejection.[66] These studies underscore the complexities and challenges of developing therapeutic mAbs and that multiple mechanisms may play a role in efficacy and impact their safety.

Ipilimumab (MDX010) is a fully human IgG1 mAb specific for human CTLA-4 being developed for the treatment of melanoma. Ipilimumab disrupts CTLA-4 interaction with its ligands, CD80 and CD86, expressed on antigen-presenting cells, augmenting immune responses. *In vivo* CTLA-4 blockade induced regression of established tumors and enhanced anti-tumor immune responses in several murine tumor models.[67,68,69,70] Blockade of CTLA-4-mediated signals is also effective in inducing rejection of immunogenic cancers in mice. Poorly immunogenic cancers in mice are rejected when anti-CTLA-4 mAb is used in conjunction with granulocyte macrophage-colony stimulating factor-secreting tumor vaccines, suggesting that CTLA-4 blockade alone, or in combination with vaccines, can induce a potent anti-tumor response. However, the selective blockade of CTLA-4 in CTLA-4-deficient mice and other murine models resulted in lymphoproliferative disease or autoimmunity. In the clinic, the treatment of stage IV metastatic melanoma patients with ipilimumab resulted in cancer regression, including complete responses.[71] Consistent with adverse effects observed in animal models, several of these patients also experienced grade 3/4 autoimmunities, including dermatitis, colitis, hepatitis, uveitis and hypophysitis, which were successfully managed by supportive care and/or corticosteroid therapies. Interestingly, there was a strong correlation between the induction of tumor regression and grade 3/4 autoimmune toxicities.

TGN1412 is a humanized super-agonistic IgG4 mAb specific for human CD28 expressed on T cells, intended for the treatment of B-cell chronic lymphocytic leukemia (BCLL) and RA. The binding of TGN1412 to the CD28 molecule on humans and nonhuman primate T cells bypasses the requirement for TCR signaling (Signal 1) and activates T cells regardless of their TCR specificity.[72] TGN1412 binds to the CD28 molecule at a site different from other anti-CD28 mAb that act in concert with TCR engagement.[73] While the Fc region of the IgG4 molecule does not mediate complement-dependent or antibody-dependent cytotoxicity, cross-linking via the Fc receptor is required for the efficient activation of T cells.

In vitro TGN1412 caused a profound, polyclonal T-cell proliferation of human peripheral blood mononuclear cells, including those from patients with BCLL. It also induced a profound activation and proliferation of T-cell subsets including CD4+ and CD8+ T cells, naive and memory T cells, and regulatory T cells. TGN1412 was shown to induce a transient, well tolerated expansion of T cells in nonhuman primates treated with TGN1412 and efficacy was demonstrated in a rhesus monkey collagen-induced arthritis model.

The safety of TGN1412 was evaluated in the cynomolgus monkey. TGN1412 was well tolerated following intravenous administration as doses up to 50 mg/kg weekly for 4 weeks. No drug-related toxicities were observed including evidence of hypersensitivity or systemic immune system deviation. With regard to the immune system, there were no adverse hematological changes or changes in globulin levels and no treatment-related alteration in lymphoid organs. Consistent with the pharmacology, a transient increase in CD4+ and CD8+ T-cell numbers were observed between Days 13 and 17 after dosing, and an increase in CD25+CD4+ T-cell numbers that correlated with the CD4+ cell count. Although CD69 is upregulated *in vitro*, an increase in activated CD4+CD69+ or

CD8+CD69+ T cells was not observed *in vivo*. NK cells appeared to increase during the recovery period and an expansion of B cells was observed that persisted longer than that observed for T cells in individual animals. Transient, moderate elevations of serum IL-2, IL-5 (anti-inflammatory TH2-type cytokine), and IL-6 (inflammatory cytokine) was observed in individual animals at 2 or 24 hours following the initial dose but were not observed on Days 17 or 62; no changes in TNFα or IFNγ (major pro-inflammatory cytokines) were observed. Thus, TGN1412 did not appear to be associated with a cytokine-release syndrome that has been observed in humans with upon administration of agonistic anti-CD3 antibodies.[78]

In the Phase 1, first-in-man trial, all six subjects who were administered the starting dose of 0.1 mg/kg/day, estimated to be 1/500th of the no-observable-adverse effect dose in the monkey, developed severe headaches, vomiting, back pain, fever, and swelling of soft tissue of the head, immediately following dosing, which progressed to multi-organ failure and coma in several patients. The United Kingdom Medicines and Helathcare Regulatory Agency immediately suspended the filing and launched an investigation. Their interim report concluded that the drug was not contaminated and that the trial was run according to protocol. Rather, the data suggests that the most likely cause is that the drug induced a pharmacological effect, stimulating T cells in a way that caused the release an overwhelming flood of inflammatory cytokines, a cytokine-release syndrome, that was not observed in preclinical animal models despite apparently adequate preclinical testing.[74] The full impact of this event on clinical trials world wide has yet to be determined.

T-CELL TARGETED THERAPIES

Muromonab-CD3 (Orthoclone OKT3®) is a murine IgG2a mAb used in the treatment of acute allograft rejection, which binds to CD3 expressed on the surface of mature T cells in a complex with the TCR.[75,76] *In vitro*, OKT3 activates T cells resulting in the release of numerous cytokines, including IL-1, IL-2, IL-6, TNFα, and IFNγ.[77] In vivo, the release of these cytokines following the first dose can result in a flu-like syndrome, characterized by fever and chills, and occasionally dyspnea and hypotension.[78,79] In addition, complement can be activated leading to a rapid and profound depletion of mature peripheral T cells from the peripheral blood and the CD3/TCR complex is down modulated, leading to immunosuppressive activity.[80] Upon treatment discontinuation, peripheral blood T-cell numbers and their surface expression of CD3 return to normal levels.

Priliximab (cM-T412) is an anti-CD4 chimeric monoclonal antibody that was evaluated in the clinic for the treatment of autoimmune diseases. Priliximab binds to CD4 on the surface of T cells and leads to a profound and sustained decrease in circulating CD4+ T cells; decreased counts have been reported to be below normal levels at 18 and 30 months following single- and multiple-infusions.[81] Similar findings were observed in preclinical studies in chimpanzees.[82] The administration of priliximab was also associated with a cytokine-release syndrome that caused transient fever, myalgia, chills, headache, nausea, and/or hypotension that was accompanied by an increase in serum IL-6. Although evidence of efficacy was observed in clinical trials for CD, the

long-term, possibly irreversible, decrease in CD4+ cells was of significant concern and no further studies have been conducted. Several non-depleting anti-CD4 antibodies were also evaluated in CD and ulcerative colitis (UC), but demonstrated less efficacy than the depleting antibodies.[83,84,85]

Alefacept (Amevive®) is a recombinant fusion protein consisting of the first extracellular domains of human LFA-3 fused to a fragment of the Fc domains of human IgG1. The LFA-3 portion of alefacept binds to the CD2 receptor, which is expressed on all T-cell subsets, preventing their interaction with LFA-3 on antigen-presenting cells.[86] However, the greater the expression of CD2, the greater the binding avidity. Since CD2 expression is greater on activated and memory-effector T cells (characterized by the CD45RO+ phenotype) than naive T cells,[87] alefacept preferentially binds to memory-effector T cells and consequently inhibits their activation and the associated inflammatory response. In addition, the IgG1 Fc domains of alefacept bind to receptors on macrophages, NK cells, and neutrophils, resulting in the release of pro-apoptotic mediators by these cells, resulting in targeted apoptosis of CD2-bearing cells, especially memory-effector T cells, which further contributes to its biological activity.[88]

Alefacept was the first biologic agent approved for the treatment of psoriasis. It is administered IM or IV once weekly for 12 weeks with a minimum of 12 weeks between treatment courses. Alefacept reduces the number of total lymphocytes and CD4+ and CD8+ lymphocyte counts. This reduction in T cells is selective for memory T cells, sparing naive T cells, as well as CD19+ B cells and CD16+/CD56+ NK cells. By 12 weeks after the last dose and prior to the next course of treatment, total lymphocyte levels return to normal. Due to the profound effect on lymphocytes, CD4+ counts are monitored weekly during treatment. Patients responding to alefacept have consistent reductions in the number of T cells infiltrating diseased skin, as well as dendritic cells, and a marked reduction in several key inflammatory molecules. This activity may be due to the depletion of activated clones of disease causing memory T cells in tissue or alternatively, there may be an increase in regulatory T cells via CD2 stimulation.

Alefacept was generally well tolerated in clinical trials. Despite its effect on T-cell counts, there was no evidence of an increased risk of infection or malignancies and no opportunistic infections were reported.[89] Furthermore, patients had a robust antibody response to both neoantigens and recall antigens despite its mechanism of action leading to a reduction in T cells.[90] In a 1-year toxicity study in monkeys, B-cell lymphoma was noted in 1 high-dose animal treated with alefacept after 28 weeks of dosing, and additional monkeys in this study developed B-cell hyperplasia of the spleen and lymph nodes. Evaluation of the lymphoid tissue of these and other monkeys treated with alefacept documented B-cell hyperplasia, presumably mediated by a gamma herpesvirus (LCV) that was detected in lymphocytes by molecular analysis.[91,92] Latent LCV infection is generally asymptomatic, but has been associated with B-cell lymphomas in immunosuppressed Old World primates.[93]

B-CELL TARGETED THERAPIES

Rituximab (Rituxan®) is a chimeric IgG1 anti-CD20 mAb that selectively targets B lymphocytes. CD20 is a transmembrane phosphoprotein that is densely expressed on

pre-B cells and mature B cells, but not on precursor cells or plasma cells.[94] It is also expressed on the majority of B-cell lymphoma cells. CD20 is essential in the differentiation and proliferation of B-lymphocytes. The binding of rituximab to CD20 leads to the rapid depletion of B cells from the blood, bone marrow and lymphoid tissue for up to 3 to 6 months.[95] The selective depletion allows for the reconstitution of the B-cell compartment after therapy by the precursor cells and the continuous production of immunoglobulins by the plasma cells. The lysis of B cells is mediated by multiple mechanisms that include antibody-dependent cellular cytotoxicity through the recruitment of natural killer cells, macrophages and monocytes via CD20-bound Fc receptors and complement-mediated toxicity by the activation of complement. In addition, binding of rituximab has been demonstrated to lead to calcium influx and B-cell apoptosis. CD20 is not shed into the plasma after binding of rituximab, nor is it internalized after antibody engagement. Rituximab is approved for the treatment of relapsed or refractory, low grade, or follicular CD20+ B-cell non-Hodgkin's lymphoma and RA.[96] In open-labeled studies, rituximab appears to be efficacious in other autoimmune diseases, including SLE, idiopathic thrombocytopenia purpura, thrombotic thrombocytopenia purpura, and hemolytic anemia.[97]

Importantly, rituximab does not appear to compromise humoral immunity despite B-lymphopenia and a significant drop of serum IgM levels. The overall incidence of infections does not appear to be increased even after prolonged treatment with the antibody[98] and the incidence of serious infections appears to be similar to the range observed with anti-TNF agents.[99] However, rituximab is associated with an infusion-related toxicity profile, which is characterized by fever, chills, hypotension and nausea. This reaction which is most frequently observed following the first infusion, is greatly reduced following subsequent infusions, and is likely the result of the lysis of B cells/lymphomas and the ensuing release of cytokines.

ANTI-ADHESION MOLECULES

The recruitment of lymphocytes from the blood into tissue at sites of inflammation or injury is a multi-step process regulated by the sequential engagement and activation of adhesion molecules on leukocytes and endothelium. Initially, leukocytes flowing in the axial core of the blood stream slow down and begin to roll along the inflamed blood vessel endothelium through the interactions of selectins and integrins with their respective ligands. The rolling leukocytes are now able to respond to chemokines secreted by inflammatory cells in the diseased or inflamed tissue, activating adhesion molecules in the integrin family that mediate adhesion to endothelial cells. Once the cells are attached, the process of diapedesis begins.[100,101,102] The crucial role of adhesion molecules in inflammatory disorders makes them an attractive therapeutic target for a number of diseases.

Natalizumab (Tysabri®) is a recombinant humanized IgG4 mAb that binds to the α4-integrin subunit of α4β1 (VLA-4) and α4β7 (LPAM-1) integrins expressed on the surface of all leukocytes except neutrophils, and inhibits the α4-mediated adhesion of leukocytes to their respective receptors.[103,104] The receptor for α4β1, vascular cell adhesion molecule-1 (VCAM-1), is expressed on activated endothelial cells and is

believed to be involved in the entry of leukocytes into sites of inflammation, including the central nervous system (CNS). The receptor for α4β7 is mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is expressed on vascular endothelial cells of the gastrointestinal tract. The inhibition of this interaction is thought to be important in preventing the further recruitment of activated leukocytes into the inflamed tissue, thereby reducing the inflammatory state in diseases such as MS, inflammatory bowel disease (IBD), and CD.

Natalizumab was approved by the U.S. FDA for the treatment of MS in November 2004, and is being evaluated in Phase 3 clinical trials for IBD and CD.[105] Based on Phase 3 clinical trials in MS patients, natalizumab appeared to be safe, since an increased rate of infection was not observed after 1 year of treatment.[106] The safety of combined treatment with immunosuppressants, other than short courses of corticosteroids, was not evaluated and was not recommended because of the potential for an increased risk of infections. However, barely three months after its approval, natalizumab was voluntarily withdrawn from the MS market by its manufacturer and all ongoing clinical trials were suspended due to the occurrence of a rare neurological disease, progressive multifocal leukoencephalopathy (PML), in 3 out of approximately 3,000 patients enrolled in clinical trials (2 in MS and 1 in CD).[107,108,109]

PML, a fatal CNS disorder associated with opportunistic infection or reactivation of a clinically latent JC polyoma virus. This virus infects and destroys oligodendrocytes, leading to multifocal demyelination and neurologic dysfunction. After infection, the virus remains latent but can become activated in immunocompromised individuals, such as patients with AIDS, leukemia, or organ-transplant recipients who have profoundly impaired cell-mediated immunity. All three patients that developed PML had been treated for at least 18 months with natalizumab and had received concomitant treatment with other immunosuppressive agents. Although the mechanistic link remains to be elucidated, it is possible that the combination of suppressant(s) and the targeted inhibition of lymphocyte trafficking allowed the endogenous virus to evade surveillance. Subsequently, the FDA suspended all clinical trials that involve α4β1, but not α4β7, as the target until the full results of the Tysabri® safety data are completed. However, on March 8, 2006, the FDA advisory panel unanimously recommended that natalizumab should be returned to the market but with certain restrictions. While the FDA had removed the hold on clinical trials, its return to the market is still pending FDA decision as of this writing.

CONCLUSION

The impact of immunomodulatory protein therapeutics on diseases having unmet medical need has already proven remarkable. These new treatments have been credited with a favorable clinical profile from slowing the disease progression to outright cures. Although the cost of these advanced medicines may be offset by the enormous improvement in the quality of life of numerous patients, we must remain vigilant throughout their clinical development.

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9 Immunoaugmenting Therapeutics: Recombinant Cytokines and Biological Response Modifiers

James E. Talmadge

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INTRODUCTION

Immunotherapy has a long history in the treatment of cancer. Immunoaugmenting drugs were popularized by William B. Coley, who treated cancer patients with mixed bacterial toxins early in the 20th century.[1] These early studies resulted in the clinical approval of microbially derived substances such as Bacille Calmette-Guerin (BCG) (bladder cancer, USA), Krestin, Picibanil, and Lentinan (gastric and other cancers, Japan), and Biostim and Broncho-Vaxom (recurrent infections, Europe). While these "crude" drugs have numerous immune augmenting activities, they also have regulatory difficulties due to impurities, lot-to-lot variability, and adverse side effects. Similarly, traditional herbal medicines (Asia) may contain constituents with immunotherapeutic

activity. The purification, characterization, and synthetic production of active moieties from natural products (Bestatin, Taxol) and culture supernatants (FK-506, Rapamycin, Deoxyspergualin, and Cyclosporin) have provided valuable drugs. The current developmental focus is on recombinant proteins (cytokines and monoclonal antibodies (mAbs)), although the utility of these drugs are limited due, in part, to bioactivity and pharmacologic deficiencies (see chapter 8 of this volume for a discussion of cytokines and monoclonal antibodies as therapeutic agents).

The 20th anniversary of the first approved biopharmaceutical-recombinant insulin (Humulin: Genotech, USA) was in 2002. Today, biotechnological drugs incorporate not only immunoregulatory proteins, enzymes, and biologics derived from natural sources, but also engineered (manipulated) mAbs and cytokines, in addition to gene therapy, and tissue and cellular engineering strategies. The growth in the number of approved biopharmaceuticals, as well as the number that are currently undergoing clinical evaluation, has been rapid. Thus, we have limited our discussion of biotherapeutics to immune augmenting agents, including recombinant, natural, and synthetic drugs that are currently approved for clinical use. However, we have not included vaccines and mAbs, and will not discuss nucleic or engineered tissue gene therapeutic products.

The approach taken herein is to subcategorize the various immune augmenting agents and identify if they are approved for sale in either the United States or other countries. Select recombinant proteins, natural products, and synthetic immune augmenting drugs are discussed in more detail, predominantly to make a point or discuss general characteristics. The figures provide a review immunoregulatory cascades, illustrating a general concept while using one or more specific examples in the text. In this way, a few overall concepts are discussed, using specific examples, while providing information on the majority of recombinant, synthetic, and natural product drugs.

RECOMBINANT PROTEINS

Recombinant proteins (Table 9.1) have emerged as an important class of drugs for the treatment of cancer, immunosuppression, myeloid dysplasia, autoimmunity, inflammation, and infectious diseases. However, our limited understanding of their pharmacology and mechanism of action (MOA) has hindered development. To facilitate development, information is needed on their pharmacology, toxicology, and MOA.[2,3] One approach to development is to identify a clinical hypothesis based upon therapeutic surrogate(s) identified during preclinical studies.[4,5] A surrogate for clinical efficacy may be a phenotypic, biochemical, enzymatic, functional (immunologic, molecular or hematologic), or quality-of-life measurement that is believed to be associated with therapeutic activity. Phase I clinical trials can then be designed to identify the optimal immunomodulatory dose (OID) and treatment schedule that maximizes the augmentation of surrogate end point(s). Subsequent Phase II/III trials can be established to determine if the changes in the surrogate levels correlate with therapeutic activity. Table 9.1 lists the recombinant proteins that are approved for use in the United States.

Table 9.2 catalogs the recombinant proteins that are immunologically active and have been or are being studied in Phase I/II clinical trials in the United States. Several

TABLE 9.1
Licensed Cytokines

Drug	Corporation	Target	Approval
Actimmune® IFN-γ-1b	InterMune Pharmaceuticals, Inc.	Chronic granulomatous disease Severe, malignant osteopetrosis	Dec. 1990 Feb. 2000
Alferon N Injection® IFN alfa-n3, human leukocyte derived	Interferon Sciences, Inc.	Human papilloma virus and genital warts	Oct. 1989
Amevive® Alefacept, LFA-3-Fc, LFA-1/IgG1	Biogen Idec	Moderate to severe chronic plaque psoriasis	Jan. 2003
Avonex, IFN-β1a	Biogen Idec	Relapsing-remitting multiple sclerosis Exacerbation of multiple sclerosis	May 1996 Feb. 2003
Betaseron® Recombinant IFN-β1b	Berlex Laboratories and Chiron Corp.	Relapsing-remitting multiple sclerosis Exacerbation of multiple sclerosis	Aug. 1993 March 2003
BMP-2 bone morph. protein-2	Medtronic Sofamor Danek	Treatment of spinal degenerative disc disease	July 2002
Enbrel® TNFR:Fc	Immunex	Active rheumatoid arthritis Active rheumatoid arthritis in juveniles	Nov. 1998 May 1999
Enbrel® TNFR:Fc	Amgen	Active ankylosing spondylitis Chronic moderate to severe plaque psoriasis Psoriatic arthritis	July 2003 April 2004 June 2005
EPOGEN® Epetin alfa (rEPO)	Amgen	Anemia, chronic renal failure, anemia in Retrovir® treated HIV-infected Anemia caused by chemotherapy Surgical blood loss Chronic renal failure, dialysis	June1989 April 1993 Dec. 1996 Nov. 1999
Infergen® IFN alfa-con-1	InterMune Pahrmaceuticals, Inc. and Amgen	Chronic hepatitis C viral infection	Oct. 1997

(*Continued*)

TABLE 9.1 Continued

Drug	Corporation	Target	Approval
Intron® A IFN alfa-2b	Schering-Plough	Hairy cell leukemia	June 1986
		Genital warts	June 1988
		AIDS-related Kaposi's sarcoma	Nov. 1988
		Non-A non-B hepatitis	Feb. 1991
		Hepatitis B	July 1992
		Malignant melanoma	Dec. 1995
		Chronic viral hepatitis C	March 1997
		Follicular lymphoma with chemotherapy	Nov. 1997
Kepivance, Palifermin, KSA-2	Amgen, Inc	Oral mucositis in cancer patients	Dec. 2004
Kineret™, Anakinra IL-1Rα	Amgen, Inc	Active rheumatoid arthritis	Nov. 2001
		Sepsis	
Leukine®/Leukine® Liquid, sargramostim (GM-CSF)	Berlex Laboratories	Autologous bone marrow transplant	March 1991
		Neutropenia resulting from chemotherapy	Sept. 1995
		Allogeneic bone marrow transplant	Nov. 1995
		Peripheral blood progenitor cell mobilization	Dec. 1995
		Leukemias (leukopenias and fungal infection)	Nov. 1996
Macugen®, PEG anti-VEGF aptamer	Eyetech Pharmaceuticals, Inc. and Pfizer	Neovascular age-related macular degeneration	Dec. 2004
Neumega Oprelvekin rHu IL-11	Wyeth	Severe thrombocytopenia and reduction of the need for platelet transfusions; Chemotherapy-induced thrombocytopenia	Nov. 1997
NEUPOGEN® Filgrastim (rG-CSF)	Amgen	Chemotherapy-induced neutropenia	Feb. 1991
		Neutropenia during bone marrow transplant	June 1994
		Severe chronic neutropenia	Dec. 1994
		Autologous or allogeneic bone marrow transplantation	Dec. 1995
		Mobilization of autologous PBPCs after chemotherapy	April 1998
Ontak, Denileukin diftitox, IL-2	Ligand Pharmaceuticals, Inc.	Persistent or recurrent cutaneous T cell lymphoma	Feb. 1994

Drug	Corporation	Target	Approval
Orencia, Abatacept	Bristol-Myers Squibb Comp.	Rheumatoid arthritis	Feb. 1999
Procrit®, Epoietin alfa	Ortho Biotech, Inc.	Anemia in AZT-treated HIV patients	Dec. 2005
Procrit®, Epoietin alfa	Ortho Biotech, Inc.	Anemia in chemotherapy treated cancer patients	Dec. 1990
Proleukin, IL-2® Aldesleukin	Chiron	Anemia for elective noncardiac, nonvascular surgery	April 1993
		Renal cell carcinoma	Dec. 1996
Proleukin, IL-2® Aldesleukin	Chiron	Metastatic melanoma	May 1992
Rebetron™ ribavirin/IFN alfa-2b	Schering-Plough Corp.	Chronic hepatitis C with compensated liver disease after α-IFN treatment	Jan. 1998
Rebetron™ ribavirin/IFN alfa-2b	Schering-Plough Corp.	Chronic hepatitis C with compensated liver disease without α-IFN treatment	June 1998
Rebif®, IFN-β-1a	Serono S.A. and Pfizer, Inc.	Relapsing forms of multiple sclerosis	Dec. 1999
Regranex®, Becaplermin, rHP-DGF-BB	Ortho-McNeil and Chiron Corp	Platelet-derived growth factor treatment of diabetic foot ulcers	March 2002
Roferon® IFN alfa-2a	Hoffman-La Roche	Hairy cell leukemia	Dec. 1997
Roferon® IFN alfa-2a	Hoffman-La Roche	Chronic myelogenous leukemia	June 1986
Stemgen (stem cell factor)	Amgen	Hepatitis C	Nov.1988
		Mobilization (Australia, New Zealand, Canada)	Oct. 1995
Targretin®, Bexarotene	Ligand Pharmaceuticals, Inc	Cutaneous T cell lymphoma	July 1998
Wellferon® IFN-alfa-n1	GlaxoSmithKline	Hepatitis C without decompensated liver disease	Dec. 1999

TABLE 9.2
Cytokines

Cytokine	Corporation	Mechanism	Target
AMD-3100 (MO2O BIL)	AnorMED	CXCR4 chemokine inhibitor	Stem cell mobilization
AMG 951 (APo2L/TRAIL)	Amgen	Apoptosis Induction	Anti-cancer
BAY 50-4798	Bayer	Synthetic form of IL-2 3,000-fold selectivity for T vs. NK cells	HIV infection
CD40L	Immunex/Amgen	Increased DC activation	Renal cell carcinoma
CTCE-0214, SDF-1 agonist	ChemoCentryx	SDF-1 agonist may increase mobilization by G-CSF	Mobilization
CTCE-9908, SDF-1 antagonist	ChemoCentryx	Inhibits metastasis via SDF-1 binding	Inhibit metastasis
Flt3L	Amgen	Stem cell and DC growth factor	Stem cell mobilization, DC expansion
IL-1, Trap	Regeneron	Removes IL-1	CIAS1 associated periodic syndromes
IL-4	Schering Plough	Immunosuppression and DC manufacture	Dendritic cells
IL-6, Aitexakin	Serono		Peripheral neuropathy
IL-7, CYT 99 007	Cytheris Corp	Immune augmenting and recovery	MTD
IL-10, SCH 52000 Ilodecakin	Schering Plough	Immunosuppression	Wegener's granulomatosis
IL-12	Genetics Institute, Inc.	Immune augmenting	Vaccine adjuvant
IL-13	Cytheris Corp	DC manufacture	Dendritic cells
IL-15	Amgen	Immune augmenting and recovery	Cancer
IL-18, SB-485232	GlaxoSmithKline	Immune augmenting	Melanoma renal cell cancer
IL-21	Zymogenetics	Immune augmenting	Melanoma renal cell cancer
IL-24	Introgen	Tumor suppressor and Th1 inducer	Head and neck, lung, and breast cancer
IL-29	Zymogenetics	Anti-viral activity via receptor different to type-1 IFN	Hepatitis C

INCB3284 inhibits CCR2/MCP-1	ChemoCentryx	Anti-inflammatory	Rheumatoid arthritis and obese insulin-resistant patients
Leukine (GM-CSF)	Berlex	Phase I/II	Crohn's disease
Maraviroc, UK-427857, CCR5 agonist	Pfizer	Blocks HIV-1 infection	HIV
MLN1202,			
CCR2 agonist	Millennium	CCR2 receptors found primarily on monocytes and macrophages	Multiple Sclerosis
NGR-TNF	MolMed	TNF fused to CNGRCG, a tumor vasculature-targeting (CD13) peptide	Advanced solid tumors
NUVANCE™ (soluble IL-4 receptor)	Immunex Corporation	Immune augmenting	Asthma
Pegaldesleukin (PEG-IL-2)	Berlex	Slow release IL-2	Renal cell cancer
PEG IL-2	Cetus/Chiron Corp.	Slow release IL-2	Stage B-2 and C prostate carcinoma
rHuLIF, emfilermin, AM424	Zenyth Therapeutics/ Sereno		Prevent chemotherapy peripheral neuropathy
SCH-417690 (SCH-D, Vicriviroc)	Schering Plough	Oximepiperidine derivative, CCR5 antagonist	HIV
SLC (CCL21)	Academic	Chemoattractant	DC chemoattractant
Traficet-EN, CCR9	ChemoCentryx	Orally bioavailable, anti-inflammatory agent	IBD

of these have been dropped from active study for a variety of reasons, including toxicity, lack of desired activity and/or a change in prioritization by the intellectual property holder. Further, due to the breadth of recombinant cytokines and growth factors that are currently under study, it is likely that some are not listed herein.

Cytokines have in several instances shown increased bioactivity by continuous infusion or administration with a slow release formulation.[6] The covalent attachment of polyethylene glycol (PEG) to cytokines (pegylation) (Table 9.3), including interferon-alpha (IFN-α) and granulocyte colony-stimulating factor (G-CSF), has demonstrated significantly improved biologic activity due, in part, to their improved pharmacologic profile.[7,8] Another approach that has been taken to prolong the half-life of cytokines is the modification of IFN-α using albumin fusion technology. Albuferon has exhibited a median half-life of 148 hours as compared to 22–60 hours for PEG-Interon.[9] In addition, both of these half-lives are significantly longer than the half-life of IFN-α, which is approximately 10 hours.[10] The pharmacologic attributes of recombinant biotherapeutics are also improved with targeted delivery, which prolongs their short half-life and can increase biologic activity.[11] Further, there can be unexpected relationship between the dose administered and the biologic effect in recombinant biotherapeutics, including a nonlinear-dose relationship described as a "bell-shaped" curve.[12] This lack of a linear dose-response relationship may be due to a nonlinear dispersal throughout the body, a poor ability to enter into a saturatable receptor-mediated transport process, chemical

TABLE 9.3
Slow Release Variants

Drug	Corporation	Target	Approved
Aranesp, Darbepoetin alfa	Amgen, Inc.	Anemia associated with chronic renal failure	Sept. 2001
		Chemotherapy-induced anemia in patients with non-myeloid malignancies	July 2002
Neulasta, Pegfilgrastim, G-CSF-PEG	Amgen, Inc.	Infection, as manifested by febrile neutropenia, in patients with non-myeloid malignancies receiving myelosuppressive anti-cancer drugs	Jan. 2002
Oncaspar PEG L-asparaginase	Enzon, Inc. and Rhone-Poulenc Rorere	Acute lymphoblastic leukemia hypersensitive to native forms of L-asparaginase	Feb. 1994
Pegasys, PEG IFN α-2a	Hoffman-La Roche, Inc.	Chronic hepatitis C in patients not previously treated with IFN-α	Oct. 2002
		Combination therapy with Ribavirin	Dec. 2002
		Combination therapy with Copegus	Feb. 2005
PEG-Intron™, PEG IFN alfa-2b	Enxon, Inc. and Shering-Plough Corp	Monotherapy for chronic hepatitis C	Jan.. 2001
		Combination therapy with Rebetol	Aug. 2001

instability, sequence of administration with other agents, an incorrect time of administration, an inappropriate location, and/or response of the target cells. Further, a "bell-shaped" dose-response curve may be associated with a tachyphylaxis of the receptor expression or a signal transduction mechanism, whereby the cells become refractory to subsequent receptor-mediated augmentation.

Interferon-alpha (IFN-α)

Randomized trials with IFN-α demonstrated significant therapeutic activity against hairy cell and chronic myelogenous leukemia (CML), and a few types of lymphoma, including low-grade non-Hodgkin's lymphoma and cutaneous T cell lymphoma. The list of responding indications also includes malignant melanoma, acquired immune deficiency syndrome (AIDS), Kaposi's sarcoma, genital warts, and hepatitis B and C.

It has taken almost three decades to translate the concept of IFN-α as an anti-viral to its routine utility in clinical oncology and infectious diseases. Despite extensive study, the development of IFN-α is still in its early stages, and basic parameters such as optimal dose and therapeutic schedule remain to be determined.[13,14] The MOA is also controversial since IFN-α has been shown to have dose-dependent antitumor activities *in vitro*, yet be active at low doses for hairy cell leukemia.[13,15] Immunomodulation as the mechanism of therapeutic activity with IFN-α is perhaps best supported by its action against hairy cell leukemia. A 90–95% response rate is observed; however, this is not fully achieved until the patients have been on the protocol for a year, and it appears that low doses of IFN-α are as active as higher doses.[16] It should be noted that the clinical use of IFN-α has been partially supplanted by Gleevac, a tyrosine kinase inhibitor that has greater therapeutic activity and less toxicity. Despite the success with Gleevac, it can result in the therapeutic resistance such that even patients receiving Gleevac may ultimately need to receive IFN-α.

The initial dose-finding studies determined that a dose of 12×10^6 U/M^2 of IFN-α was not tolerable in patients with hairy cell leukemia. Subsequently, it was demonstrated that highly purified natural IFN-α at a dose of 2×10^6 U/M^2 was both well tolerated and effective when administered three times per week for 28 days;[17] however, it did retain some toxicity, including myelosuppression, neurotoxicity, and cardiotoxicity. A dose-response relationship exists for IFN-α in CML. The intended dose to induce a significant cytogenetic response and survival improvement is 5×10^6 U/M2 daily. However, the average dose delivered is approximately 5×10^6 U daily, resulting in an inferior patient outcome. Thus, the initial suggestion that lower doses of IFN-α are as efficacious as standard doses has not been confirmed in randomized trials.[18] Unfortunately, the clinical utility of IFN-α is limited by its toxicity profile. Fifteen percent to 25% of CML patients discontinue treatment with IFN-α because of intolerable side effects, and another 30–50% of patients in various trials have required dose reductions because of poor treatment tolerance. In CML, sustained therapeutic responses are found in more than 75% of patients,[18,19] and a higher dose (5×10^6 U/M^2) than that used in hairy cell leukemia is required to achieve optimal therapeutic efficacy. In addition to reducing leukemic-cell mass, there is also a gradual reduction in the frequency of cells bearing a 9-22 chromosomal translocation.[20]

The unique cellular and molecular activities of IFN-α can potentially compliment the MOAs by other therapies.[21] The MOA by IFN-α, as well as the optimal dose, remains controversial. A meta-analysis of twelve clinical studies for high risk melanoma showed a significant recurrence-free survival following treatment with IFN-α. However, the benefit of IFN-α therapy on overall survival (OS) is less clear.[22] In this study, there was a significant trend between increasing dose and recurrence-free survival, but not OS. Similarly, studies of immune augmentation in melanoma patients receiving high or low dose IFN-α revealed no association between immune response and baseline phenotypic and functional immunity.[23] However, numerous immune surrogates are augmented by IFN-α treatment and are associated with dosage. Natural killer (NK) cell and T cell functions are augmented in a dose dependent manner by IFN-α as are changes in T cell phenotypes. Further, high dose IFN-α regulates immune parameters more rapidly than low dose IFN-α. In addition, IFN-α has been shown to significantly upregulate class II major histocompatibility complex (MHC) and intercellular adhesion molecule- (ICAM-) 1 expression on tumor cells in a dose dependent manner. However, there was no relationship between immune augmentation and relapse-free survival.

Interferon-gamma (IFN-γ)

IFN-γ has multiple potential mechanisms that may be involved in tumor protection and therapy. These include, but are not limited to: (1) tumor cell anti-proliferative activity, (2) induction of angiostasis, and (3) augmentation of both innate and adoptive immunity (Figure 9.1). However, it is unclear which, if any, of these potential mechanisms are critical for the therapeutic activity of this pleiotropic cytokine. The anti-proliferative and anti-metabolic effects of IFN-γ on tumor cells include the induction of apoptosis via conventional signaling mechanisms, resulting in the expression of genes that promote cellular apoptosis, including caspase-1 and Fas ligand (FasL). The anti-tumor activity of IFN-γ is also mediated by an inhibition of neo-angiogenesis. IFN-γ can upregulate the expression of chemokines with potent angiostatic actions, including inducible protein- (IP-) 10,[24] which belongs to a family of CXC non-ELR chemokines that have angiostatic activity.

IFN-γ also has potent activating properties for augmenting macrophage mediated tumoricidal activity *in vitro* and *in vivo*.[25,26] IFN-γ activated macrophages have multiple tumoricidal mechanisms, including reactive oxygen and/or nitrogen intermediates and upregulating expression of tumor necrosis factor- (TNF-) α, FasL,[27] and TNF-related apoptosis-inducing ligand (TRAIL).[28] In addition, IFN-γ significantly enhances interleukin (IL-) 12 secretion by macrophages and dendritic cells (DCs).[29] IFN-γ is also a potent activator of NK cells that have direct cytocidal activity through mechanisms involving perforin[30] and TRAIL.[31] In addition, IFN-γ can markedly enhance adaptive T cell responses to tumors. IFN-γ has also an important role in regulating the Th1/Th2 balance during the host response to a tumor.[31]

Preclinical studies have suggested that IFN-γ has significant therapeutic activity with a reproducible "bell-shaped" dose-response curve.[12] Studies of immune response in mice have revealed a "bell-shaped" dose-response curve for the augmentation of macrophage

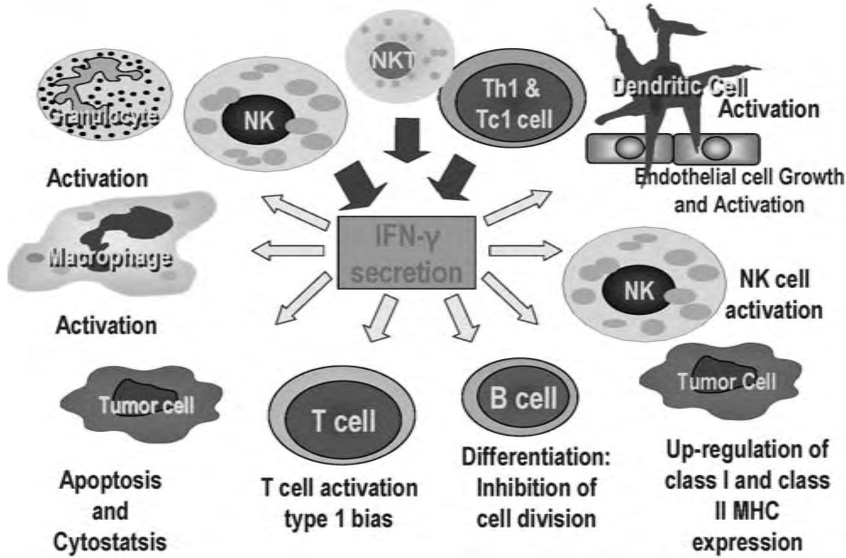

FIGURE 9.1 Regulation of Immune Responses by Interferon-Gamma (IFN-γ). IFN-γ is produced predominately by T_H1, T_c1, NKT and NK cells, resulting in the activation of T cells, NK cells, macrophages, dendritic cells (DCs) and granulocytes. In addition, it upregulates class I and class II MHC expression on tumor cells as well as the expression of a wide variety of receptors on both tumor cells and epithelial cells. IFN-γ: interferon-gamma; NK: Natural killer cell; T_H1: T-helper cell type 1; T_c1: T cytotoxic cell type 1; MHC: major histocompatibility complex.

tumoricidal activity.[12,17] Further, optimal therapeutic activity is observed in rodents at the same dose and protocol of IFN-γ, and significantly less therapeutic activity occurs at lower and higher doses. A significant correlation between macrophage augmentation and therapeutic efficacy has been reported,[17] suggesting that immunological augmentation is one mechanism for the therapeutic activity of IFN-γ. This observation supports the hypothesis that treatment with the maximum tolerated dose (MTD) of IFN-γ may not have optimal therapeutic activity in an adjuvant setting. The preclinical observation of a "bell-shaped" dose-response curve for IFN-γ has been confirmed clinically.[32,33] In general, the OID for IFN-γ occurs between 0.1 and 0.3 mg/M^2 following intravenous (i.v.) or intramuscular injection.[32] In contrast, the MTD for IFN-γ ranges from 3 to 10 mg/M^2, depending upon the source of IFN-γ and/or the clinical center. The identification of an OID for IFN-γ in patients with minimal tumor burden has resulted in clinical trials to test the hypothesis that immunological augmenting by IFN-γ will result in prolongation of disease-free survival (DFS) and OS in the adjuvant setting.[33]

IFN-γ has therapeutic activity in chronic granulomatous disease (CGD).[34] In CGD, the mechanism of therapeutic activity by IFN-γ appears to be associated with enhanced phagocytic oxidase activity and increased superoxide production by neutrophils. However,

the majority of CGD patients obtain clinical benefit by prolonging IFN-γ therapy and the MOA may not be due to enhanced neutrophil oxidase activity, but rather to the correction of a respiratory-burst deficiency in a subset of monocytes.[35] In addition, IFN-γ administration induces nitric oxide (NO) synthetase activity by polymorphic neutrophils (PMN) in patients with CGD.[36] Following two days of IFN-γ administration, a significant increase in PMN-produced NO was measured, as well as increased bactericidal capacity by the PMN.[36] As PMNs from patients with CGD lack the capacity to produce superoxide anions, it is possible that the increased NO release and *in vitro* bactericidal activity could be reducing the morbidity of CGD.[36] Similarly, IFN-γ increases the expression of Fc gamma receptor I (FcγRI) by PMNs and the MOA critical to reducing the frequency of infections in patients with CGD may not be associated with phagocytic oxidase activity and increased superoxide production, but rather with other mechanisms of granulocyte activity, including NO production.

In addition to its approval for CGD, IFN-γ has also been approved for the treatment of rheumatoid arthritis (RA) in Germany and severe malignant osteoporosis in the United States. In a randomized Phase III trial, IFN-γ was also reported to have activity in women receiving first line platinum based chemotherapy against ovarian cancer.[37] In this study, there was a significantly higher response rate and longer progression-free survival in woman receiving IFN-γ plus chemotherapy when compared to chemotherapy alone. However, there was no statistically significant improvement in OS. Currently, IFN-γ is being studied for the treatment of idiopathic pulmonary fibrosis.[38] Phase III clinical studies with IFN-γ for idiopathic pulmonary fibrosis (IPF) with mild to moderate lung function impairment have shown prolongation of survival. Subjects received IFN-γ at 200 mcg three times a and followed for at least 24 months. Adverse effects observed included flu-like symptoms (e.g., fever, headache, chills, myalgia, fatigue) and erythema or tenderness at the injection site.

Interleukin-2 (IL-2)

IL-2, a T cell growth factor, has a significant role in regulating immunity to infectious and neoplastic diseases (Figure 9.2). IL-2 is produced primarily by activated DCs, NK and T cells, and stimulates the growth of naïve T cells following antigen (Ag) activation, and later induces activation-induced cell death (AICD).[39,40] IL-2 also has effects on several other immune cells, including NK cells,[41] B cells,[42] monocyte/macrophages,[43] and neutrophils.[44] The ability of IL-2 to stimulate NK and T cell lysis of tumor cells has stimulated an interest in IL-2 as a therapeutic.[45] NK cells are part of the innate immune system and comprise 10–15% of peripheral blood lymphocyte (PBL) population. *In vivo* IL-2 promotes thymic development and the peripheral expansion of $CD4^+CD25^+$ T cells known as T regulatory (Treg) cells. Loss of Treg activity in IL-2- or IL-2R-deficient mice results in a severe lymphadenopathy and autoimmune disease. The IL-2-dependent Treg cells regulate homeostatic and Ag-induced T cell proliferative responses that can result in pathological autoreactivity.[46] IL-2 production by DCs has also been shown to be essential for the initiation of both $CD4^+$ and $CD8^+$ T cell responses and expansion of Treg cells.[47]

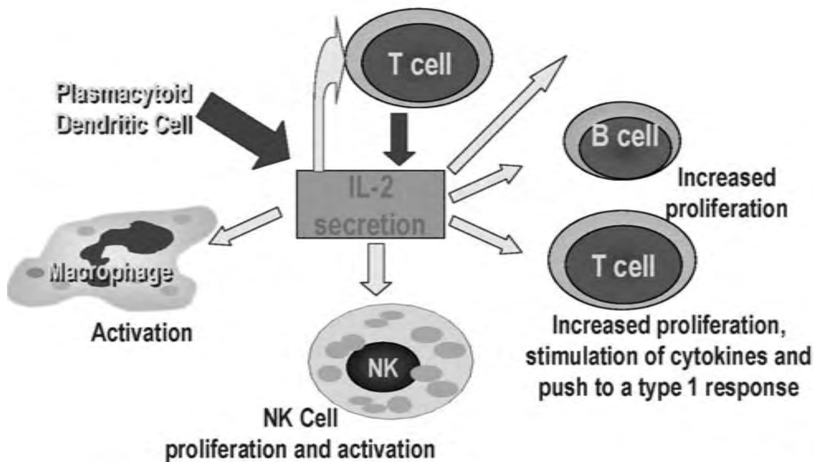

FIGURE 9.2 Regulation of Immunity by Interleukin-2 (IL-2). IL-2 production by T cells and DCs supports the proliferation of T cells, B cells, T reg cells, and NK cells, in addition to establishing a bias towards a Type 1 T cell response. IL-2 can also activate monocytes and NK cells, resulting in increased cytotoxicity. DC: dendritic cell; IL-2: interleukin-2; NK: natural killer cell.

IL-2 has been approved for use as a single agent in the treatment of renal cell carcinoma and metastatic melanoma. It has also been administered in conjunction with lymphokine activated killer (LAK) or T cell infiltrating lymphocytes (TILs) in adoptive cellular therapy protocols. However, it has been questioned whether the adoptive transfers of LAK or TIL cells are necessary or adds to the clinical efficacy of IL-2. When the clinical trials with IL-2 are rigorously examined, neither strategy has impressive (as opposed to significant) therapeutic activity.[48,49] A 7–14% overall response rate is induced by IL-2 therapy and is associated with considerable toxicity;[50] however, most of these responses are durable. Many of the clinical trials with IL-2 in metastatic renal cell carcinoma have used an MTD of IL-2. A study by Fefer et al.[51] compared maintenance IL-2 therapy at the MTD of 6×10^6 U/M^2/day as compared to a dose of 2×10^6 U/M^2/day. They found that it was possible to maintain the patients for a median of 4 days at the high dose, but with severe hypertension and capillary-leak syndrome. In the lower-dose protocol, none of the patients experienced severe hypertension or capillary-leak syndrome, and a median of nine days of maintenance IL-2 therapy. Further, there was a total response rate of 41% in the lower dose protocol compared to a 22% response rate for the higher dose protocol that has a shorter duration of administration. These investigators suggest that there may be an improved therapeutic activity associated with the longer IL-2 maintenance protocol that can be achieved at lower doses. Thus,

the optimum dose and protocol of IL-2 administration remains unknown. High-dose therapy appears to be associated with higher response rates, but more toxicity. Low-dose regimens retain efficacy with fewer toxic effects, especially hypotension.[52] Further, at low doses, outpatient subcutaneous (s.c.) administration can be used, resulting in therapeutic responses and with acceptable toxic effects.[53]

Dose-response studies have also examined the IL-2 induced transcriptional regulation of cytokine messenger ribonucleic acid (mRNA) levels in the PBL of cancer patients. The results from one study suggested that low IL-2 doses ($\geq 3 \times 10^4$ U/day) could augment T cell function, while higher IL-2 doses ($\geq 1 \times 10^5$ U/day) increases not only T cell, but also macrophage function.[54] The latter was measured as an upregulation of TNF by the higher dose of IL-2, which when combined with the increased levels of IFN-γ, are induced at the lower dose of IL-2 and may be responsible for toxicity.[55] The effect of low dose IL-2 administration has also been studied in healthy males at 1,000 or 10,000 international units (IU)/kg administered s.c.[56] No consistent changes were observed with IL-2 doses of 1,000 IU/kg; however, phenotypic and immunoregulatory changes were observed following doses of 10,000 IU/kg of IL-2. These included depressed numbers of circulating lymphocytes (CD4, CD8, and activated T, B, and NK cells) and increased numbers of neutrophils and monocytes. There was also a significant increase in IL-4 serum levels, while IFN-γ and IL-2 receptor levels were significantly depressed. These effects vary with time, but occurred at IL-2 serum levels sufficient to saturate the high affinity receptor by three hours following injection of 10,000 IU/kg. Thus, IL-2 mediated immune augmentation can occur with very low doses of IL-2 administered s.c. or by continuous infusion.

The potential for IL-2 to be therapeutically active at low doses was shown in patients with squamous cell carcinoma of the oral cavity and oropharynx.[57] In this study, IL-2 was injected perilymphatically (5,000 units daily) for 10 days prior to and following surgery, and also monthly post surgery for 5 days (×12). A significant increase in DFS and OS was observed in the IL-2 treated patients. Therapeutic activity in renal cell cancer was also shown in a protocol in which patients were randomized to receive either a high-dose i.v. IL-2 regimen or a low dose regimen using one-tenth the dose (72,000 IU/kg/8 h) both administered on a cycle of days 1–5 and 15–20 that was repeated every 4 to 6 weeks.[52] A third arm using the low dose of IL-2, but given daily s.c., was also added. In the most recent interim report,[49] toxicities were less frequent with low dose IL-2, especially hypotension, but there were no IL-2 associated deaths in any arm. A higher response rate was observed with the high dose of i.v. IL-2 (21%) versus low dose i.v. IL-2 (13%), but no OS differences were observed. The response rate of s.c. IL-2 (10%) was similar to that of the low dose i.v. IL-2, but was significantly different from high dose i.v. IL-2 therapy ($P = 0.033$). The response duration and survival of complete responders was significantly better in patients receiving high dose i.v. IL-2 as compared to low dose i.v. IL-2 therapy ($P = 0.04$). Thus, tumor regressions, as well as complete responses, are seen with both high and low dose IL-2 regimes. However, this one study suggests that IL-2 is more clinically active at the maximum dose, although no OS benefit was induced and only a small percentage of patients achieved durable clinical response.

Granulocyte Monocyte Colony Stimulating Factor (GM-CSF)

GM-CSF was initially defined by its ability to support the growth of both granulocyte and macrophage colonies from hematopoietic precursor cells.[58] GM-CSF can also potentiate the functions of mature granulocytes and macrophages,[59,60] in addition to its role as a hematopoietic regulator (Figure 9.3).[61] Similar to other proinflammatory cytokines, the production and activity of GM-CSF occurs at the site of inflammation and increased levels of GM-CSF mRNA are observed in skin biopsies from allergic patients. GM-CSF is considered an important regulator (proliferation, maturation and activation) of granulocyte and macrophage lineage populations and DCs.

GM-CSF was approved in 1991 by the United States Food and Drug Administration (FDA) to support transplant associated neutropenia and mobilize stem cells. In Europe, it is also approved for prophylactic treatment following dose intensive chemotherapy. However, the rate of absolute neutrophil count (ANC) recovery in response to treatment with GM-CSF in patients receiving myelosuppressive chemotherapy or in the

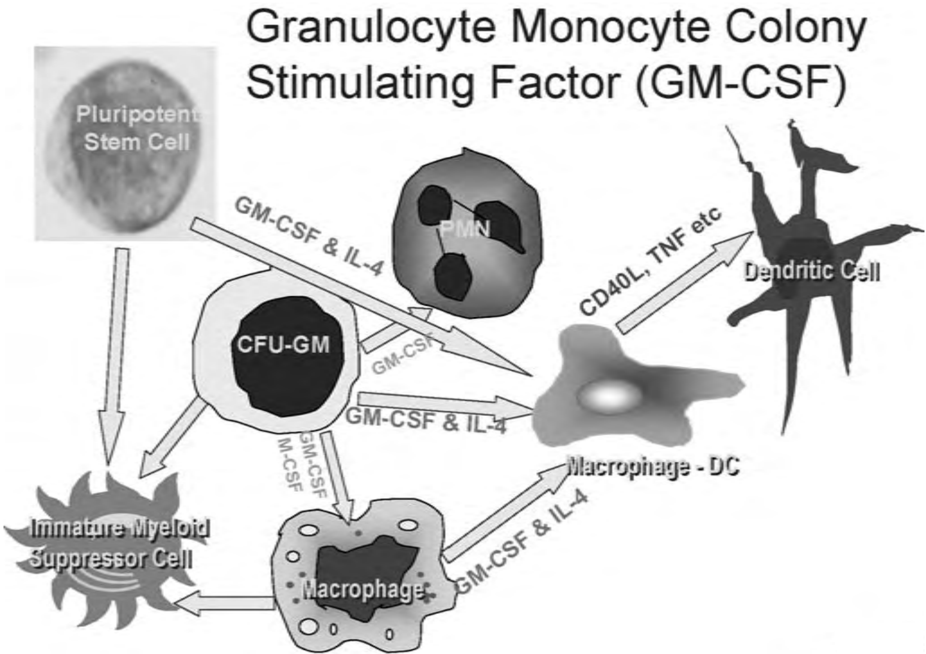

FIGURE 9.3 Maturation of Hematopoietic Precursors Into Dendritic Cells (DCs) by Granulocyte Monocyte Colony Stimulating Factor (GM-CSF). GM-CSF and IL-4 can drive pluripotent stem cells, PMNs, monocytes, and CFU-GM genetic precursors into pre-DCs, which following activation can become mature activated DCs. It can also result in the expansion of immature myeloid cells. DC: dendritic cell; GM-SCF: granulocyte monocyte colony stimulating factor; IL-4: interleukin-4; PMN: polymorphonuclear neutrophil; CFU-GM: colony forming unit-granulocyte monocyte; CD40L: CD40 Ligand; TNF: tumor necrosis factor.

mobilization of stem cell into the PB is one day slower than that observed with granulocyte colony-stimulating factor (G-CSF). The toxicity profile of both growth factors includes low grade fever, myalgias, and bone pain. Patients receiving chronic GM-CSF therapy post marrow graft failure have been shown to have significantly improved survival as compared to historically matched controls,[62] which significantly reduced hospital time and duration of antibiotic therapies have been shown with both growth factors.[63]

In addition to effects on hematopoiesis, GM-CSF also affects macrophages and DCs. DCs are antigen presenting cells (APCs) that are critical to the induction of T cell responses. GM-CSF is a significant mediator of proliferation, maturation, chemotaxis, and migration of DCs.[64] Because of theses activities, it has been used as a vaccine adjuvant to enhance the induction of Ag-specific cytotoxic T cells.[65,66] In addition, GM-CSF alone or in combination with IL-4 is used both *in vitro* and *in vivo* to expand DCs for use as vaccines.[67,68] Primarily, this is undertaken *ex vivo*, although GM-CSF alone or GM-CSF and IL-4 can also mobilize and expand DCs clinically.[69] Thus, the use of GM-CSF outside its approved indications is rapidly expanding and represents a significant new area of clinical development. The potential utility is receiving significant attention at present not only to address neutropenia, but also as a direct therapeutic agent within an adjuvant protocol and use in the development of vaccines.

Cancer patients have defective macrophages and DCs, including ones infiltrating the tumor.[70] GM-CSF can expand and activate the hisotcytes to become cytotoxic against tumor cells.[71] It has been studied either alone or in combination with IL-2 for adjuvant therapeutic activity. In one study, 48 cancer patients with surgically resected stage III or stage IV melanomas received GM-CSF s.c. for 14 days with treatment monthly for 1 year or until disease recurrence and outcomes compared to matched historic controls. A median survival time of 37.5 months was observed with GM-CSF therapy versus 12.2 months for historical controls.[72]

Engineered Recombinant Proteins

Recombinant biopharmaceuticals have also been engineered to improve their pharmacologic properties and several have been approved for clinical use (Table 9.3). The primary strategy has been pegylation, which can prolong the half-life and bioavailability of a protein, reducing the number of injections required. This results in improved patient compliance, as well as protecting against binding of mAbs to monocytes and PMNs, enzyme degradation and antibody (Ab) induction.

Pegylation improves the bioactivity by decreasing systemic clearance, which reduces the frequency of dosing, improves patient compliance, lowers cost and allows the extension of the patient life. Pegylated IFNs have also obtained regulatory approval. Native IFNs have relatively short half lives, typically one to four hours, and pegylation can increase this to twenty-four hours or longer. As an example, IntronA (IFN-α2b) is typically administered three times a week, whereas pegylated material is administered as a single weekly dose. Similarly, thrice weekly injections of Roferon (IFN-α2a) have been replaced by a single weekly injection of Pegasys. Pegylated G-CSF has also been approved for single injection to replace daily or twice daily injections of G-CSF for five or more days, and pegylated IL-2 is under clinical development (Table 9.2).

Erythropoietin has been genetically manipulated to increase glycosylation, resulting in altered oligosaccharide sequences and improved pharmacokinetics. The sialic acid content of the glycoproteins has a significant effect on a protein. Thus, Aranesp, an analog of recombinant human erythropoietin (EPO), has two extra glycosylation sites in the EPO backbone and a longer half life than native EPO. Additional strategies are being used to prolong the half life of proteins, including nanoparticles, liposomes, and poloxamer matrixes, which allow the slow release of a protein. These formulations have not yet been approved by the FDA; however, such formulations result in not only a slow release, but can also be targeted to organs or tumors via modification(s) of the formulation.

NATURAL AND SYNTHETIC BIOLOGICAL RESPONSE MODIFIERS (BRMs)

Natural BRMs

The use of BRMs to treat human disease has its origins in the use of bacterial toxins to treat cancer by William B. Coley.[73] These early studies resulted in the use of microbially-derived substances such as BCG, Picibanil, carbohydrates from plants or fungi such as Krestin and Lentinan, other products such as Biostim and Broncho-Vaxom, as well as thymic extracts (Table 9.4). However, the lot-to-lot variation in the manufacture of these drugs has dampened enthusiasm. Equally, the focus on MOAs in drug development strategies has also dampened developmental efforts. The particulate nature of some BRMs can also result in pulmonary thrombosis and respiratory distress following i.v. injection. However, BRMs are commonly used to treat bladder cancer and derivatives of natural products are routinely used clinically.

TABLE 9.4
Natural BRMs

Agent	Chemical Nature	Action	Clinical Use
BCG (U.S. & Europe)	Live mycobacteria	Macrophage activator	Bladder cancer (March 2000)
Picibanil (OK432) (Japan)	Extract Strep. Pyogenes	Macrophage activator	Gastric/other cancers
Krestin (PSK) (Japan)	Fungal polysaccharide	Macrophage activator	Gastric/other cancers
Lentinan (Japan)	Fungal polysaccharide	Macrophage activator	Gastric/other cancers
Biostim (Europe)	Extract Klebsiella penum.	Macrophage activator	Chronic or recurrent infections
Thymostimulin (Europe)	Thymic peptide extract	T cell stimulant	Cancer & infection
T-activin (Russia)	Thymic peptide extract	T cell stimulant	Cancer & infection
Thym-Uvocal (Germany)	Thymic peptide extract	T cell stimulant	Cancer & infection

BCG

The most commonly used microorganism for cancer therapy in the United States is BCG, which was approved by the FDA in March 2000. It has been used systemically for the treatment of metastatic disease or adjuvant therapy, intralesionally (especially for cutaneous metastatic malignant melanoma), topically for superficial bladder cancer, and in combination with other immune modulators, tumor vaccines, and chemotherapy. When given intravesically, it can treat superficial bladder cancer in residual disease and in the adjuvant setting, resulting in a prolonged disease-free interval and time-to-progression.[74] The mechanism by which BCG mediates its antitumor response is not known, but BCG treatment induces granulomatous inflammation in the bladder[75] and elevates IL-2 levels in the urine of treated patients,[76] suggesting that an augmented local immune response may be important. There appears to be a relationship between cytokine production and therapeutic efficacy as a multivariate logistic analysis demonstrated that IL-2 induction provided a discriminating parameter for remission in patients receiving BCG treatment for their superficial bladder carcinoma.[77]

Synthetic BRMs

Nonspecific immunostimulants (Table 9.5) can have widespread effects on the immune system similar to microbially-derived agents, including side effects akin to infection (e.g., fever, malaise, myalgia, etc.). These agents can enhance nonspecific resistance to microbial or neoplastic challenge when administered prior to challenge (immunoprophylactic), but rarely when administered following challenge (immunotherapeutic). This is an important distinction in that the primary objective for the oncologist is the treatment of preexistent metastatic disease. This class of biologically active agents includes drugs approved in many countries, including several synthetic agents with U.S. approval. Analoging of several of the approved drugs is ongoing with a focus on retinoic acid and thalidomide.

Levamisole

Levamisole was the first chemically defined, orally active immunostimulant to be licensed (USA) for clinical use.[78,79] It was approved for the treatment of Duke's C colon cancer in combination with 5-fluorouracil (5-FU). It was shown to promote T lymphocyte, macrophage and neutrophil function in these patients. It stimulates T cell function *in vivo*, particularly in immunodeficient individuals, presumably through the action of its sulfur moiety. A dose-response study with Levamisole demonstrated that a significant increase in the frequency of PB mononuclear cells expressing CD16 (NK cell) were noted at all dose levels, although lower toxicity was observed at the lower doses of Levamisole.[80] The authors suggested that short-term Levamisole administration was only minimally immunomodulatory and that chronic administration at low doses may be better tolerated and provide similar levels of immune modulation as that observed with higher doses.[80] It is relatively nontoxic (flu-like symptoms, gastrointestinal upset, metallic taste, skin rash, and Antabuse reaction), but can produce an agranulocytosis. It is noted that the adjuvant therapeutic activity of Levamisole has been questioned

TABLE 9.5
Chemically Defined BRMs

Agent	Chemical Nature	Action	Clinical Use
1018-iss	Immunostimulatory phosphorothiolate oligodeoxyribonucleotide	Toll receptor agonist	Vaccine adjuvant
Aldara, Imiquimod	Imidazoquinoline heterocyclic amine	TLR7, TTR8	Actinic keratoses, BCC, genital warts
AMG531	Peptibody (Fc conjugate)	Stimulates the TPO receptor	ITP
Ampligen	Polyribonucleotide	Toll receptor agonist	Cancer
AVE-7279	Oligonucleotide	Toll-like receptor agonist	Asthma
AVR118, reticulose	Peptide nucleic acids	Immune augmentation	AIDS Related Anorexia/Cachexia
Bestatin (Japan)	Dipeptide	Macrophage and T cell stimulant	Acute myelosis leukemia
CC4047, Actimid	Thalidomide analogue	Antiangiogenesis	Myelofibrosis
CC5013, Lendlidomide	Thalidomide analogue	Antiangiogenesis	MDA, AML, anemia
CPG7909, Promune	Toll receptor agonist	Augment immature immunity	Vaccine adjuvant
Cytosine-phosphate-guanine	Nucelotide	Binding to TLR9 and DC activity	Vaccine adjuvant
HMR 1726, Teriflunomide	Dihydro-orotate dehydrogenase inhibitor	Reduced number of active lesions	Multiple Sclerosis
Isoprinosine (Europe)	Inosine:salt complex	T cell stimulant	Infection
Leflunomide	Isotazole analogue	Suppressive for TNF, IL-17	RA, cGVHD
Levamisole (USA)	Phenylimidothiazole	T cell stimulant	Cancer
MDP-PE, muramyl tripeptide phosphatidyl, Ethanolamine	Lipoidal tripeptide	Macrophage activating agent	Osteosarcomas
Murabutide	MDP analogue	Macrophage augmenting agent	HIV/vaccine adjuvant
Resveratrol	Naturally occurring phytoalexin	COX-2 inhibitor	HIV/Cancer
Retinoid analogs		Immune augmenting	Vaccine adjuavant
Romurtide (Japan)	18 lys MDP	Macrophage stimulant	Bone marrow recovery
Thalidomide (USA)	a-(N-phthalimido) glutarimide	TNF expression inhibitor	Erythema nodosum leprosum, cGVHD
Thymalfasin (Approved in ~30 countries)	Thymosin, α1	Thi1, NK, and DC activation	Hepatitis C
Thymopentin TP-5 (Italy & Germany)	Pentapeptide	T cell stimulant	Rheumatoid arthritis infection and cancer
Tucaresol (589C80)	4-[2-formyl-3-hydroxyphenoxymethyl]benzoic acid	Augmentation of a Th-1 response	HIV immunosuppression
Veldona (nIFN-a	Natureal protein	Anti-inflammatory	Sjogren's syndrome

in recent years. In one Phase III trial comparing 5-FU with leucovorin to 5-FU with Levamisole, it was found that the 5-FU and Levamisole significantly prolonged DFS and OS in patients with type III colon cancer who had undergone curative resection relative to adjuvant therapy with Levamisole.[81]

Muramyl Dipeptides (MDPs)

One of the largest and best-studied class of synthetic agents is the muramyl dipeptides (MDP). MDP was discovered based on the isolation of the minimally active substitute for intact BCG in Freund's adjuvant.[82] Unfortunately, as with many of the polypeptides that have low molecular weights, MDP has a short serum half-life and bioactivity requires frequent high doses. In addition, MDP is strongly pyrogenic, presumably due to their induction of IL-1. MDP has been incorporated into multilamellar vesicles (MLV) for higher stability and to facilitate monocytic phagocytosis of the MLV.

The first MDP to be licensed, Romurtide (Japan), induces bone marrow recovery following cancer chemotherapy.[83] Its MOA is suggested to be the activation of macrophages to secrete colony-stimulating factors (CSF), IL-1 and TNF, resulting in the stimulation of marrow precursors to produce increased numbers of progenitor and mature granulocytes and monocytes. Therefore, the period of granulocytopenia and the risk of secondary infections are reduced, allowing more frequent and/or intense chemotherapy. Murabutide, an orally active form of MDP that does not induce fevers, is currently in clinical trials in cancer and infection (France) (Table 9.5). In order to further stabilize the incorporation of MDP into MLV, lipophilic analogs of MDP such as MTP-PE have been developed. MTP-PE encapsulated in liposomes is in an ongoing Phase III clinical trial for osteosarcoma,[84,85] and has been studied in patients with resectable melanoma[86] (USA and Europe). Preclinically it has shown protection to the mucosal epithelium from cytoreduction therapy.[87] MDPs are also potent adjuvants alone and/or with oil, and are under consideration for use with HIV vaccines employing various synthetic peptide epitopes. ImmTher® is a liposome-encapsulated lipophilic disaccharide tripeptide derived from the MDP family that has the capacity to activate macrophages. It has been evaluated in Phase I and II trials in large tumors in advanced colorectal cancer patients, and is being evaluated in randomized Phase II clinical trials for the treatment of Ewing's sarcoma and osteosarcoma.[88]

Bestatin

Bestatin (ubenimex) is a potent inhibitor of aminopeptidase N and aminopeptidase B,[89] which was isolated from a culture filtrate of *Streptomyces olivoreticuli* during the search for specific inhibitors of enzymes present on the membrane of eukaryotic cells.[90] Inhibitors of aminopeptidase activity are associated with macrophage activation and differentiation, Bestatin has shown significant therapeutic effects in several clinical trials.[91] In a multi-institutional study,[92] patients with acute non-lymphocytic leukemia (ANLL) were randomized to receive either Bestatin or placebo orally after completion of induction and consolidation therapy, and concomitant with maintenance chemotherapy. Remission duration was prolonged in the Bestatin group, although this difference did

not reach statistical significance; however, OS was prolonged in the Bestatin group. In a multi-center study, Bestatin was administered to acute leukemia and chronic myelogenous leukemia patients who did not develop any graft-versus-host disease (GVHD) within 30 days following bone marrow transplate (BMT).[93] Bestatin-treated acute leukemia patients had an increased incidence of chronic low grade GVHD compared with the control arm and a lower relapse rate. Recently, a Phase III study of resected stage 1 squamous cell lung cancer patients treated with either Bestatin or placebo daily per OS for 2 years revealed that 5 year cancer-free survival was significantly greater in the Bestatin group as compared to placebo group. In this study, the 5 year cancer-free survival was 71% for the Bestatin cohort and 62% for the placebo group. OS was also significantly improved as was cancer free survival.[94]

Oligonucleotides (ODNs)

Bacterial extracts can activate both innate and adaptive immunity. Recently, it was determined how the innate immune system detects infectious agents and distinguishes different classes of pathogens. Originally thought of as nonspecific immune activators as discussed above, these molecules are now known to be specifically recognized by receptors that are expressed in a cell-specific and compartmentalized manner.[95] The best-characterized family of pattern-recognition receptors is the toll-like receptor (TLR) family. One of these, TLR9 is expressed in the endosomal compartment of plasmacytoid DCs and B cells,[96] and is essential for the recognition of viral and intracellular bacterial DNA.[97] Now that specific ligands have been identified, immunotherapy has begun to grow beyond the nonspecific effects of whole bacterial extracts, and to develop synthetic TLR ligands (TLRLs).

One example of such synthetic immunomodulatory molecules is the short ODNs that mimic the innate immune response to microbial DNA and contain one or more cytosine-phosphate-guanine (CpG) dinucleotide-containing motifs with unmethylated cytosine residues recognized by TLR-9. The immune effects of CpG ODNs occur in two stages: an early stage of innate immune activation and a later stage of enhanced adaptive immunity (Figure 9.4). Within minutes of B cells or plasmacytoid DC exposure to CpG, the expression of costimulatory molecules, resistance to apoptosis, upregulation of the chemokine receptor CCR7 that causes cell trafficking to the T cell zone of the lymph nodes, and secretion of T_H1-promoting chemokines and cytokines, including macrophage inflammatory protein-1, IP-10, and other IFN-inducible genes, occurs.[98] The CpG-induced secretion of IFN-α, TNF-α, other cytokines and chemokines induce secondary effects within hours, including NK cell activation and enhanced expression of Fc receptors, resulting in increased Ab dependent cellular cytotoxicity. This innate immune activation and pDC maturation into myeloid DCs is followed by the induction of adaptive immune responses. B cells are strongly costimulated if they bind specific Ag at the same time as CpG, which selectively enhances the development of Ag-specific Abs.[99] CpG binding also enhances APC function and upregulates expression of costimulatory molecules, including CD40, CD80, and CD86.[100] The efficient activation of APCs and induction of IL-12, IL-18, IFN-α, and IFN-γ explains their ability to induce T-helper cell 1 (T_H)1–polarization adjuvant,[101] inhibit T_H 2 responses,[102] and stimulate CD8 T cell responses.[103]

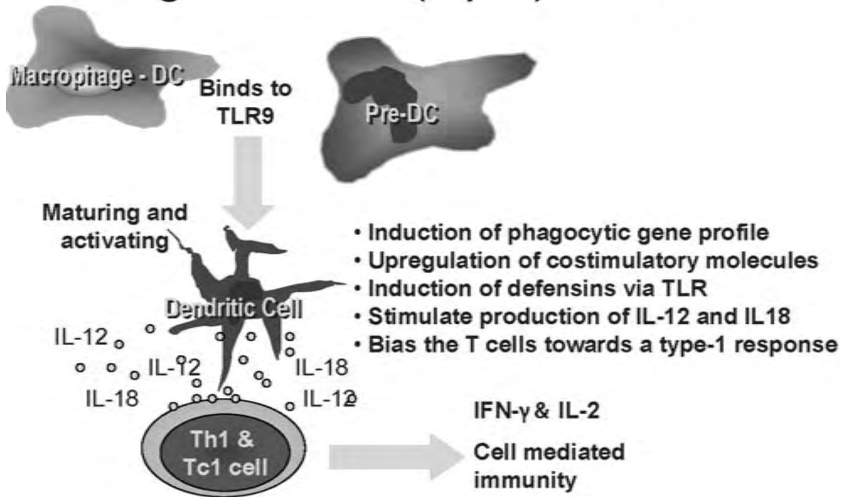

FIGURE 9.4 Regulation of Innate and Adaptive Immunity by Cytosine-Phosphate-Guanine (CpG) Motifs. Co-incubation in the presence of or injection of unmethylated CpG motifs results in binding to TRL9, ensuing the activation and maturation of DCs, which can interact with T_H1 or T_c1 cells and upregulate cell mediated immunity. In addition, CpG can induce phagocytosis, upregulation of co-stimulatory molecules, defensins, and IL-12. CpG: cytosine-phosphate-guanine; TLR9: toll-like receptor 9; DC: Dendritic cell; T_H1: T helper cell type 1; T_c1: T cytotoxic cell type 1; IFN-γ: interferon-gamma; IL-2: interleukin-2; IL-12: interleukin-12.

CpG ODNs are also effective as vaccine adjuvants to enhance adaptive T_H1 cellular immune responses.[104] In mice, CpG ODNs can trigger strong T_H1 responses,[105] enhancing the number and function of tumor-specific Cytotoxic T lymphocytes (CTLs) and IFN-γ secreting T cells.[106] This has resulted in therapeutic vaccines in mouse tumor models where no other approach has shown comparable efficacy, even with large (1 cm) established tumors.[107,108] Even without a vaccine, CpG ODNs can induce $CD8^+$ T cell-mediated regression of established tumors with durable memory responses.[109]

CONCLUSIONS

The goal of regulating the host's immune responses has been achieved as a viable therapeutic strategy for some indications. The optimism for this approach has fluctuated; however, currently immunotherapeutics represent a quarter of all drug approvals in the United States. During the last decade, we have observed an explosion in the cloning of immunoregulatory genes and their receptors, as well as the development of novel therapeutic approaches. These critical advances represent the culmination of efforts with

crude and fractionated natural products, supernatants, and cellular proteins. Thus, immunotherapeutics can be subdivided into recombinant proteins and natural or synthetic products. The latter do not currently share the enthusiasm directed towards recombinant proteins, yet have given us many therapeutically important drugs.

In the last 30 years, nonspecific immunostimulation has progressed from initial trials with crude microbial mixtures and extracts to more sophisticated uses with a large number of targeted immunopharmacologically active compounds having diverse actions on the immune system. Further, a body of immunopharmcologic knowledge has evolved with the finding that immune active drugs can have a pharmacology divergence from convention, especially in terms of dosing schedules and responses. This knowledge is important in evaluating agents and predicting appropriate use and efficacy. While much remains to be learned and new compounds to be extracted and/or cloned, the future of immunotherapy seems bright. A number of the cytokines have been approved, as well as numerous supplemental indications,[110] in the United States, Europe, and Asia. However, it is apparent that the combinations of cytokines and BRMs will have optimal activity when used as adjuvant therapeutics with more traditional therapeutic modalities.

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10 Opioid-Induced Immunomodulation

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INTRODUCTION

The health consequence of opioid use has gained increased attention over the last several years. There are numerous studies detailing the effects of opioids on immune status and some investigations have begun to identify the mechanisms involved in the effects. In addition to these recent developments, there is an historical record containing interesting observations of the effects of opioids on disease states and health. The purpose of this chapter will be to provide some of the historical information that pertains to health-related effects of opioid use and to examine some of the recent scientific evidence documenting the effects of opioids on immune status. Furthermore, we will report some of the studies identifying the mechanisms of the effects of opioids on the immune system. Those studies show both direct effects of opioids on cells of the immune system and indirect effects that involve an interaction of the central nervous and immune systems. There are also recent data showing that one important pathway in the central nervous system involves the dopaminergic system. Finally, we examine the relationship between opioid-induced immune alterations and changes in host defense against infection.

OPIOID-INDUCED IMMUNOMODULATION: HISTORICAL PERSPECTIVE

Although opioids have been used for thousands of years for their analgesic, euphoric, antitussive, and gastrointestinal effects, only relatively recently has their impact on

normal immune function come under controlled investigation in the laboratory. Of particular interest is the role that opioid use may have on susceptibility to infectious disease and the impact that opioids have on normal immune status in users of these drugs, whether in a clinical or recreational setting. Among the earliest observations of the effects of opioids on the occurrence and severity of infectious diseases occurred in the mid-1500s. Fallopious, a professor of anatomy at Pisa, administered opium to a condemned prisoner suffering from malaria. Although the prisoner died, this finding was the first documented experiment undertaken to examine the effects of opioids on an infectious disease [1]. It would not be until the latter half of the 19th century that the association between opioid use and susceptibility to infectious diseases became more documented and investigated.

Interestingly, Thomas De Quincey's 1821 publication *Confessions of an Opium Eater*, first brought to attention the potential harmful effects of chronic opioid use, as detailed in a chapter titled "Miseries of Opium." Another 19th century author, Alonzo Calkins argued in his book, *Opium and the Opium Appetite*, that opiate addiction was "plagued with disease."[1] Crothers [2] brought attention to the fact that morphine users were often the subjects of a multitude of serious infections, including pneumonia, nephritis, and other diseases. It was also noted that among 19th century opiate addicts in China, a number of disease complications were apparent such as tuberculosis, pneumonia, bronchitis, chronic abscesses at injection sites, diarrhea, and jaundice [3]. In a review by Risdahl and colleagues, [1] they suggest that throughout the early 20th century the association between a growing number of infections experienced by opioid users and the immunomodulatory effects of opioid use was strengthening. As intravenous administration of opiates increased in popularity, reports began to emerge of an increased incidence of infections in the drug addicted population. Biggam [4] was one of the first to call attention to the high incidence of malaria in heroin addicts in Cairo. An additional report in 1934 by Helpern [5] confirmed that this was also the case in New York City, in which an outbreak of fatal estivo-autumnal (*Plasmodium falciparum*) malaria was confined to the drug addicted population. Among the most important studies of the first half of the 20th century examining the impact of opioid use on infectious complications was performed by Hussey and Katz [6].In this landmark investigation of heroin use and susceptibility to infectious diseases, Hussey and Katz reported that complications found in a group of 102 addicts included skin abscesses, thrombophlebitis, septicemia, bacterial endocarditis, tetanus, and malaria. Reports of the high rate of infections in opiate addicts began to proliferate, with infective complications including skin sepsis, bacterial and fungal endocarditis, septicemia, viral hepatitis, bacterial bronchopneumonia, malaria, and tetanus [6–9].

While it was becoming widely accepted that opioid use was associated with increased susceptibility to an extensive variety of infectious diseases, few people questioned the deleterious impact that the drugs themselves were having upon the immune system. Rather, initial hypotheses regarding the close association between opioids and disease focused on the confounding effects of lifestyle, nutritional deficit, societal issues, and contaminated needle use in the drug-abusing population. For example, equipment was often passed from person to person with little attempt to sterilize the drug equipment or the site of injection. An additional factor was the typical lifestyle

associated with injection drug use. Injection drug users frequently followed practices resulting in malnutrition, stress, reversed sleep-wake patterns, sleep deprivation, and excessive alcohol consumption, many of which are known to impair immune function. Although several researchers were interested in the effects of abused opioids such as heroin on the immune system, a more controlled, mechanistic approach to the issue did not come about until the HIV/AIDS epidemic of the early 1980s [10,11]. Given the high rate of drug abuse (especially heroin) in this population, a new directive emerged to elucidate the precise mechanism by which opioids might affect the immune system. Additionally, the increased prevalence of viral, bacterial, and parasitic infections in heroin users suggested that immunologic impairment was a real entity that should be more thoroughly explored. The stage was set for new investigations into the cellular and molecular mechanisms that might explain the high incidence of infectious diseases amongst heroin users, and the fairly common observation of enhanced susceptibility to pathogens within this population.

OPIOIDS AND IMMUNOMODULATION

Observations

Based upon recent controlled studies, there is considerable evidence that opioids such as morphine induce substantial effects on immune status. For example, it has been shown that morphine administration is associated with alterations in a number of immune parameters, such as natural-killer cell activity [12,13], proliferation of lymphocytes, [13, 14] antibody production [15,16], and the production of interferon [17]. Studies in our laboratory have shown that acute morphine treatment in rats suppresses splenic lymphocyte proliferative responses to both T- and B-cell mitogens, splenic natural-killer cell activity, blood lymphocyte mitogenic responsiveness to T-cell mitogens, and the *in vitro* production of the cytokines interleukin-2 and interferon-γ [18-22]. Furthermore, the immune alterations induced by morphine are dose-dependent and antagonized by the opioid-receptor antagonist, naltrexone (e.g., [22]).

Like morphine, heroin also produces a dose-dependent, naltrexone-reversible suppression of the proliferation of T- and B-cells, production of interferon-γ, and cytotoxicity of natural killer (NK) cells in the spleen [23, 24]. In addition, heroin has been shown to have effects on inducible nitric oxide synthase (iNOS) expression, the enzyme responsible for nitric oxide formation [25, 26]. This study showed that heroin injection results in pronounced and widespread reductions in iNOS expression in rats. The generation of nitric oxide by cells of the immune system, particularly the macrophage, provides a substantial degree of microbial resistance [27–31]. Indeed, mice lacking the gene for iNOS have markedly reduced resistance to parasitic and bacterial infections [32–34]. The nitric oxide produced by macrophages also provides resistance to viral infections and exert tumor cytotoxicity.[28,35,36] In addition to its role in the control of infectious organisms, nitric oxide produced by iNOS appears to be involved in controlling the onset and duration of cellular immune responses. For example, numerous studies have shown that nitric oxide limits the proliferative activity of lymphocytes [37–39], mediates apoptosis

in macrophages [40–42], and regulates antibody formation [43, 44]. Thus, nitric oxide serves an important role in orchestrating the timed execution of immune responses. Given the pronounced effects of opioids on immune status, it is not surprising that opioid use has potential and apparent deleterious impacts on health.

Mechanisms

Although the immunomodulatory effects of opioids have been studied extensively, the mechanisms by which opioids exert these effects are only beginning to be understood. One of the major focuses of research on the mechanisms of opioid-induced immunomodulation has been to determine the site of action of opioids. One direction of this work tests whether opioids alter immune status by acting directly on cells of the immune system, possibly *via* opioid receptors present on the surface of these cells. Wybran and colleagues [45] provided some of the first evidence for the presence of opioid receptors on human peripheral blood T-lymphocytes by showing a naloxone-reversible suppression of the percentage of active T-cell rosette formation by morphine. Radioligand binding studies using ^{3}H-naloxone yielded results consistent with the presence of mu-opioid receptors on human blood lymphocytes, human blood platelets, and normal and mitogen-stimulated rat splenocytes [46, 47]. There is also evidence that different subtypes of opioid receptors are found on immunocytes (e.g., [48–50]).

The opioid receptors found on immunocytes appear to be functional, since *in vitro* exposure to morphine produces concentration-dependent and opioid antagonist-reversible alterations in several assays of immune status. For example, the addition of morphine to cultures of human peripheral blood mononuclear cells suppresses Con A-stimulated interferon-γ production [51] and enhances the growth of HIV-1 [52]. Furthermore, addition of morphine to cultures of murine peritoneal macrophages suppresses phagocytic activities [53–55]. In contrast, there are some studies that report no direct effect of morphine or an effect only at very high concentrations [56–64]. For example, Bayer et al. [56] showed in rats that only high concentrations of morphine, when added directly to mitogen-stimulated splenocyte and blood cultures, suppress lymphocyte proliferation. In that same study, the suppressive effects of morphine were not antagonized by the simultaneous addition of naltrexone to culture. These results make it clear that there are direct effects of morphine on immune status, but the effects are complex. These findings indicate the existence of opioid receptors on cells of the immune system and provide one pathway in which opioids such as morphine modulate the immune system.

Another site of action for opioids is through the regulatory actions of the central nervous system (CNS) on the immune system. Substantial evidence supports the existence of a complex, bidirectional link between the CNS and the immune system (e.g., [65]). Experimental evidence indicates that morphine's immunomodulatory effects involve central opioid receptors. An initial study by Shavit and colleagues [12] found that systemic administration of morphine, but not N-methylmorphine (a form of morphine which does not readily penetrate the blood-brain barrier), produces a naltrexone-reversible suppression of splenic natural killer cell activity in the rat. That same study showed that intracerebroventricular (icv) administration of morphine dose-dependently suppresses

splenic natural killer cell activity, and prior administration of naltrexone blocks the effect of icv morphine. Along similar lines, microinjections of morphine directly into the periaqueductal gray region of the rat brain produce a highly significant, naltrexone-sensitive suppression in splenic natural killer cell activity [66]. Bayer and colleagues showed that microinjection of morphine into the anterior hypothalamus inhibits blood lymphocyte proliferation to mitogen [67]. In addition, studies from our laboratory have shown that a single icv microinjection of morphine dose-dependently alters lymphocyte proliferation to T- and B-cell mitogens and natural-killer cell cytotoxicity in the spleen [68]. Our studies also showed that icv administration of N-methylnaltrexone dose-dependently antagonizes the immunomodulatory effects of systemic morphine administration, whereas systemic administration of N-methylnaltrexone (at doses that do not act centrally) are ineffective in blocking morphine's effects [20]. Thus, there is an overwhelming amount of evidence implicating a role of the central nervous system in opioid-induced immune alterations. Given that the central nervous system is involved in opioid-induced alterations of immune status, there is tremendous opportunity for behavioral processes to influence opioid-immune interactions.

Role of Conditioning Processes

The study of Pavlovian conditioning of immune responses has provided some of the most convincing evidence that environmental factors regulate the immune system. The conditioning of immune responses is well established in a number of paradigms. One of the earliest demonstrations of conditioned immune responses involved pairing the taste of saccharin with the immunosuppressive drug, cyclophosphamide. That study showed that the taste of saccharin acquired immunosuppressive properties through its association with cyclophosphamide [69]. In related studies, presentation of a conditioned stimulus that had previously been paired with electric shock induced alterations in a number of immune responses (e.g., [70–75]). These findings showed that aversive conditioned stimuli induce activation of neural circuitry involved in the modulation of immune status. Subsequent work discovered that the immunomodulatory effects of an aversive conditioned stimulus, one based on pairings with electric shock, were mediated by opioid-receptors, as evidenced by the ability of naltrexone to block conditioned effects [73,75]. These studies indicate that aversive stimuli and exogenous opioids act through a common pathway to regulate immune responses. Furthermore, these findings suggest the immunomodulatory effects of exogenous opioids can be conditioned to environmental stimuli as well.

Several investigators have shown that many of the physiological and behavioral effects of opioids can be conditioned. For example, environmental stimuli that have been paired with morphine administration can elicit morphine-like effects, such as hyperthermia, when presented in the absence of morphine [76–79]. In line with these studies, our laboratory provided the first demonstration that alterations of immune status can be conditioned to environmental stimuli that have been paired with morphine administration [80–82]. In that investigation, rats received subcutaneous injections of morphine in a distinctive environment. When rats subsequently were re-exposed to the distinctive

environment in the absence of morphine, immunological alterations occurred that were similar to those produced by morphine alone, including decreased mitogen responsiveness of lymphocytes, decreased interleukin-2 production, and decreased natural killer cell activity in the spleen. Extensive control procedures showed that these effects were the direct result of the conditioning process [80].These results are important because they provided the first demonstration that immunologic alterations can be conditioned to environmental stimuli paired with morphine administration. There is also evidence that conditioned stimuli previously paired with heroin induce a pronounced reduction of LPS-induced nitric oxide expression [83].The reduction of nitric oxide expression was widespread with the effect being found in spleen, lung, and liver. The conditioned effect was evident in both at the level of mRNA and protein expression. Control procedures showed that the effect was specific to exposure to conditioned environmental stimuli and was not related to ancillary effects of the conditioning procedure. Overall, the implication is that the host response to opioids will not be static, but will change as the organism learns to predict the administration of opioids on the basis of distinctive environmental stimuli. In other words, these results demonstrate that alterations of immune status can be induced by stimuli associated with the administration of opioids, indicating that the detrimental health consequences of opioid use may also be conditioned to the environmental stimuli and not solely the pharmacological property of the drug.

Role of the Dopaminergic System

Although it is known that the activation of CNS opioid receptors by morphine impacts immune status, there is a paucity of literature delineating the specific brain structures and neurotransmitter systems involved in opioid-induced immunomodulation. Our laboratory has recently provided new data indicating that the regulation of central dopaminergic pathways is one mechanism by which morphine modulates immune status. Increases in dopamine signaling elicited by morphine have been implicated in mediating many of morphine's physiological and behavioral effects, but the role of dopamine in morphine-induced immunomodulation had not been examined until recently by our laboratory [84].We began testing the hypothesis that a dopaminergic mechanism mediates morphine-induced immunosuppression by examining the effect of the D_2-like receptor agonist 7-OH-DPAT on acute morphine treatment in rats. 7-OH-DPAT was selected for use in our initial study because it has been shown to functionally antagonize dopamine signaling at the behavioral, cellular, and molecular level, possibly as a result of activity at dopamine autoreceptors. For example, 7-OH-DPAT inhibits stimulated endogenous dopamine release [85] and attenuates a diverse set of opioid-elicited behaviors (e.g., antinociception [86], conditioned place preference [87], locomotor activity [88]) that are associated with increased dopamine signaling. Moreover, we have found that 7-OH-DPAT exerts a functionally antagonistic effect on morphine-induced increases in dopamine signaling at the molecular level as evidenced by data showing that 7-OH-DPAT completely blocks morphine-induced expression of c-Fos protein in several dopamine terminal regions (unpublished data). Results from our study showed that intracerebroventricular administration of 7-OH-DPAT dose-dependently antago-

nized morphine-induced reductions of splenic NK cell activity at several-fold lower doses than were effective following systemic administration [84]. These findings were important in that they provided the first evidence for the direct involvement of central dopaminergic mechanisms in morphine-induced immunomodulation.

To extend our initial findings and begin to characterize the dopaminergic mechanisms involved in morphine-induced immunomodulation, we examined the role of dopamine D_1 receptors in the nucleus accumbens specifically. The mesolimbic system is perhaps the most widely studied dopaminergic system with respect to its interactions with opioids because it is an integral component of the so-called reward circuitry of the brain. Indeed, the activation of dopamine D_1 receptors in the nucleus accumbens is necessary to produce the rewarding/reinforcing effects of morphine [89], but it also appears to be necessary for supraspinally mediated opioid analgesia [90].The latter finding is of great significance, as neural pathways subserving morphine's effect on immune functions have been suggested largely based on studies of the analgesic effects of morphine. For example, microinjection of morphine into the PAG produces both analgesia [91] and suppression of NK activity [66], and the onset of morphine's immunologic and analgesic effects follows a similar time course [92]. However, although similar neural structures appear to mediate certain aspects of opioid analgesia and immunomodulation, discrepancies do exist. For instance, morphine infusions into the anterior hypothalamus produce immune alterations but not analgesia [67], and despite the fact that opioids such as hydromorphone and oxycodone produce potent analgesia, they do not obligatorily suppress immune status [93]. Nonetheless, the large degree of similarity between mechanisms of opioid analgesia and immunomodulation has proven useful in attempting to elucidate neurotransmitter systems that mediate the effects of morphine on the immune response.

It is known that the nucleus accumbens participates in the regulation of peripheral immunity, as lesions [94] or pharmacological manipulation [95] of mesoaccumbens dopamine neurons results in altered immune responses. Given that morphine administration increases extracellular dopamine in the accumbens, we hypothesized that the nucleus accumbens may comprise a critical neural substrate of opioid-mediated immunomodulation. In a recent study by our laboratory, we have shown that morphine's suppressive effect on splenic NK cell activity requires the activation of dopamine D_1 receptors, as systemic administration of the D_1 selective antagonist SCH-23390 blocked morphine's effect on NK activity, while the D_2 antagonist raclopride had no effect [96]. That study also showed that microinjection of SCH-23390 into the nucleus accumbens shell, but not the core, completely prevented morphine-induced reductions of NK activity, indicating that the activation of D_1 receptors in the shell of the nucleus accumbens is an essential mechanism involved in the modulation of NK activity by morphine. Moreover, it appears that direct activation of D_1 receptors in the nucleus accumbens shell is alone sufficient to suppress NK activity, as we found that administration of the selective D_1 agonist SKF-38393 into the shell of the accumbens suppressed splenocyte NK activity similarly to morphine. Hence, it is suggested that D_1 receptor activation in the nucleus accumbens shell is both a necessary and sufficient mechanism of opioid-induced NK cell suppression.

Given that a common property of virtually every abused drug is the ability to

increase dopaminergic transmission in the nucleus accumbens, it is plausible that in addition to morphine, other drugs of abuse should depress NK cytolytic responses via a similar mechanism. Reductions in splenic NK activity have been reported following *in vivo* administration of heroin [24], amphetamine [97], ethanol [98], and cocaine [99]. Although it is unknown whether dopamine receptors in the nucleus accumbens mediated the observed immunosuppression in these studies, the notion that such diverse classes of abused drugs may act through a common central mechanism to modulate host immunity is an intriguing hypothesis. Preliminary studies have also indicating that accumbens D_1 receptors are involved in the conditioned effects of morphine on NK cell activity suggesting that conditioned and unconditioned effects are mediated through a common pathway. Overall, there is substantial evidence to suggest that opioids alter components of host defense by modulating central dopaminergic signaling pathways. These data may have important implications regarding the neural mechanisms whereby drugs of abuse compromise host defenses and provide further clues as to how conditioning processes modulate immune status.

Infection Outcome

The suppressive effects of opioids on measures of immune status are well-established, and the mechanisms of those effects are being identified. However, the literature regarding the impact of opioid on infectious pathology and disease in controlled animal models is surprisingly limited and the reported findings are seemingly contradictory. For example, studies have shown that morphine administration can exacerbate [100–107] or produce no effect [108] on infection severity and outcome in various animal models. Regarding the detrimental effects of morphine on infection susceptibility, MacFarlane and colleagues [100] demonstrated that mice receiving morphine by osmotic minipump were more susceptible to mortality induced by oral inoculation of *Salmonella typhimurium*. Likewise, morphine has been shown to increase mortality to *Streptococcus pneumoniae* lung infection [102]. Alternatively, Alonzo and Carr [103] reported a protective effect of morphine such that mice receiving morphine prior to ocular herpes simplex virus-1 (HSV-1) infection had decreased mortality compared to infected control animals. Furthermore, morphine has been shown to potentiate some aspects of infection severity while other infectious complications are attenuated. Risdahl and colleagues [109] demonstrated that morphine delivered before Swine Herpes Virus-1 and *Pasteurella multocida* inoculation developed more advanced pneumonia than control animals, but were protected from severe infection consequences such as HSV-1 associated neurologic disease and mortality. Moreover, evidence suggests that the protective versus detrimental effects of morphine against *Plasmodium berghei* infection may be biphasic and dose dependent [110]. Collectively, these studies demonstrate the complexity of the effects of morphine on infection resistance.

In our preliminary studies, we assessed the effects of morphine on infection susceptibility using a subcutaneous chamber model of Gram-negative sepsis. The subcutaneous chamber model was employed based on the well-characterized proinflammatory immune response involved in pathogenesis of the infection and its clinical relevance

in modeling sepsis arising from dissemination of bacteria from a focal site of infection challenge. The results showed that morphine decreased dissemination and mortality associated with *Porphyromonas gingivalis* strain A7436 (Pg A7436) infection. The data also showed that morphine decreases the enhancement of vascular permeability and inflammation of the subcutaneous chamber, an important process in progression of the infection from the local site to the periphery. These results suggest that a protective effect of morphine on a disseminating Gram-negative infection may relate to the suppression of alterations in vascular permeability and the proinflammatory immune response. In other words, the protective effect of morphine on a live Gram-negative infection in the subcutaneous chamber model is related to the reduction of proinflammatory events essential for bacterial dissemination from the chamber. Thus, these results suggest that morphine reduces the development of proinflammatory events required for pathogenesis of the infection. The effect of morphine on infection outcome is likely determined by a variety of factors that include dose employed, schedule and duration of administration, species, and infectious disease model. However, the challenge is to identify how opioid-induced immune alterations interact with the pathogen to determine the effect that opioids have on infection outcome.

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11 Immunomodulation by Nutraceuticals and Functional Foods

David M. Shepherd

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INTRODUCTION

Over the last decade, there has been a steady increase in the popularity and usage of natural products to enhance overall health. These "nutraceuticals" and "functional foods" modulate the function of various physiological systems including the immune system. By altering immunity, it is possible to augment an individual's ability to ward off infection, or suppress autoimmunity and chronic inflammatory diseases. Thus, the renaissance of herbal extracts as well as the increased consumption of other dietary components has afforded the public a relatively inexpensive way to self-medicate.

Definitions and Overview

By definition, a nutraceutical (derived from the term "nutritional pharmaceutical") is a foodstuff (fortified food or dietary supplement) that is held to provide health or medical benefits in addition to its basic nutritional value [1]. Nutraceuticals derived from botanicals deliver a concentrated form of presumed bioactive agents from plants that are not generally part of the food supply. The term nutraceutical has no regulatory definition. Similarly, functional foods, as defined by the International Life Sciences Institute (ILSI), are foods that by virtue of physiologically active food components, provide health benefits beyond basic nutrition [2]. For the purposes of this review, these two terms will be differentiated by the form in which they are consumed. "Nutraceuticals" refers to dietary supplements most often found in pill or capsule form; "functional foods" are ingested as part of a normal food pattern. Both are intended to provide beneficial effects beyond their nutritional value, and contribute to an improved state of health and/or reduction of risk of disease.

Current Regulatory Environment

The general public's overall perception of most natural products is that they are safe and efficacious—although neither may be true nor have been determined scientifically. Currently, the regulations governing the production, distribution and sale of dietary supplements and functional foods in the United States are primarily provided by several pieces of legislation and enforced by the U.S. Food and Drug Administration (USFDA) [3, 4]. First, dietary supplements fall under the regulation of the Dietary Supplement Health and Education Act of 1994. Accordingly, they are viewed differently from conventional pharmaceutics and do not require USFDA approval prior to marketing and selling of these products. Second, the 1990 Nutrition Labeling and Education Act permits the USFDA to authorize health claims for foods or dietary supplements based on their review of the scientific literature. Third, health claims can be made for foods based on an authoritative statement by a scientific body of the U.S. government or the National Academy of Sciences, as described in the 1997 Food and Drug Administration Modernization Act.

In other parts of the world, different views and regulations often exist for the production and sale of natural products. In Canada, where sales of functional foods and natural health products (NHPs; aka nutraceuticals) have increased 130% over the last 4 years, any supplement or functional food that carries a health claim, or levels of ingredients not found in conventional foods, are currently regulated as drugs [5]. In Europe, herbal products are known as phytomedicines and strictly regulated as drugs under the European Scientific Cooperative on Phytotherapy (ESCOP) [6]. In addition, the European scientific community continues to develop the concept of what functional foods are, and how they should be regulated. It appears that some, but certainly not all, functional foods will eventually be classified as "novel foods" and, consequently, will require safety assessment as described in the EU Novel Food Regulation. In Asia and many developing countries, nutraceuticals and functional foods are considered traditional

medicines. Japan regulates some natural products as foods and others as drugs [7]. In China, the Ministry of Public Health has legally approved some functional foods for medicinal use [7]. Thus, distinguishing between foods and traditional medicines can be difficult. On the same hand, the World Health Organization reports that in developing countries approximately 80% of the world populations rely on traditional medicines, mostly from botanical sources, for their primary healthcare [8, 9]. Taken together, there exists a pressing global need for a more thorough understanding of the potential uses and safety concerns relating to natural products.

Lack of Credible Scientific Data

Although many of the natural products currently produced and sold throughout the world have been used for centuries, relatively little credible scientific information is available describing the specific bioactive ingredients of these products, their mechanisms of action, or their potential to generate adverse effects. This is particularly true when considering the use of natural products to specifically modulate the immune system. Prior to 1980, very limited information was available describing the effects of nutritional supplements on immunity. However, over the last 25 years, there has been an explosion of research conducted in this area encompassing immunology, toxicology, nutrition, and alternative medicine [10, 11]. The goal of this review is to present current findings on the effects of select herbal extracts and functional foods on the immune system in relation to human health. To this end, significant preclinical and clinical studies that describe immunomodulation by natural products are featured.

IMMUNOMODULATION BY NATURAL PRODUCTS

Herbal Extracts

Echinacea

For the last 10 years, Echinacea has consistently been one of the top selling botanicals in the United States, reaching sales of greater than 5.2 million dollars in 2004. Three species of Echinacea are used medicinally: *E. angustifolia*, *E. pallida*, and *E. purpurea*. All three varieties exhibit immunomodulatory activity although they differ chemically in composition (see the review by Percival [12]). As with most herbs, variation in chemical composition and biological activity may exist due to geographical location and time of harvest, as well as the developmental stage, and growth conditions of the plant. Another critical factor in the production of most herbs is the portion of the plant that is extracted. For the preparation of Echinacea, the roots, leaves, or whole plant may be used although the composition of these extracts differs significantly [13]. Typically, the roots possess higher concentrations of volatile oils and pyrrolizidine alkaloids, while in contrast, the leaves and upper parts of the plant yield complex polysaccharides, and caffeic acid derivatives upon extraction [12, 14]. At present, the significance of employing different methods of extraction to produce water-soluble, ethanol-soluble, alkaline, lipophilic,

and polar fractions has not been fully defined, although each generates active components. The public's increasing use of this herb over the last century suggests that it is relatively safe at commonly consumed doses. However, additional studies designed to evaluate the potential for Echinacea to produce adverse effects on the immune system and other organ systems are still needed.

Extracts of Echinacea have been shown to significantly modulate the *in vitro* function of immune cells from laboratory animals and humans. All three species of Echinacea are immunostimulatory in nature, and have been shown to activate various immune cells *in vitro* including monocytes, macrophages and natural killer cells [15]. Several studies examining the effects of low concentrations of *E. purpurea* on cultured mouse and human macrophages reported significantly augmented production of IL-1, TNF-α, IL-6, IL-10, and nitric oxide (NO) when compared to untreated cells [16, 17]. Phagocytosis of yeast particles by human granulocytes was stimulated by commercial extracts of *E. angustifolia* and *E. purpurea* [13]. Moreover, ethanolic root extracts of *E. purpurea* stimulated phagocytosis to a greater extent than did *E. angustifolia* and *E. pallida*. In addition, significantly enhanced natural killer (NK) cell function and antibody-dependent cell-mediated cytotoxicity (ADCC) was observed following Echinacea treatment of human peripheral blood mononuclear cells (PBMC) from both healthy subjects and patients suffering from chronic fatigue syndrome or AIDS [18]. *E. purpurea* extracts were shown to be potent activators of human NK cell cytotoxicity, resulting in increased CD69 expression in the $CD16^+$ and $CD16^+CD56^+$ NK subsets [19]. In contrast, *E. angustifolia* had no effect on the production of NO by mouse macrophages although it did increase the proliferation and IFN-γ production by anti-CD3-stimulated T cells [20]. Furthermore, studies have demonstrated that preparations of *Echinacea* are strongly involved in the reduction of inflammatory processes and possess potent antioxidant activity *in vitro* [12, 21, 22].

Treatment of laboratory rodents with preparations of Echinacea significantly affects the generation of innate and adaptive immunity. In a study describing the oral treatment of female Swiss mice with a glycerine extract of *E. purpurea*, the authors reported enhancement of the primary antibody forming cell (AFC) response to sheep red blood cells (SRBC) [23]. An enhancement of KLH-specific humoral immunity was also noted in rats treated for 6 weeks with a commercially available root extract of *E. angustifolia* in their water [24]. The Echinacea-treated rats showed a significant augmentation of their primary and secondary IgG response to the antigen when compared to controls. Likewise, aging rats fed an Echinacea extract (containing 1% cichoric acid) over an 8-week period had increased numbers of circulating leukocytes (with an increased frequency of monocytes and decreased level of granulocytes) and plasma IL-2, but no increase in phagocytic activity of PBMC [25]. Echinacea also affects the generation of immunity against infectious disease. The survival rate of healthy and immunocompromised mice injected with lethal doses of *Candida albicans* or *Listeria monocytogenes* was significantly increased following the intravenous injection of polysaccharides isolated from *E. purpurea* [26, 27]. From these studies, the authors concluded that the polysaccharide fraction of their herbal extract was at least partially responsible for the immunoprotective effects in mice via stimulation of cells such as granulocytes and macrophages that

mediate innate immunity. Overall, the prevailing literature supports the premise that Echinacea possesses immunostimulatory activity in laboratory animals.

In contrast to the effects of Echinacea on both the innate and adaptive arms of the immune system in laboratory rodents, humans treated with extracts of this herb benefit primarily from enhanced phagocytic activity. The intravenous or oral administration of alcohol extracts from the roots of *E. purpurea* resulted in a significant increase in PMN activity [28]. In addition, intravenous injection of purified polysaccharides extracted from this herb also induced acute phase reactions and activation of phagocytic cells in the blood. However, studies that examine the ability of Echinacea to prevent illness show little to no significant benefits. In a double-blind, placebo-controlled crossover study, oral administration of freshly pressed juice of *E. purpurea* failed to stimulate nonspecific immunity in healthy young men [29]. Furthermore, Melchart and colleagues summarized the results of five randomized, placebo-controlled studies that evaluated the effects of all three species of Echinacea on 134 subjects [30]. In this report, only two of the five studies showed any positive effects of Echinacea treatment on the measured outcome (phagocytic activity of peripheral blood neutrophils). The authors concluded that Echinacea may have no effect on young, healthy volunteers and should instead be examined primarily in patients with immune disorders.

The traditional usage of Echinacea by native populations was primarily to treat colds, sore throats, or coughs. It is this indication as a treatment for upper respiratory illness (URI) that has been tested in several clinical studies. As reviewed by Barret and colleagues, seven studies published between 1984 and 1997 demonstrated a significant effect of Echinacea in the treatment of URI symptoms [31]. The measured outcome for two studies was flu-like symptoms, whereas for the other five trials, URI symptoms were reported [12]. In all seven studies, Echinacea significantly modified the severity and duration of cold symptoms. In two separate clinical trials reviewed by Borchers and colleagues, positive effects for both *E. purpurea* and *E. angustifolia* were observed for the treatment of URI [13]. Both studies were designed as placebo-controlled, double-blind clinical trials. In the first study involving 180 patients with URI, patients receiving a 900 mg/day dosage of an ethanolic extract from the root of *E. purpurea* experienced significantly diminished symptoms of shorter duration than those receiving the 450 mg/day dose, or the placebo [32]. In the second study, 303 volunteers were treated with a liquid tincture of mostly *E. angustifolia*, and 306 subjects treated with a placebo [33]. The Echinacea tincture effectively diminished the frequency of URI symptoms, even when given prophylactically. Taken together, these studies support the use of Echinacea to treat colds and flu in humans, although much more research is needed to elucidate specific mechanisms of action of this herb.

Ginseng

Another popular herbal dietary supplement that has a long history of use for medicinal purposes is ginseng. Much like Echinacea, three primary species of ginseng exist for therapeutic consumption: *Panax ginseng* (Asian ginseng), *Panax notoginseng* and *Panax quinquefolius* (American ginseng). These herbal varieties can be readily authenticated

via the use of restriction fragment length polymorphism (RFLP) procedure [34]. Extraction of ginseng is almost exclusively performed on the root of the plant, as that portion contains the highest concentration of bioactive compounds. The major purported bioactive components of ginseng are the ginsenosides of which there are two major classes, the Rb and Rg groups. The Rb group includes Rb1, Rb2, Rc, Rd, and Rh2, while the Rg group includes Re, Rf, Rg1, Rg2, and Rh1. These steroidal saponins have diverse effects in multiple target organs including the immune system. In addition, a polysaccharide fraction has been isolated from ginseng (named "ginsan") that has associated immunomodulatory activity. Much like Echinacea, ginseng has been used for thousands of years with no apparent adverse effects on the immune system reported in the literature [35]. Recent reports, however, have suggested that ginsenosides may be developmental toxicants, so it will be of interest to evaluate the effects of ginseng extracts on the developing immune system [36].

Ginseng and its components can significantly affect various *in vitro* immune responses. Using a polysaccharide-rich extract of *P. ginseng* on mouse immune cells, significant increases have been reported for phagocytosis, the production of pro-inflammatory cyokines, and the generation of lymphokine-activated killer (LAK) cells [37–39]. Interestingly, purified ginsenoside extracts from *P. ginseng* containing exclusively Rh1 or Rg1 inhibited the activation of RAW264.7 cells, and murine $CD4^+$ T cells, respectively [40, 41]. In the latter study, ginsenoside Rg1 was also shown to effectively polarize the $CD4^+$ T cell response towards a Th2 response. In my own laboratory, as shown in Table 11.1, a standardized extract of *P. notoginseng* (NotoG) significantly affected the inflammatory responsiveness of murine bone marrow-derived dendritic cells (BMDC). This extract, like most from *P. notoginseng*, contains very high concentrations of ginsenosides (ginsenosides comprise approximately 95% of the extract). Additional investigations in my laboratory have demonstrated similar anti-inflammatory effects of NotoG in RAW 264.7 cells [42]. A recent study by Oh and colleagues also demonstrated the anti-inflammatory effects of 20(S)-Protopanaxatriol, a ginsenoside metabolite generated by intestinal bacteria [43]. In this study, iNOS and COX-2 expression was inhibited in LPS-stimulated RAW264.7 cells. In contrast, the production of pro-inflammatory cytokines by mouse splenocytes and peritoneal macrophages was increased following treatment with the standardized extract, CVT-E002 [44]. However, this extract was derived from *P. quinquefolius* that may contain a very different chemical composition. Finally, ginseng extracts from both *P. ginseng* and *P. quinquefolius* were shown to exhibit potent anti-oxidant activities resulting in decreased erythrocyte hemolysis and lipid peroxidation of murine immune cells [45]. Collectively, these studies demonstrate the effectiveness of ginseng extracts to alter the function of cultured immune cells from laboratory animals.

Ginseng extracts have also been shown to modulate the immune responsiveness of human leukocytes *in vitro*. For example, extracts from *P. ginseng* were reported to increase NK cell function and ADCC in PBMC from both healthy and immunocompromised individuals [18]. In addition, similar extracts and their degradation products displayed anti-complement activity [46]. In contrast to the mouse data described above, a standardized ginseng extract (Gerimax) by itself (no inflammatory stimulus) induced the production of IL-12 (but not IL-10) by PBMC from healthy test subjects [47].

TABLE 11.1
Immunomodulatory Effects of *Panax Notoginseng* Extract on Murine BMDC

	No LPS		LPS	
	No herb	**NotoG**	**No herb**	**NotoG**
TNF-α	0.1 ± 0.0	0.2 ± 0.1	17.9 ± 1.0	14.7 ± 0.0
IL-6	ND	ND	37.3 ± 0.2	24.0 ± 1.7*
IL-12	0.1 ± 0.0	0.1 ± 0.0	4.1 ± 0.2	1.7 ± 0.1*
CD40	717 ± 20	ND	9705 ± 2021	5005 ± 330*
CD86	1144 ± 68	ND	5110 ± 231	5595 ± 200
No LDL	23.0 ± 2.0	25 ± 2.0	ND	ND
LDL	8965 ± 1259	2979 ± 209*	ND	ND

Mouse bone marrow cells were treated with GM-CSF for 7 days to induce differentiation into dendritic cells. Cells were either unstimulated or stimulated with (1 μg/ml) LPS and treated with 0 or 200 μg/ml Panax notoginseng (NotoG) for 24 hours. Alternatively, BMDC were pre-treated with NotoG (0 or 200μg/ml) for 24 hours and incubated with acetylated-LDL for 1.5 hours.
Data shown are representative of three independent experiments. Cytokine concentrations are in ng/ml. Expression of co-stimulatory molecules and LDL uptake is represented as Mean Channel Fluorescence. Data represent mean ± SEM. *, $p < 0.05$ for the comparison of untreated and NotoG-treated BMDC, or untreated and LDL-treated BMDC.

Furthermore, in a recent study by Takei and colleagues, the ginsenoside metabolites M1 (Compound K) and M4 (20(S)-Protopanaxatriol) were demonstrated to promote the maturation of human monocyte-derived DCs [48]. M4, and to a lesser extent M1, increased the DC expression of key costimulatory molecules, decreased their endocytic activity, and induced their production of IL-12. Moreover, these effects had the end result of generating mature DCs that could induce potent Th1 polarization. Unfortunately, it was not clearly defined in this study if the ginsenoside metabolites were used at physiologically relevant concentrations, a variable that would be expected to have significant implications on the interpretation of these results.

The overwhelming majority of reports found in the literature that describe the effects of ginseng on the *in vivo* treatment of laboratory rodents indicate that ginseng extracts stimulate immune function. In a model of bacterial lung infection, rats were found to have decreased bacterial loads following oral treatment with an extract of ginseng [49]. Surprisingly, these animals also displayed decreased titers of Ag-specific IgM and IgA suggesting that ginseng was preferentially stimulating innate immunity. In C3H.HeN mice, an extract from the root of *P. ginseng* induced an increase in the expression of iNOS and the production of TNF-α, IFN-β and IFN-γ by splenocytes and peritoneal macrophages [50]. This effect was dependent on expression of toll-like receptor 4 (TLR4) as similar results were not seen in TLR4-knockout mice. Ginseng has also been reported to possess anti-carcinogenic properties. In a model of urethane-induced lung adenoma, treatment of mice with ginseng led to enhanced NK cell activity and a decreased number of lung tumors [51]. Treatment of ICR mice with purified ginsenosides from *P. notoginseng* resulted in a significant adjuvant effect on the humoral response to ovalbumin (OVA) [52]. In these studies, mice immunized subcutaneously with OVA

and ginsenosides together exhibited significantly higher serum OVA-specific IgG, IgG1 and IgG2b antibody titers when compared to the control group that received OVA alone. Splenocytes from these animals also displayed enhanced proliferation following mitogenic stimulation. Similar immunostimulatory effects have been demonstrated following the treatment of mice with isolated ginsenosides. Rb1 treatment increased immune function in immunocompromised mice as determined by enhanced peritoneal macrophage activity and an increased SRBC response [53]. In addition, purified Rg1 caused an increase in the number of splenic SRBC-specific PFC and $CD4^+$ T cells, as well as the production of macrophage-derived IL-1 in treated mice [54]. Collectively, these studies demonstrate the potential for ginseng to enhance immunity in laboratory rodents.

Evidence for ginseng extracts to modulate human immune function also exist but are much more limited. In a double-blind, placebo-controlled, randomized study reported in 1996, a standardized ginseng root extract (Ginsana) was given to volunteers who were being vaccinated against influenza to determine if this treatment would induce higher immune responses to that virus [55]. Individuals were treated with either a placebo or 100 mg Ginsana (once daily) for 12 weeks. On the 4th week, participants received an anti-influenza polyvalent vaccination. Ginseng extract treatment significantly reduced the number of cases of influenza as well as the common cold when compared to the control. Moreover, antibody titers to influenza and NK cell activity were significantly increased in those individuals receiving Ginsana. Similarly, in a recent double-blind, placebo-controlled, randomized clinical trial of198 subjects, CVT-E002 (a proprietary extract of *P. quinquefolius*) was demonstrated to prevent acute respiratory illness (ARI) in institutionalized, older adults [56]. The treatment of subjects (average age of 82) with CVT-E002 (200 mg, twice daily) for 8 to 12 weeks resulted in decreased ARI due to infection with influenza or respiratory syncytial virus. The ginseng extracts that were tested were found to be safe, well tolerated and effective for preventing disease. Overall, ginseng appears to hold great promise as a natural product that can improve health by modulating immunity and potentially inhibiting inflammatory conditions.

Functional Foods

Fish Oil (n-3 PUFAs)

Dietary polyunsaturated fatty acids (PUFAs), especially the n-3 series that are found in marine fish oils, modulate a variety of normal and disease processes, and consequently affect human health. PUFAs are classified based on the position of double bonds in their lipid structure and include the n-3 and n-6 series. Dietary n-3 PUFAs include α-linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) whereas the most common n-6 PUFAs are linoleic acid, γ-linolenic acid, and arachidonic acid (AA). AA is the primary precursor of eicosanoids, which includes the prostaglandins, leukotrienes, and thromboxanes. Collectively, these AA-derived mediators can exert profound effects on immune and inflammatory processes. Mammals can neither synthesize n-3 and n-6 PUFAs nor convert one variety to the other as they do not possess the appropriate enzymes. PUFAs are required for membrane formation and function

and thus are considered essential components of the mammalian diet. PUFAs are ubiquitously distributed throughout the body and dietary ingestion of these fatty acids can have significant effects on cellular function, especially on cells of the immune system. PUFAs, especially the n-3 series from fish oil, generate immunomodulatory and anti-inflammatory effects. For a summary of the well-established effects of PUFAs on the immune system, the reader is directed to several comprehensive reviews [15, 57–59]. For this chapter, a brief synopsis of PUFA-induced immunomodulation will be provided followed by a discussion of the potential mechanisms of action of PUFAs on immune cells.

Fish oil has been shown to be anti-inflammatory and immunosuppressive in a large number of animal studies. Although dietary fish oil has limited effects on rodent macrophage phagocytosis, it has been reported to significantly reduce the production of inflammatory mediators such as reactive oxygen species, nitric oxide and cytokines (TNF-α, IL-1β and IL-6) [57]. Among the PUFAs tested, EPA appears to be the most inhibitory. Furthermore, T cells treated with PUFAs produce lower levels of Th1 (IL-2 and IFN-γ) but not Th2 (IL-4 and IL-10) cytokines, and display reduced expression of CD25 [57]. Antigen presentation by monocytes, macrophages, and dendritic cells is also diminished following exposure to fish oil, perhaps by the resulting downregulation of accessory molecules including MHC class II, CD18, CD11a, and CD54 [59].

Feeding animals fish oil results in decreased immune- and inflammatory-mediated diseases. NK cell activity, CTL activity, and DTH responsiveness were all significantly reduced in laboratory animals fed high levels of fish oil [57]. Suppression of both the graft-versus-host and host-versus-graft responses has been reported following fish oil treatment of mice. Not surprisingly, PUFAs also effectively produced immunosuppressive effects in several rodent models of organ transplantation. In addition, dietary fish oil has significant effects on several other animal models of autoimmune and inflammatory disease including IgA nephropathy, amyloidosis, collagen-induced arthritis, and ulcerative colitis [59]. On the other hand, studies characterizing the effects of PUFAs on antibody production in laboratory animals have produced conflicting results. In rats fed 25% fish oil, an enhanced production of OVA-specific IgG and IgE titers was reported [60]. However, mice immunized with rat erythrocytes displayed a significant reduction in antibody production following ingestion of a diet containing 20% fish oil [61]. Of note in this study, as with most animal studies investigating the effects of fish oil on the immune system, PUFAs were administered in amounts beyond those that can be consumed by humans.

Consumption of fish oil in excess can generate immunotoxic effects in laboratory animals. Rats fed a 17% fish oil diet had reduced wound-healing responses when compared to corn oil [59]. In a mouse model of bacterial resistance to *S. typhimurium*, lower survival rates were reported for those animals that ingested a 20% fish oil diet over 15 days [59]. Similar fish oil-induced effects in guinea pigs were noted in a study of experimental tuberculosis leading the authors to conclude that this treatment resulted in decreased resistance to infectious disease. The consumption of fish oil has also been reported to result in alterations of hemostatic parameters such as platelet production and function. However, there is no indication that at doses normally consumed by humans, immunotoxicity will occur.

A number of studies in humans show that PUFAs can generate significant immunomodulatory effects. Generally, these studies have utilized considerably lower amounts of fish oil to treat subjects than found in most animal studies. Numerous clinical trials have examined the effects of fish oil on rheumatoid arthritis and many have reported statistically significant benefits such as decreased morning stiffness and numbers of tender joints [57]. Several other studies have reported that PUFAs can provide therapeutic benefits for patients with IgA nephropathy, the most common primary human glomerulonephritis [57]. Other studies describe similar beneficial effects for PUFA-enriched diets to treat Crohn's disease, other inflammatory bowel diseases such as ulcerative colitis, as well as psoriasis, asthma, systemic lupus erythematosus, and multiple sclerosis [57]. Thus, immunomodulation by PUFAs appears to be a promising intervention for the treatment of many autoimmune and inflammatory diseases.

A variety of biochemical and molecular mechanisms have been described to explain how PUFAs can modulate immune cell fate and function. The primary mechanism of action of dietary n-3 PUFAs involves the replacement of AA in the lipid membrane of the cells with either EPA or DHA. This, in effect, competitively inhibits the oxygenation of AA by the COX enzymes. For example, the EPA-induced suppression in the production of AA-derived eicosanoids is followed by a subsequent increase in the production of those from EPA. Generally, the EPA-derived eicosanoids are considered to be much less potent than those from AA, thus explaining, at least partially, the anti-inflammatory effects of PUFAs. A similar mechanism of action can be demonstrated for DHA, either directly or by retroconversion to EPA.

Recently, eicosanoid-independent mechanisms of action of PUFAs on immune cells have been reported that include effects on gene expression, and membrane structure and function. PUFAs regulate gene expression either directly via interaction with nuclear receptors, or indirectly by altering other signaling pathways that are initiated at the plasma membrane. To this end, PUFAs have been shown to bind and activate peroxisome proliferator-activated receptors (PPARs) [57]. Fatty acids can influence the activity of several PPARs including PPAR-α and PPAR-δ. However, it is the interaction of PUFAs with PPAR-γ that produces the most significant effects on immune cells due to the ability of PPAR-γ to interfere with lymphocyte activation and modulate macrophage differentiation. Moreover, PPAR-γ agonists have been demonstrated to inhibit the production of pro-inflammatory mediators such as TNF-α, IL-1β, IL-2 and IL-6 as well as the expression of iNOS [57]. Although PUFAs can effectively bind and activate PPARs, evidence of their direct involvement in PUFA-mediated immunomodulation remains to be formally proven, especially *in vivo*.

Alteration of membrane structure in immune cells by PUFAs can lead to defective signal transduction and decreased function. PUFA treatment of CD3-activated human T cells results in decreased calcium mobilization, proliferation and IL-2 production [58]. Recently, PUFAs were shown to inhibit T cell activation by blocking key signal transduction events, specifically the activation of c-Jun NH2-terminal kinase (JNK) and NF-AT [62]. Furthermore, the expression of CD25 (but not CD69) as well as the production of IL-2 and IL-13 (but not IFN-γ, IL-4 and IL-10) was significantly reduced in PUFA-treated peripheral blood T cells. In a separate study, Li and colleagues reported

that DHA altered the lipid components of lipid rafts and modified the IL-2-induced JAK-STAT signaling pathway via partial displacement of CD25 from the lipid rafts [63]. Lipid rafts are distinct microdomains of the plasma membrane that are critically involved in signal transduction, especially in lymphocytes. Many of the signaling components involved in T cell receptor-mediated signal transduction are localized in lipid rafts, including Fyn, Lck, and LAT Src family kinases. Recently, Geyeregger and colleagues reported that PUFA treatment resulted in the modification of lipid rafts in human T cells, ultimately leading to signaling defects and decreased activation [64]. Moreover, T cells treated with PUFAs form incomplete and unstable immunological synapses with antigen presenting cells, an effect that was mediated by LAT displacement from lipid rafts. Taken together, these studies demonstrate the potential for PUFAs to act via eicosanoid-independent, as well as eicosanoid-dependent mechanisms to alter immune cell function, and provide biochemical and molecular explanations for the immunomodulatory potential of fish oil.

Green Tea (EGCG)

Tea is one of the most widely consumed beverages in the world. It is made from the leaves of the plant *Camellia sinesis*, and manufactured as green, oolong, or black tea. Green tea is the non-fermented form of this "functional food" and contains several polyphenolic compounds including epicatechin, epicatechin gallate, epigallcatechin, and epigallocatechin gallate (EGCG). Many health-promoting properties such as antioxidant, antiproliferative, and chemopreventive effects are believed to result from green tea consumption. Similarly, ingestion of green tea extracts containing high concentrations of EGCG have been reported to produce health benefits [65]. Recently, several Phase I studies have evaluated the potential toxicity of administering green tea extracts orally to humans and found them to be safe and tolerable at doses resulting in relatively high blood concentrations of EGCG [66–69].

Green tea extracts and/or pure EGCG modulate the function of both rodent and human immune cells *in vitro*. In two separate studies by Shimamura and colleagues, EGCG (1-50 μg/ml) displayed mitogenic effects on murine B lymphocytes [70, 71]. In these reports, the direct plaque-forming cell (PFC) response to SRBC was strongly enhanced in splenic B cells following EGCG treatment. Conversely, maturation of murine BMDC was suppressed in a dose-dependent manner following exposure to EGCG (10-100 μM), an effect that was mediated via inhibition of mitogen-activated protein kinases and NF-κB [72]. Furthermore, in a recent study by Rogers and colleagues, EGCG was also shown to modulate cytokine production by BMDC following stimulation with LPS, muramyldipeptide, or infection with *Legionella pneumophila* [73]. In a dose-dependent fashion, EGCG suppressed IL-12 production by BMDC while increasing their secretion of TNF-α. However, the authors noted that the concentration of EGCG (50 μg/ml) that consistently generated these results would be difficult to achieve in human plasma solely by consumption of green tea but may be attainable following the ingestion of pharmaceutically prepared EGCG formulations. Similar suppressive effects of EGCG on the generation of pro-inflammatory mediators such as IL-12 and iNOS (but not

COX-2) have been reported for murine macrophages [74–76]. In humans, neutrophils from healthy donors were reported to down-regulate ROS activity, caspase 3 expression, and IL-8-induced migration following *in vitro* exposure to EGCG (1-100 μM) [77]. In contrast, EGCG at similar concentrations (12.5-50 μM) enhanced apoptosis in Jurkat T cells, an effect that was mediated primarily by increased production of hydrogen peroxide [78]. Other effects of EGCG on human T cells include the direct binding of this polyphenol to CD4 leading to decreased binding of gp120 and presumably inhibition of HIV-1 infection, and the attenuation of adhesion and migration of $CD8^+$ T cells following the binding of EGCG to CD11b on these cells [79, 80]. Taken together, these studies demonstrate the potential for EGCG to modulate the *in vitro* fate and function of immune cells.

Green tea polyphenols, EGCG in particular, also affect the generation of immunity in laboratory animals. At a dose of 0.5 g/kg body weight, green tea polyphenols completely inhibited LPS-induced lethality in Balb/c mice, an effect that was mediated at least in part through decreased TNF-α production (presumably by down-regulation of NF-κB activation) [81]. In another study involving the generation of innate immunity in mice, oral administration of both green tea extract (containing 3.5 mg/ml EGCG) and pure EGCG (0.5 mg/ml) were shown to inhibit neutrophil-dependent angiogenesis and enhance the resolution of pulmonary inflammation [77]. Using a model of collagen-induced arthritis, Haqqi and colleagues reported a significantly reduced incidence and severity of arthritis in mice orally administered green tea polyphenols [82]. Subsequent analysis showed a reduction in the expression of pro-inflammatory mediators including COX-2, interferon-γ, and TNF-α in the arthritic joints of mice fed green tea polyphenols. Likewise, EGCG treatment led to suppressive but protective effects in a mouse model of multiple sclerosis [83]. Oral treatment of mice with EGCG (300 μg/mouse twice daily) significantly inhibited and reversed relapsing-remitting experimental autoimmune encephalomyelitis (EAE). Moreover, proliferation of encephalitogenic T cells in the EGCG-treated mice was impaired as was their production of TNF-α which ultimately resulted in both limiting brain inflammation and reducing neuronal damage. Finally, in perhaps the best characterized animal model demonstrating the immunomodulatory effects of green tea polyphenols, EGCG has been shown to protect mice against the UVB-induced local as well as systemic immune suppression. This beneficial effect in mouse skin is primarily due to decreased UVB-induced infiltration of leukocytes, depletion of antigen-presenting cells, and oxidative stress following EGCG treatment [84, 85]. However, altered production of IL-12 and IL-10 is another important immune function regulated by EGCG and may be critical for the prevention of solar UVB light-induced skin disorders associated with immune suppression and DNA damage.

Unfortunately, to date, no formal clinical studies have been conducted to specifically evaluate the effects of green tea, green tea extracts, or EGCG on immune function. However, based on several Phase I studies that have evaluated EGCG and/or Polyphenon E (a defined, decaffeinated green tea polyphenol mixture) and found them to be safe and tolerable at relatively high doses, it is likely that future clinical studies may follow [66–69].

ADDITIONAL AREAS OF INTEREST

NUTRACEUTICAL-DRUG INTERACTIONS

Many nutraceuticals and functional foods have been reported to interact with pharmaceutics to produce adverse effects. Although medicinal herbs and drugs are therapeutic at one dose, they often are toxic at another. Interactions between the two may increase or decrease the pharmacological or toxicological effects of either product. For the purposes of this review, examples of reported nutraceutical-drug interactions are provided for two dietary supplements previously discussed; however, for more comprehensive information on this topic the reader is directed to several recent reviews [8, 86, 87].

Herbal nutraceuticals and functional foods can significantly interact with several commonly used pharmaceutics. Ginseng alters the anticoagulant effects of Warfarin in healthy patients and is discouraged prior to surgery [88]. It has also been reported to reduce blood concentrations of ethanol following the induction of alcohol dehydrogenase and aldehyde dehydrogenase, and to lower blood glucose levels via a mechanism involving the ginsenoside Rb2 [89]. Ginseng's use with estrogens and corticosteroids is discouraged due to potential additive effects it may have with these compounds [87]. However, ginseng has been shown to enhance the efficacy of influenza vaccinations indicating that it can interact beneficially with some therapeutics [89]. In a prospective, randomized and placebo-controlled, double-blind study involving kidney graft recipients, fish oil supplements, containing high concentrations of n-3 PUFAs, reduced tissue rejection by increasing the absorption and metabolism of cyclosporine [90]. Fish oil-enriched diets and supplements have also been reported to reduce the effects of aspirin on lymphocyte function, synergize with celecoxib, a COX-2 inhibitor, to significantly reduce pro-inflammatory mediators in a model of LPS-induced experimental periodontitis, and to combine with statins to lower LDL and triglyceride levels in patients [91–93]. Taken together, the potential for nutraceuticals or functional foods to interact with pharmaceutics and alter their therapeutic activity highlights the need for intensive investigations in this area of toxicology.

NUTRACEUTICAL-NUTRACEUTICAL INTERACTIONS

Relatively few reports have examined the benefits or perils of herb combinations. Because the activity of a particular herb in a mixture might differ from its activity as a single component, it would be prudent to test the effects of "prescribed" formulas. The potential for herbal formulae to affect the immune system are increasingly being found in the use of Kampo (Japanese herbal) medicinals as described in an excellent review by Borchers and colleagues. [94]. For example, Shosaiko-to is a seven-herb mixture that has been shown to protect patients from viral hepatitis via modulation of several cytokines such as IFN-α/β, G-CSF, and TNF-α. Animal studies have been conducted to evaluate the immunomodulatory effects of the various herbs in Shosaiko-to. Interestingly, four of its individual constituents induced several-fold higher levels of TNF-α when

compared to the complete herbal mixture. These results led the authors to conclude that the remaining botanicals were effectively downregulating TNF-α production. Thus, the potential exists for multiple, complex interactions to occur between individual herbal ingredients in medicinal mixtures that ultimately may produce enhanced therapeutic activity, or alternatively, unwanted adverse effects.

Quality Control Issues

Finally, with regard to the increasing consumption of nutraceuticals in this country, a critical problem in this industry involves quality control of the products being sold on the market. Labeling of herbal products may not accurately reflect their contents, and adverse interactions attributed to specific herbs or mixture of herbs may actually be due to misidentified plants, or adulteration, or contamination with pharmaceutics or heavy metals. The addition of pharmaceutical drugs to "herbal" products is a serious problem with Asian patent medicines. In addition, heavy metal contamination, especially with lead or mercury, is not uncommon in Asian herbal products. Although the federal regulations (or lack thereof) in the United States currently do not require standardization and monitoring of nutraceuticals, it would significantly provide added protection for the consumer. Improved quality control is also necessary to limit the present problem of wide interproduct and intraproduct (lot-to-lot) variation in the composition of bioactive constituents.

CONCLUSIONS

The rise in popularity of nutraceuticals and functional foods in the last decade has provided the consumer with expanded options for self-medication. The majority of these natural products are generally considered to be safe and effective by the general public. However, as with pharmaceutical drugs, there exists the potential for adverse effects to develop if not used properly, or if quality control of the product is poor or lacking. Based on the lack of current regulations governing the dietary supplement industry, and the relative paucity of pre-clinical and clinical research on the efficacy or toxicity of the majority of natural products on the market today, ingestion of these products by the consumer may be risky. More scientifically based studies evaluating safety and efficacy issues on the use of nutraceuticals and functional foods are needed. Additionally, studies directed at defining nutraceutical-drug and nutraceutical-nutraceutical interactions would greatly serve public safety.

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Part III

Environmental Agents

12 Lead Immunotoxicity

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INTRODUCTION

Currently, the Centers for Disease Control and Prevention defines an elevated blood lead (Pb) level, or threshold for intervention level, to be 10 μg/dL or greater in a child ≤ 6 years old. Blood Pb levels (BLL) have declined steadily since the late 1970s, but exposure to Pb remains a significant problem at the local level where it is more common among low-income children, urban children, and those living in older housing. The most recent National Health and Nutritional Examination Survey data indicate that the geometric mean BLL in American children has decreased to 2 μg/dL [1]. However, despite the continued lowering of BLLs nationally, complacency about adverse health effects of Pb is not warranted. According to recent CDC statistics, ~ 11.5% of children ≤6 years old have BLLs in excess of 10 μg/dL. The social and economic factors contributing to Pb exposure are well known and are reflected by a disproportionate percentage of African-American children (21.6%) versus non-Hispanic White children (8.9%) afflicted by Pb poisoning. The main source of Pb exposure for children is deteriorating paint and paint dust found in antiquated housing. It is estimated that 42 million homes, most of them in inner city neighborhoods, contain Pb paint somewhere in the dwelling, and 1.8 million children live in homes where there is deteriorating paint [2]. Pb toxicity is an international public health issue; it affects children worldwide [3–7], and it has recently been realized that refugee children resettling within the United States are a particularly vulnerable [6].

Pb toxicity is more than just a children's health issue; it remains an important occupational issue in many countries including the United States, and there are numerous citations in the literature where the immunological status of workers exposed to Pb on the job has been found to be abnormal [8–13]. Probably the most important

health issue concerning Pb is that the legacy of past Pb usage remains with us—either because anthropogenic activities have redistributed Pb in the environment such that we're continually exposed to it, or more importantly because our past exposure to Pb has resulted in a significant body burden. Legislation lowering Pb usage (e.g., in gasoline) has proven to be instrumental in reducing exposure. However, much of the Pb that most of us were exposed to during our formative years is now stored in our bones [14]. Significant quantities of Pb are released from the maternal skeleton during pregnancy and lactation [15], and Pb is released from bone during menopause and aging [16–18]. Thus, endogenous as well as exogenous sources of Pb may contribute to its adverse health affects. Data also suggest that there are effects of Pb on cognitive function at levels lower than previously thought to be of concern [19], meaning that there may not be a threshold level of Pb toxicity on many organ systems. The immune system may be an important target of subclinical lead toxicity.

As more has been learned about Pb, it has become clear that Pb toxicity is mediated by perturbations of normal physiological functions at the cellular and molecular levels, and emphasis has shifted toward understanding these mechanisms in greater detail, at lower exposure levels, and in organ systems that historically have not been considered part of the clinical features of Pb poisoning. The immune system is one such organ system where a clearer understanding of the effects of Pb on it is needed. Metals, including Pb, are known to modulate the activities of immunocompetent cells by a variety of mechanisms. Depending on the metal, its concentration and biological availability, and a host of other factors, the outcome of this modulation may be either immunoenhancement or immunosuppression. The general premise and object of concern is that metals, by modulating immunoregulatory activities, may disrupt immune homeostasis leading to either immunodeficient or autoimmune states.

Many studies have shown that Pb exposure can lead to immunodeficiencies [reviewed in 20]. The most compelling studies show an increase in susceptibility to a variety of infectious agents in Pb intoxicated animals suggesting that Pb compromises protective immunity. It is fairly well-established that Pb intoxication lowers host resistance to pathogens and exacerbates morbidity and mortality associated with such infections. More recently, the spectrum of immunopathologies affected by Pb has widened; reports have suggested that Pb exposure contributes to the heightened incidence of atopic diseases [21, 22].

Pb exposure at relevant levels does not produce overt cytotoxicity of immunocytes. Instead, immune-associated health effects result from dysregulation and shifts in functional capacity rather than profound lymphoid deficiencies. As a result, the most sensitive biomarkers and indicators of Pb-induced immunotoxicity are those associated with specific functional capacities as opposed to more classical measures of cell enumeration and/or lymphoid organ pathology.

EFFECTS OF Pb ON HUMORAL IMMUNITY

Antibody levels can remain normal or even increase in Pb-exposed animals and humans [reviewed in 20]; however, the nature and spectrum of the antibodies produced is the

more significant concern. Because Pb can alter T lymphocyte-driven B cell maturation, it may skew class switching in Pb-exposed animals and humans. With sufficiently prolonged exposure to Pb, antibody production may be depressed overall. An additional concern is the potential for Pb to enhance autoantibody production [23]. In retrospect, the apparent discrepancy may have been caused by differences in both the concentrations of Pb administered as well as the duration of exposure. Mudzinski and colleagues [24] studied Pb exposure in several strains of mice and reported that Pb acetate administered in the drinking water (10 mM for 8 weeks) elevated the response in one strain (Balb/c mice) but failed to alter the humoral response to sheep erythrocytes in all other strains. McCabe and Lawrence [25] demonstrated that Pb caused an elevation in B cell expression of MHC class II molecules thereby influencing B cell differentiation. Pb seemed to increase class II molecules by influencing a translational and/or a posttranslational mechanism [26].

Epidemiological and occupational studies have reported differences in circulating levels of immunoglobulins associated with Pb burden. However, the studies lack consistency beyond the effect of Pb on IgE. In studies reporting depressed antibody levels, Sun and colleagues [27] reported that IgM and IgG were lower but IgE was higher among females within the highest dose of Pb examined while Basaran and Undeger [28] and Undeger and coworkers [10] found that IgM, IgG, and some complement proteins were reduced among battery workers with high Pb exposure. In contrast, Sarasua and colleagues [29] reported an elevation in IgA, IgG, and IgM associated with environmental Pb exposure. Pinkerton and colleagues [30] reported a significant Pb-associated decline in serum IgG and an elevation in B cell percentage. High dose and/or long duration of Pb exposure may reduce serum Ig levels; however, the more critical issue is the distribution of Ig classes and subclasses following early life stage and/or chronic exposure to low levels of Pb.

One of the major hallmarks of Pb-induced immunotoxicity is an increase in IgE levels. This can arise both with antigen-specific responses or when measured as total serum IgE. In humans, Karmous and coworkers [31] examined second-grade children living near Pb-emitting sources and reported a positive association of BLLs with serum IgE concentration. Among children in Taiwan, Sun and colleagues [27] also found a positive association of BLL and serum IgE level. For children in Missouri from 9 months to 6 years of age, Lutz and colleagues [21] found a correlation of BLLs and serum IgE levels. In a study of adults, Heo and coworkers [32] reported that Pb-exposed battery workers with BLLs greater than 30 μg/dL had significantly higher serum IgE levels than those with a BLL less than 30 μg/dL.

Animal data support this relationship between BLL and IgE level and suggest that even low-level exposure to Pb early in life may produce elevated IgE production in the juvenile. For example, gestational exposure of rats to 100 ppm Pb acetate in the drinking water was shown to elevate IgE in the adult offspring [33]. Snyder and colleagues [34] found that gestational exposure of mice to Pb acetate produced neonatal BLLs not significantly different than those of control animals (5.0 μg/dL) but with elevated IgE production in the gestationally-exposed mouse. In many of these cases, Pb exposures producing elevated IgE were also associated with increases in IL-4 production by T cells [33, 34].

Because a relationship has been established between relative Th2 cytokine levels and serum IgE levels and the risk of allergic airway inflammation [35], the disease implications of Pb-induced increases in IgE production are potentially significant. In fact, Pb-induced increases in IgE production may help explain, in part, the asthma epidemic that has occurred in the last several decades [36]. Joseph and colleagues have recently published findings implicating heightened BLL with an observed trend toward elevated risk of developing asthma in Caucasian children [37]. Elevated IgE levels may persist long after BLLs have returned to normal, meaning that Pb exposure in early life might alter risk of later life allergic disease with no residual evidence of the Pb exposure. Therefore, future human studies may need to consider the entire history of Pb exposure within an individual rather than simply the BLL at the time of immunological assessment.

EFFECTS OF Pb ON T CELL-MEDIATED IMMUNITY

Cells of the T cell lineage appear to be more sensitive to the immunomodulatory effects of Pb compared to other lymphoid populations. In addition, there are considerable differences in sensitivity across various T cell subpopulations [38–41]. This differential sensitivity has become another major hallmark of Pb-induced immunotoxicity, although most data implicate T cells as indirect targets of Pb immunotoxicity. Both *in vivo* and *in vitro* observations of T-dependent immune responses in the presence of Pb suggest that T helper function and Th-dependent cytokines are skewed preferentially toward Th2 reactivities. Smith and Lawrence [42] found that Pb inhibited antigen presentation and stimulated a T cell clone of the Th1 phenotype. McCabe and Lawrence [38] were the first to show that Pb inhibited Th1 stimulation while it promoted presentation to Th2 clones. Heo and colleagues [39–41] provided both *in vitro* and *in vivo* results supporting this immunomodulation by Pb.

Using naïve splenic CD4+ T cells derived from D11.10 ovalbumin-transgenic mice, Heo and colleagues [41] found the T cells significantly skewed toward the Th2 helper phenotype and away from the Th1 phenotype. The effects of Pb were largely overcome if IL-4 was inhibited by the addition of anti-IL-4 to the cultures or if the Th1- promoting cytokine IL-12 was added exogenously to the cultures. This study provides evidence that Pb can directly promote Th2 development among precursor Th(0) cells and impair development of Th1 cells. In addition to the Th biasing of immune responses, Pb has the capacity to bias usage of certain murine T cell receptor Vβ genes [41]. This is of concern as it suggests that exposure to Pb may alter the T cell receptor repertoire thereby skewing the T cell response toward self reactivity and risk of autoimmune diseases.

Suppression of the DTH response is another hallmark of Pb-induced immunotoxicity first demonstrated by Muller and colleagues [43]. In a subsequent study, developing Sprague-Dawley rats were exposed to Pb acetate in the drinking water (lowest dose at 25ppm) first via the dams during gestation and through weaning and then by direct exposure of the offspring until six weeks of age [44]. Rats administered the lowest evaluated dose of Pb (producing a BLL of 29.3 μg/dL) had a significantly reduced delayed type hypersensitivity (DTH) response to the purified protein derivative (PPD) of tuberculin.

Lasch-Loquire and coworkers [45] evaluated the contact hypersensitivity reaction against picryl chloride (0.5 mg/Kg Pb s.c.) in mice with Pb administered just before or during the sensitization period. Pb exposure suppressed the DTH response regardless of the window (before or during sensitization) in which it had been administered.

More recently, Miller and colleagues [33] found that female rats gestationally-exposed to 250 ppm of Pb acetate in drinking water had a persistently reduced DTH reaction against keyhole limpet hemocyanin (KLH) protein. Similar findings were obtained with both F344 and CD strains of rats [46–49]. In F344 rats a BLL of 6.75 μg/dL (measured at 4 weeks of age) after gestational exposure to Pb acetate was associated with depressed DTH against KLH in the 13-week-old adult female offspring [49]. McCabe and colleagues [50] drew attention to the relationship between Pb-induced suppression of DTH and the prior observations of Pb-induced Th skewing. These authors gave Balb/c mice various doses of Pb acetate in the drinking water for 3 weeks prior to measuring the DTH against sheep erythrocytes. In this study, a BLL of 87 μg/dL significantly correlated with an impaired DTH response. Pb acetate (200 μg) administered *in ovo* to chicken embryos at 9 days of incubation failed to depress juvenile DTH against BSA, but when the same dose of Pb was administered three days later producing the same BLL juvenile DTH was severely reduced [51]. Using the latter model, embryonic administration of exogenous thymulin was found to partially restore juvenile DTH function following embryonic exposure to Pb [52]. Regarding developmental sensitivity of the DTH response to Pb-induced immunosuppression, parallel findings were obtained in the rat [48] and the chicken [51]. Administration of 500ppm Pb acetate during gestational days 3–9 or 15–21 produced no DTH effect compared with DTH suppression in the corresponding adult offspring. It should be noted that in several studies, Pb-induced suppression of the DTH response was associated with reduced capacity to produce the Th1 cytokine, IFN-γ [51, 52].

The effects of Pb on the mixed lymphocyte response (MLR) have been examined in prior studies. McCabe and colleagues [53] and Farrer and colleagues [54] demonstrated that Pb *in vitro* at very low concentrations (0.1 μM = ~2 μg/dL) significantly enhanced the proliferation and expansion of murine alloreactive CD4+ T lymphocytes in the MLR. The expanded T cell population was found to have a high density of CD4 molecules on the cell surface making them phenotypically similar to memory/effector T lymphocytes. In a study using Lewis strain rats, Razani-Boroujerdi and coworkers [55] also found evidence for Pb-induced stimulation of the *in vitro* MLR.

Table 12.1 illustrates studies reporting shifts in T lymphocyte cytokine production induced by Pb. Most studies found that Pb exposure at low to moderate levels causes a significant shift in the production of Th1 versus Th2 cytokines with the bias toward the latter. Production of IL-4, IL-6 and frequently IL-10 is elevated while production of IFN is decreased and production of IL-12 is below the levels needed for effective host resistance to *Listeria.* These results occur even at low levels of exposure and the effects are persistent. The only exceptions to Pb-induced biasing in favor of Th2 are found in the reports by Goebel and colleagues [56] and Mishra and colleagues [57]. In the former case, an autoimmune prone strain of mice was employed; these authors found different results upon using a normal strain of mice [58].

One ramification of Pb-induced biasing toward Th2 cells is the impact of elevated

TABLE 12.1
Examples of Lead-Induced Shifts in Th1 vs. Th2 Cytokines

Species	Strain/Gender	Age	In vivo/ Ex vivo	Cytokine Alterations	Lead dose/ Concentration	Duration of Exposure	References
Mouse	Balb/c ByJ female or male	Adult	No	↓IFN-γ ↑IL-4	10 μM – 50 μM	2 days	41
Mouse	Balb/c and DO11.10 ova-tg mice	Adult	Yes	↓IFN-γ ↓IFN-γ/IL-4 ratio	50 μg each injection (s.c.) 3 per week	2 weeks	39
Mouse	Balb/c ByJ female	Adult	Yes	↓IFN-γ ↑IL-6	2 mM	3 weeks	39
Rat	F344 females	Embryo-fetal	Yes	↓IFN-γ ↑IL-10	250 ppm to Dams	2 weeks before and 3rd week of gestation	103
Rat	CD females	Fetal	Yes	↑IL-10	500 ppm in water to Dams	6 days via gestation of Dam	48
Rat	F344 females	Embryo-fetal	Yes	↑IL-4 ↓IFN-γ splenic lymphocytes	250 ppm in water to Dams	2 weeks prior and 3rd week of gestation for Dam	49
Chicken	Cornell K females	Embryonic	Yes	↓IFN-γ	50 μg	Single injection	51
Chicken	Cornell K females	Embryonic	Yes	↓IFN-γ stimulated thymocytes	400 μg	Single injection E12	52

IL-4 on IgE. Excessive IL-4 correlates with elevated IgE and risk of atopy. Kishikawa and coworkers [59] demonstrated that administration of IL-12, a potent Th1-promoting cytokine, to Pb-exposed mice restored the balance of Th1 (IFN-γ) versus Th2 cytokines (IL-6), which reduce corticosterone levels and enhanced host resistance in *Listeria*-infected mice. This observation links the role of Th1/Th2 balance in host resistance against disease and the potential significance of Pb in the disruption of that balance.

EFFECTS OF Pb ON MYELOMONOCYTIC CELL FUNCTION

Many reports have indicated that macrophages are targeted by Pb and that many of the immunomodulatory effects of Pb can be attributed to alteration of macrophage functions [reviewed in 20]. Most recently, Pace and colleagues [60] demonstrated the importance of the effects of Pb on macrophage-influenced tissue homeostasis. Neonatal exposure of mice to Pb acetate via the drinking water produced a significant reduction in the testicular macrophage population, which correlated with increased estradiol levels in the testis. The authors hypothesized that this Pb-induced alteration of testicular macrophages may be linked to male infertility.

One of the most sensitive alterations induced by Pb is the capacity to impair NO production by macrophages. Several groups have shown that exposure to Pb significantly reduces NO production [51, 61, 62], and this impaired NO production may be the basis of certain aspects of Pb immunotoxicity including reduced host resistance to *Listeria* [23] and increased T cell proliferation in response to allo-antigen [Farrer and McCabe, unpublished data].

Early research showed that Pb exposure could increase sensitivity to bacterially-derived endotoxins [63] as well as increase production of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) by macrophages [64–67]. Studies in several species indicate that Pb boosts production of TNF-α both immediately following adult exposure and in later life following gestational exposure. Flohe and coworkers [67] reported that Pb-induced elevation in TNF-α production is sensitive to both protein kinase C signaling as well as protein production. Not only can the production of TNF-α be elevated following exposure to Pb, but also the expression of the receptor for TNF-α (TNF-R) is elevated [68]. Therefore, the combined effect of elevated cytokine production by macrophages as well as increased receptor expression would be expected to contribute to problematic inflammatory responses.

Increased production of IL-6 following exposure to Pb has been demonstrated by several investigators [59, 67–70]. Since IL-6 is a proinflammatory cytokine, increased production due to Pb exposure may influence many different tissues. Dylatov and Lawrence [68] provided evidence that Pb, IL-6 and LPS can combine to exert a significant impact on the permeability of the blood brain barrier as well as the properties of brain neurons and endothelial cells. Another proinflammatory cytokine, IL-1β, has been reported to increase following exposure to Pb [71]. It seems probable that enhanced production of both IL-1β and IL-6 would increase the potential for local tissue inflammation.

Results from several studies suggest that Pb exposure of macrophages can increase the release of superoxide anion and/or hydrogen peroxide, at least shortly after exposure.

Pineada-Zavaleta and colleagues [62] reported that production of superoxide anion by monocytes activated with IFN-γ plus LPS was directly correlated with BLL in Pb-exposed children in Mexico. Similarly, Zelikoff and colleagues [65] demonstrated that pulmonary macrophages of rabbits exposed to Pb via inhalation produced elevated levels of both H_2O_2 and superoxide anion upon subsequent stimulation. Baykov and colleagues [72] exposed Balb/c mice to dietary Pb and found that peritoneal macrophages had an increased spontaneous release of H_2O_2. The nature of populations examined and/or the timeframe of assessment relative to exposure may be important factors in the effect of Pb on ROI production. The effect of lead on production of cyclooxygenase metabolites such as prostaglandin E_2 (PGE_2) appears to be important [67, 73, 74]. Bone marrow-derived macrophages (BMDM) can form colonies in response to certain growth factors (e.g., colony stimulating factor-1; CSF-1), and this property is related to the growth and differentiation of subsequent macrophage populations. Kowelenko and colleagues [75] found that lead-exposed CBA/J female mice had reduced macrophage colony formation in response to CSF-1 that was exacerbated by *Listeria* infection. Reduced CSF-1 responsiveness of macrophages was also obtained following *in vitro* exposure to lead [76]. These results suggest that Pb can impair the generation of macrophage populations as well as modulate the functional spectrum of fully-matured macrophages.

Antigen presentation by macrophages can be influenced by exposure to Pb. Kowelenko and colleagues [77] showed that mouse macrophages exposed to Pb (both *in vivo* and *in vitro*) can induce an increased proliferative response of T lymphocytes in co-culture but that antigen specific stimulation of primed T cells is significantly reduced. Pb-suppressed antigen presentation capabilities of mouse macrophages were also reported by both Smith and Lawrence [42]. More recently, Farrer and colleagues have demonstrated that antigen processing, as opposed to antigen presentation, is more sensitive to low levels of Pb [54].

Some studies have described significant effects of Pb on phagocytosis by macrophages, particularly phagocytosis mediated through the FC receptor. Because cell adherence to surfaces may be influenced by Pb [78], impairment of phagocytosis may also involve some deficiency with macrophages anchoring to substrates. Kowelenko and coworkers [77] studied the effect of Pb acetate at 10mM in the drinking water of CBA/J mice and reported no effect on phagocytosis of *Listeria monocytogenes* targets, but an overall decreased resistance to *Listeria.* Exposure of peritoneal and splenic macrophages to 100 μM Pb *in vitro* also produced no significant effect on phagocytic activity. Trejo and colleagues [63] reported that a single i.v. injection of Pb (5mg/rat) into male Sprague Dawley strain rats inhibited the phagocytic capacity of Kupffer cells.

For neutrophils, several groups have reported alterations in chemotactic activity following exposure to Pb [79, 80]. As was the case for macrophages, human neutrophils exposed to Pb were suggested to have increased release of ROIs [81]. Therefore, neutrophils may contribute to Pb-induced tissue inflammation and damage via increased ROI release.

EFFECT OF Pb ON HOST RESISTANCE TO DISEASE

Host resistance to disease has been used as an effective measure of the impact of environmental toxicants on immune function. To date, for virtually every agent examined,

there has been either no effect or an increased susceptibility to disease resulting from exposure to Pb [reviewed in 20]. Pb increases the susceptibility to viral infections such as encephalomyocarditis virus [reviewed in 20], Langat virus [82], and Semliki Forrest virus [83]. In chickens administered Pb acetate orally (20 and 40 mg/100g body weight) for 56 days, antibody production against Newcastle's virus vaccine was reduced while mortality against viral challenge was increased [85]. Human studies appear to reflect the results predicted from the animal studies. Pb exposure increased the incidence of influenza cases among occupationally-exposed workers [84]. It seems likely that the reduced Th1 capacity (including effective CTL generation) combined with increased TNF-α, ROI and PGE_2 production by responding macrophages would contribute to increased tissue pathology but reduced viral clearance for many infections.

The majority of Pb-associated host resistance studies have been conducted on bacterial diseases including *Salmonella typhimurium*, *Escherichia coli,* and *Staphylococcus epidermidis* [reviewed in 20]. Host resistance to *Listeria* infection has been well characterized and is an important model in Pb exposure research. This bacterial infection requires effective antigen presentation (probably involving toll-like receptor 2 involvement), a robust response by activated macrophages leading to IL-12, IFN-γ and NO production, in combination with a robust Th1 cell driven response leading to killing of phagocytized organisms. In the case of Pb-induced immunotoxicity, everything works against this type of response. Macrophages have severely suppressed NO production, overproduce TNF-α, ROIs and PGE_2 and contribute to skewing of the response toward Th2. Excessive production of IL-6 and other pro-inflammatory cytokines are part of the "sickness behavior" outcome that involves both the immune and central nervous systems [68, 71, 86]. Pb-induced impairment in host resistance to *Listeria* was reported by Lawrence in 1981 [reviewed in 20]. More recently, Kim and Lawrence [69] demonstrated that neurological circuitry as it pertains to brain lateralized behavior could impact the effect of Pb on immune responses and host resistance to *Listeria*. Using Balb/c female mice, Kishikawa and Lawrence [58] found that exogenously-administered IL-12 (1 μg each day for three days, i.p.) enhanced production of IFN-γ as well as host resistance to *Listeria* in Pb-exposed mice. However, excess IL-6 production was not reversed by IL-12 in Pb-exposed mice, which continued to display the sickness behavior phenotype. The results with IL-12 validate the importance of the Th skewing and macrophage impairment induced by Pb on host resistance to certain diseases.

Few studies have been conducted to date evaluating the effects of Pb exposure on host resistance to parasitic diseases, although one study examined the effect of Pb on the ability of mouse macrophages to kill *Leishmania enriettii* parasites *in vitro* [87]. The authors found that 30–100 μM Pb acetate interfered with the killing ability of macrophages without producing macrophage cytotoxicity.

Tumor immunity also is impaired by exposure to Pb. In lead-exposed C57Bl/6 males challenged with Maloney Sarcoma virus, primary tumor growth was enhanced in animals that received high doses of Pb (130 and 1300 ppm for 10–12 wk) in comparison to the control group [88]. Other studies involving Pb exposure and tumors indicate that Pb can exacerbate the ability of other toxins to promote tumor formation [89] or, in turn, inhibit the ability of anti-oxidants to block tumor formation [90]. The tumor promoting activity of Pb might involve depressed Th1 and macrophage function as well as excessive ROI release into tissues.

CONTRIBUTION OF Pb EXPOSURE TO HYPERSENSITIVITY AND AUTOIMMUNITY

Pb exposure at low to moderate levels appears to alter T lymphocyte responses in such a way as to increase the risk of atopic disease and some forms of autoimmunity. Increased IgE production following exposure to Pb is among the most frequently reported immune alteration, suggesting that Pb is a possible risk factor for allergic asthma [33, 34, 40, 91, 92] as well as later life allergic disease [32].

Hudson and colleagues [93] reported that exposure to Pb can exacerbate systemic lupus erythematosus (SLE) in lupus-prone strains of mice. In contrast with the effect of mercury, these authors found that for lupus, Pb exposure would not induce this autoimmune condition in genetically-resistant mice but would increase severity of the disease in genetically-prone animals. The authors noted some gender effects within certain strains (NZM88). Using early *in ovo* exposure to Pb (10 μg/egg), Bunn and colleagues [94] found that Pb acetate-exposed male chicks could be induced to produce autoantibodies against thyroglobulin, which were not present in acetate-exposed controls. No Pb-induced alteration was observed in females that were predisposed to mount anti-thyroglobulin responses. The gender effect is intriguing in that autoimmune thyroiditis in genetically-predisposed strains is always more severe in females than in males.

The production of autoantibodies to neural proteins in battery workers [95] as well as in rats exposed to low levels of Pb via drinking water [96] has been reported. Exposure to Pb may precipitate autoimmunity by altering antigenicity and/or the capacity of the immune system to respond to certain antigens. This, in turn, may contribute to the Pb-associated neuropathy.

DIFFERENCES IN SENSITIVITY BASED ON AGE OF EXPOSURE

Since the 1990s, several studies have addressed the developmental immunotoxicity of Pb [reviewed in 97–100] and distinct patterns have emerged from exposure data using animals of different ages. First, BLLs of below 10 μg/dL at or near birth are associated with juvenile and/or adult immunotoxicity (Table 12.2). Several studies report effects in the range of 5–8 μg/dL [34, 49, 51, 94, 95]. These low levels would seem to place the immune system on par with the neurological system in terms of potential sensitivity to Pb. Second, Pb-induced immunotoxic effects can persist long after blood levels and potential body burdens of Pb are significantly reduced [33, 51]. In most studies, immunotoxic alterations were present when Pb levels in exposed animals were not distinguishable from control levels. This should provide a cautionary note regarding studies in humans. Third, Pb can induce qualitatively different immune alterations depending upon the developmental window of exposure [48, 51]. Some studies in animals have noted gender differences in the effects of Pb following exposure [46–48, 93, 94] and such results have also extended to humans in terms of Pb-induced immune and inflammatory alterations [31, 101]. These studies suggest that hormonal differences among females and males may impact some outcomes of low level Pb exposure even in early life stages. Fourth, even very low levels of Pb can predispose the immune sys-

TABLE 12.2
Differential Sensitivity to Lead-Induced Immunotoxicity Based on Age

Species	Altered Endpoint	Adult *	Neonatal*	Embryo – fetal *	References
Mouse	↑IgE	38 μg/dL	12 μg/dL	~5μg/dL	34 41
Rat	↓DTH (persistent effect assessed 13 weeks post-exposure)	>112 μg/dL (measured at birth for persistent effect)	—	34 μg/dL	33 46–48
Mouse	↓DTH	87 μg/dL	29 μg/dL	—	44 50
Rat	↑TNF - α (persistent effect assessed 13 weeks post-exposure)	>112 μg/dL (measured at birth for persistent effect)	—	8 μg/dL	33 49

*Lowest BLL Reported With Effect

tem for unanticipated postnatal responses when stressed. This general phenomenon is sometimes termed latency. As an example, a single *in ovo* exposure of embryonic day 5 chick embryos to low levels of Pb (BLL one day post hatch of 8.2 μg/dL) produced leukocyte profiles that were completely normal. However, when these animals were exposed to a respiratory virus, their pattern of leukocyte mobilization was completely aberrant from that of controls [102].

SUMMARY AND CONCLUSIONS

Studies in humans and animals are in general agreement concerning both the levels of Pb associated with immunotoxicity as well as the nature of the immunotoxicity induced by Pb. Figure 12.1 summarizes the basic immunotoxic changes induced by Pb that result in Th skewing, impaired macrophage function and increased risk of inflammation-associated tissue damage.

There are three major hallmarks of Pb-induced immunotoxicity: (1) Pb can dramatically suppress the Th1-dependent DTH response, as well as production of associated Th1 cytokines; (2) Pb can dramatically elevate production of IgE while increasing production of Th2 cytokines such as IL-4; and (3) Pb can impair the production of new macrophages and shift existing macrophage populations into a hyper-inflammatory response phenotype that promotes tissue inflammation but, ironically, is not particularly effective against bacterial pathogens.

Pb-induced skewing of Th activity (biasing responses toward Th2) across a population would lead to the expectation of a greater risk of atopic disease and some forms of autoimmunity. Additionally, resistance to some infectious diseases could be reduced where reduced Th1 and macrophage functional capacities occur.

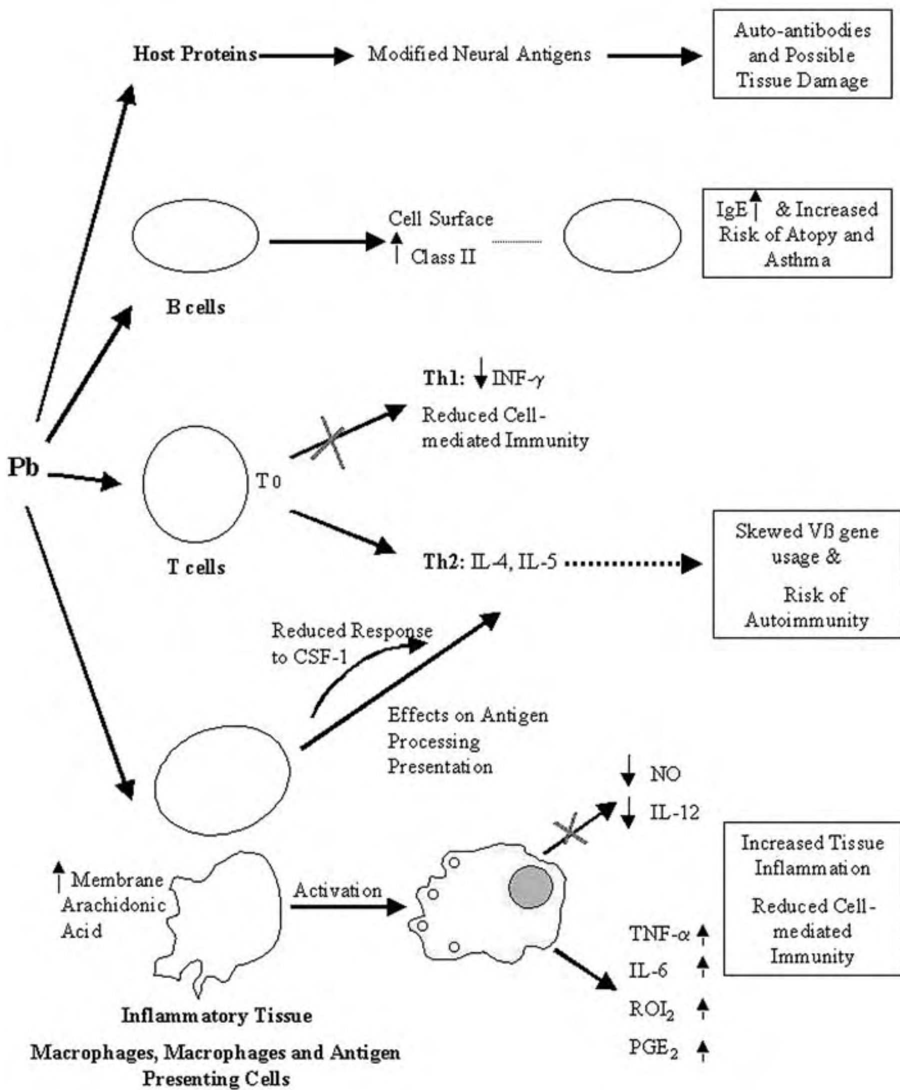

FIGURE 12.1 Key alterations of the immune system associated with exposure to Pb. Skewing of the immune response by lead can alter the risk of disease in the absence of profound loss of immunocytes.

Sensitivity of the immune system to Pb appears to differ across life stages, and studies in rodents suggest that the gestational and neonatal periods are the most sensitive. Compared to adults, the increased dose sensitivity of the embryo-fetus would appear to fall in the range of 3-12X depending upon the immune endpoint considered. Recent studies have suggested that exposure of embryos to Pb producing neonatal BLLs below 10 μg/dL can also produce later-life immunotoxicity (Table 12.2). Furthermore,

immunotoxicity persists long after any evidence of prior embryonic Pb exposure. This latter observation from several laboratories may have implications for the design of human studies.

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13 Immunotoxicology of Jet Propulsion Fuel-8

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BACKGROUND

APPLICATIONS OF JP-8

Jet Propulsion Fuel-8 (JP-8) serves as the primary fuel used by the United States Air Force and the North Atlantic Treaty Organization (NATO) at volumes exceeding 5 billion gallons yearly. During the early 1990s, JP-8 emerged as the replacement fuel for JP-4. This transition to a single versatile fuel provided logistical ease during military deployments as it fueled military aircrafts and land-based vehicles, as well as heating and lighting sources. When necessary, JP-8 also served as a dust suppressant or a carrier for insecticides, pesticides, and herbicides [1].

COMPONENTS OF JP-8

Jet fuel formulations used by the U.S. Air Force, Army, and Navy/Marines include JP-4, JP-5, JP-8, or JP-8+100, while the aviation industry utilizes Jet A or A1. Essentially all these fuels are kerosene-based, complex mixtures of hundreds of aliphatic and aromatic hydrocarbons (C6-C17) that are differentiated based on performance additives. Primary

components of JP-8 based on v/v% include dodecane (~22%), tetradecane (~17%), decane (~16%), hexadecane (~12%), trimethylbenzenes (~6%), *m*-xylene (~4%), naphthalene (~1%), toluene (<1%), benzene (<0.8%), and PAHs (<0.1%) (reviewed by Ritchie et al.[2]). JP-8 is essentially commercial Jet A with the supplementation of three additives: (1) antistatic agent, Stadis 450 (<3 mg/L); (2) a corrosion inhibitor; and (3) DCI-4A (0.85-11 mg/L), and an anti-icing additive, diethylene glycol monomethyl ether[3]. JP-8+100 is primarily used for U.S. Air Force light fighters and helicopters. Its composition is based on JP-8 with the addition of a thermal stability package that increases fuel safety by 100°F (SPEC-AID 8Q462). Additional details regarding chemical and physical properties of JP-8 and other kerosene-based fuels are available in a review published by Ritchie and colleagues [2].

Human Exposure Potential

Due to its widespread use, jet fuel is the single most common chemical exposure among military and aviation industry personnel, with greater than 2 million individuals exposed annually. Occupational exposure to JP-8 is likely to occur on a regular basis during aircraft refueling and servicing as well as during maintenance, inspection, and cleaning of fuel storage tanks [4,5]. Aerosol emissions from aircraft "cold starts" can often saturate ground crew. Spills or leaks occurring during manufacture, transport, or while in storage facilities are also a concern as contaminated soil or water may increase risk of exposure to nearby residential, wildlife, or recreational areas.

During the transition to JP-8, an increased number of reports describing adverse health effects emerged from fuel handlers, mechanics, and flight line personnel. Effects included increased incidence of fatigue, emotional dysfunction, skin irritation, headaches, postural sway imbalances, decreased attention spans, adverse effects on sensorimotor speed, and alterations in liver and respiratory function [6,7,8,9]. Recently, alterations in peripheral blood cell populations were reported in fuel system maintenance personnel. An increase in circulating neutrophils and monocytes occurred, while no changes were evident in the number of T and B cells or natural killer (NK) cells [10]. Due to the recent adoption of JP-8, there are no epidemiological data describing death or cancer among occupationally exposed civilian or military personnel [11,12].

Interim recommended exposure guidelines for JP-8 have been set at 350 mg/m^3 as an 8-hour time-weighted average or 1000 mg/m^3 for short-term 15-minute exposures [1]. However, JP-8 exists in many different phases, thereby complicating risk assessment efforts. Skin contact with raw fuel and inhalation of the aerosol phase, vapor phase, or combustion products are common routes of exposure, whereas oral ingestion by way of contaminated food or water is possible when leaks and spills from underground and above-ground fuel storage tanks occurs [11]. All phases, however, have been shown to cause some level of toxicity affecting renal, pulmonary, hepatic, neurological, endocrine, skin, and immune organ systems[1].

This chapter will provide an in-depth summary of the immunotoxicity of JP-8 as learned from human, animal, and *in vitro* studies. For additional reading regarding the toxicity to other organ systems, readers are guided to a comprehensive review recently published by the National Research Council [1].

IMMUNOSUPPRESSIVE EFFECTS

Inhalation Exposure

Although kerosene-based jet fuels have been widely used for the past 50 years, relatively modest information is available regarding potential health risks associated with daily occupational exposure. Based on early mutagenicity studies that were negative [13,14,15] and minimal toxicity reported following inhalation or oral exposures for 90 days to 1 year [16,17], JP-8 initially appeared to pose relatively little health risk in the workplace. However, mounting concerns regarding the etiology of Gulf War Illness during the early 1990s, and the onset of increasing numbers of self-reported symptoms associated with exposure to JP-8, led to expanded efforts to investigate its toxicological effects.

Beginning in 1997, studies by Harris and colleagues alerted the scientific community that the immune system was a target for JP-8 following acute inhalation exposure and that some of these effects were long lasting. Following nose-only exposure of mice to JP-8 aerosol/vapor (0-2500 mg/m^3) for 1 hour per day for 7 days, they reported dose-responsive effects such as decreased spleen and thymus weight; reduced cell numbers in lymph nodes, bone marrow, and peripheral blood [18]; and suppression of NK cell function, lymphokine-activated killer cell activity, cytotoxic T-lymphocyte generation, and T-helper cell activity [19]. Furthermore, T-cell proliferative responses were suppressed after stimulation with a T-cell mitogen, conconavalin A (Con A) with or without exogenous interleukin-2 (IL-2) [18,20] that persisted for one month following JP-8 exposure [20]. Time-course studies of effects following a single exposure to 1000 mg/m^3 JP-8 for one hour [21] indicated significant decreases in immune organ weights and numbers of viable immune cells in the spleen within two hours of exposure, and decreased bone marrow cellularity within four hours of exposure. Altogether, these studies suggested that JP-8 targets systemic immune function following acute inhalation exposure. However, alterations in body weight following exposure were not reported in some of these studies. Therefore it is not clear whether the severity of immunological effects were due solely to JP-8 or underlying overt toxicity associated with body weight changes exceeding 10%. Despite this limitation, these initial reports established that the immune system was a vulnerable target, thereby prompting further immunotoxicity studies with JP-8.

Early studies by Pfaff and colleagues [22,23] demonstrated that JP-8 lung toxicity was mediated, at least in part, by the reduction of substance P (SP) in lung lavage fluid. Consequently, mice treated with SP (1 μM or 1 nM) or an agonist of the neurokinin receptor-1 (NK1-R) during JP-8 exposure were protected against lung toxicity and immunosuppression [24,25].Conversely, treatment with an SP antagonist exaggerated lung and immunotoxicity [26]. Recently, Monteiro-Riviere and colleagues [27] investigated mechanisms of JP-8-induced pulmonary damage associated with dermal exposure. In human epidermal keratinocytes, IL-8 typically increases after JP-8 exposure; however, co-administration of substance P (SP) with JP-8 resulted in a reduction of IL-8 production, thereby decreasing toxicity[27].

To determine if JP-8 exposure influences concurrent lung infection by respiratory influenza virus, Wong and colleagues [28] challenged female C57BL/6 mice to A/Hong Kong/8/68 influenza virus (HKV) (10 μL aliquot of 2000 viral titer in the nasal passages)

following inhalation exposure to JP-8 (1023 mg/m^3, 1 h/day for 7 d). Interestingly, JP-8 exposure with HKV challenge (JP-8 + HKV) resulted in a significant increase in inflammatory cells in bronchoalveolar lavage fluid (BALF). However, pulmonary microvascular permeability, total lung compliance, and leukotriene B4 levels in BALF were not exacerbated in the JP-8 + HKV treatment group beyond that observed in HKV alone-induced responses. Although there was an increase in total BALF cell numbers, the authors report that this inflammatory cell profile did not correlate with changes in pulmonary vascular permeability and histopathology but was likely attributable to corresponding JP-8-induced increases in SP levels. This observation contrasts with previous reports demonstrating that exposure to JP-8 alone suppresses SP in BALF. However, mice were evaluated after a one week delay following chemical and/or viral challenge, thus permitting a compensatory increase in SP to occur. Overall, this study demonstrated that a synergistic effect of JP-8 exposure and challenge with influenza virus did not occur [28]. With the substantial alterations in immunological parameters formerly reported [18,20,21,29] coupled with reports of increased lung permeability [22,23,25,30,31], it is encouraging to risk assessment efforts that JP-8 did not profoundly exaggerate coexisting viral infection. However, the role of JP-8 in the onset of respiratory disease is not addressed in this study and further investigation would be required to clarify this issue.

Dermal Exposure

Local Toxicity

Dermal exposure to chemicals is one of the leading causes of job-related illness as reported by the National Institutes for Occupational Safety and Health. As jet fuel is the primary occupational exposure of military and aviation industry personnel, there is elevated concern regarding JP-8 dermal exposures in the workplace. Several anecdotal reports confirm that persons exposed to jet fuel experience itching or burning skin, skin redness or rash, skin dryness or dermatitis, skin lesions or weeping, or skin sensitization [32,33,34], yet little is known regarding possible systemic effects following dermal exposure.

Studies of jet fuel dermal exposure have identified a variety of toxicological effects [1,11,35,36]. In general, repeated exposure to JP-8 causes skin irritation as demonstrated in several studies using rats and pigs [34,35,37,38,39]. These results are consistent with early studies that identified skin irritation and hyperplasia following exposure to kerosene-based jet fuels [40,41]. Dermal exposure to JP-8 also disrupts skin barrier function and alters skin structures. Using an *in vivo* porcine skin model, fabric occluded low dose (67 μL/cm^2) exposure to JP-8 for 4 days caused increased epidermal thickness, intraepidermal and subcorneal microabscesses, and changes in enzyme histochemistry [35]. Dermal application of JP-8 (335 or 67 μl/cm^2 for 4 d) on porcine skin caused low-level inflammation in the skin, and increased the number of Langerhans cells, mitochondrial and nucleolar alterations, and disorganization of the stratum granulosum-stratum corneum interface [27]. Transepidermal water loss

(TEWL), a measure of damage to the stratum corneum, also increased after exposure to JP-8 [34,35,38,39].

A number of mechanisms have been proposed to account for JP-8 dermal irritation including the modulation of proinflammatory cytokines. Irritant dermatitis is not an immune-mediated response, but rather an immediate response by keratinocytes. Within hours following dermal application of JP-8 in F-344 male rats, acute skin irritation and elevated levels of IL-1α and inducible nitric oxide synthase (iNOS) are noted *in vivo*[34]. Keratinocytes constitutively produce IL-1α, IL-1β, and tumor necrosis factor alpha (TNF-α), which may trigger cutaneous inflammatory responses (reviewed by Corsini and Galli [42]). Furthermore, IL-1α can stimulate adjacent keratinocytes to secrete IL-1α and other proinflammatory cytokines. iNOS, which is constitutively produced by macrophages, Langerhans cells, and keratinocytes, participates in contact hypersensitivity, wound healing, irritation, and allergic contact dermatitis. *In vitro*, JP-8 exposure also increases proinflammatory cytokines such as TNF-α and IL-8 in keratinocytes[33,43]. However, levels of TNF-α did not increase following dermal exposure in rats, while both IL-6 and IL-1β did.[44] Although detection patterns of proinflammatory cytokines differ among *in vivo* and *in vitro* studies, it is apparent that their generation by keratinocytes substantially contributes to dermal irritation.

Dermal toxicity due to JP-8 may also be attributed to increased free radical formation that may be involved in the development of skin sensitization [45]. Following dermal exposure to JP-8, increasing levels of oxidative species are observed [46]. *In vitro* studies with rat lung epithelial cells demonstrated that JP-8-induced cell death is inhibited by exogenous glutathione or the thiol-containing antioxidant N-acetyl-cysteine [47]. Furthermore, mice treated with anti-oxidants (Vitamins C, or E, or beta-hydroxy toluene) prior to JP-8 dermal application exhibit a partial recovery in immune function [48]. Correspondingly, other studies identified increases protein levels of glutathione-*s*-transferase (GST) in kidney, liver, and lungs suggesting compensation against JP-8-induced cellular damage via the generation of reactive oxygen species [49,50,51].

JP-8 also selectively promotes necrosis or apoptosis in various cell types. JP-8 (>160 μg/mL JP-8 *in vitro*) induces necrosis and cell death in human primary keratinocytes and skin fibroblasts by down-regulating the prosurvival genes *Bcl-1* and $Bcl\text{-}x_L$, and increasing levels of *Bad* and *Bak* [52]. *Bad* and *Bak* promote cell death by altering mitochondrial membrane potential. JP-8-induced necrosis is independent of Poly (ADP-ribose) polymerase (PARP) which decreases ATP levels[52]. Alternatively, JP-8 causes classical apoptosis in cultured lung and hematopoietic cells (RLE, U937, Jurkat, and thymocytes) by altering cytochrome *c* release in the mitochondrial pathway[53]. Recently, macroarray analysis confirmed a number of alterations in stress and apoptosis-related genes in Jurkat cells following exposure to JP-8.[54] It is possible that activation of these genes leading to cell death may account for the loss in immune cells and alterations in immune function previously reported following JP-8 exposure.

Based on observations in JP-8 inhalation studies, the protective role of SP in dermal toxicity was recently examined. As mentioned earlier, inhalation exposure to JP-8 and SP resulted in protection from of lung toxicity and restoration of immune function [24,25,26]. Similarily, co-administration of substance P (SP) with JP-8 also decreased toxicity in human epidermal keratinocytes[27]. This suggests that a common

mechanism of JP-8-induced toxicity may bridge these organ systems. Consideration of the regulation of IL-8 production or T-cell autocrine effects following SP exposure may be provide additional understanding to mechanisms of toxicity.

There is emerging concern regarding long-term dermal exposure to JP-8 and the development of skin cancer. In early studies, Jet A (administered 3 times per week for 105 weeks) caused increased development of fibrosarcoma tumors and squamous cell carcinoma [55]. Carcinogenicity studies performed by Freeman and colleagues [56] indicated that Jet A caused skin irritation and tumors in 44% of the mice following 2 years of skin painting. Furthermore, chronic dermal exposure to longer-chain aliphatics (C14-C25) typically leads to skin tumors[57, reviewed in 2]. Jet fuels including JP-8 have not been considered mutagenic [13,14,15,57]. Recently however, studies using rodent micronucleus assay identified that dermal applications of Jet A or JP-8 (240 mg/mouse) induced slight but significant increases in micronuclei in peripheral blood at 72 hours, while Jet A only increased micronuclei in bone marrow at 72 hours [58]. Moreover, both JP-8+100 and JP-8 are capable of DNA damage in human peripheral cells [59].

Systemic Toxicity

Dermal exposure to JP-8 can affect systemic immunity and may be selective for T helper-1 driven responses affecting delayed-type hypersensitivity (DTH) [60] and possibly immunity to pathogens. In either multi-day (50 µL JP-8 daily for 4 or 5 d) or single (300 µL JP-8) exposure studies with female C3H/HeNCr mice, JP-8 dose-responsively and consistently suppressed induction of hypersensitivity, regardless of whether the contact allergen was applied locally at the site of exposure or at a distant-untreated site [36]. Additionally, JP-8 is classified as weak skin sensitizer based on guinea pig studies and the murine local lymph node assay, which are used to measure the induction phase of contact sensitization [61,62]. DTH is also suppressed in a dose-dependent manner during the elicitation phase when mice are challenged with *Candida albicans* 10 days following immunization and during the memory response when mice are challenged 30 days after immunization [63]. Furthermore, proliferative responses are decreased when splenic T-cells from JP-8 treated mice (cumulative dermal applications of 250-300 µL) are stimulated with anti-CD3 [36,60]. Based on body surface area (BSA), 300 µL JP-8 dermal exposure for a mouse (BSA ~ 0.007 m^2) is comparable to a 100 mL exposure in a 200 lb human (BSA ~ 2.17 m^2) [36]. Ramos and colleagues [63] proposed such impairment in immunological memory could lead to decreased vaccine efficacy, thereby compromising resistance to infectious diseases in military personnel.

JP-8-induced systemic immunosuppression is explained in part by modulation of cytokine and inflammatory pathways. Within 48 hours of a single dermal application of JP-8 (300 µL), serum IL-10 levels increase significantly to nearly 3000 pg/mL [36]. As IL-10 is known to suppress DTH responses [64,65], it is likely that modulation of this cytokine contributes to JP-8's immunotoxicity. Furthermore, splenic T-cell proliferative responses are significantly decreased in JP-8 exposed mice, yet this effect is reversed following neutralization of IL-10, administration of IL-12, or inhibition of prostaglandin E2 (PGE2) production [60]. Additional studies demonstrated suppression

of cell-mediated hypersensitivity is completely reversed by administration of SC 236 (a selective cyclooxygenase-2 inhibitor) prior to JP-8 exposure, indicating that inhibition of prostaglandin E2 production is required for JP-8-induced immunosuppression [63]. Only partial reversal of immunosuppression is observed when SC 236 is administered after JP-8 exposure. Additionally, JP-8- or Jet A-induced immune suppression is blocked by pretreatment with platelet activating factor (PAF) receptor antagonists both *in vivo* and *in vitro*. As PAF upregulates cyclooxygenase-2 production and PGE2 synthesis by keratinocytes, interference with these pathways reduced the immunological deficits due to jet fuel [48].

It is evident that JP-8 suppresses cell-mediated immune responses; however, there are conflicting data on its effects on humoral immunity. For example, dermal exposure to JP-8 (300 μL/d) for 7 days did not affect circulating levels of IgM and IgG antibody to keyhole limpet hemocyanin (KLH) [60]. In contrast, 7 days of exposure to JP-8 (50 μL/d) decreased the number of spleen cells secreting IgM antibody in response to immunization with sheep red blood cells (sRBC) as measured by plaque formation [66]. The plaque-forming cell (PFC) assay has long been considered an important test for evaluating systemic humoral immunity and predictive for the identification of immunotoxicants [67,68]. As the experimental exposure designs were similar, the disparity is likely due to measurement variables with these assays. Additional studies are warranted to understand the effect of JP-8 on B-cell antibody responses as components of JP-8, such as PAHs, are known to cause significant deficits in humoral immunity [69,70].

Oral Exposure

Research studies investigating exposure to JP-8 via oral administration offers an alternative examination of the systemic effects of JP-8 on immune function. Admittedly, this does not ideally mimic occupational exposures, but it does eliminate technical limitations associated with inhalation and dermal penetration of JP-8. It has been suggested that the only route available that can assess the whole mixture (JP-8 in its entirety), without fractionation due to volatilization of components, is the oral route [1]; as select components of JP-8 may have increased permeability during dermal exposure, other specific components may be enriched following inhalation exposures[71].

Oral JP-8 exposure in B6C3F1 female mice ranging from 500–2500 mg/kg for 7 or 14 days primarily results in humoral deficits, while cell-mediated function and host challenge to bacteria or tumor remain intact [71,72,73]. Humoral immunity proved to be the most sensitive endpoint in these studies, as dose-responsive suppression was consistently observed in the IgM PFC response beginning at treatment levels of 500 mg/kg/d JP-8. Oral exposure to JP-8 (500 and 1000 mg/kg/day) did not affect the total number of B-cells (B220+) in the murine spleen [72] suggesting that the decrease in anti-sRBC plaque forming cells is not simply attributable to a decrease in total splenic B-cells. The only other noteworthy change was in thymus mass and cellularity which did not occur until exposure levels reached 1500 and 2000 mg/kg/d, respectively [71]. Conversely, exposures as much as 2000 mg/kg/d JP-8 did not alter natural killer cell (NK) activity or T- and B-cell proliferative responses, while exposures as much as 1000 mg/kg/d JP-8

did not alter delayed-type hypersensitivity, and host resistance to bacterial challenge with *Listeria monocytogenes* or tumor challenge with B16F10 tumors[71,72].

Disparate effects on T-cell proliferative responses have been reported following exposure to JP-8. Significant suppression of T-cell proliferation is reported following either inhalation or dermal exposure to JP-8 [18,20,36], while the response is unaffected following either the oral or dermal exposure routes in other studies [66,71,72]. These differences may be explained by variations in exposure routes and in assay methodology, as agents used to evaluate T-lymphocyte activation and proliferation were diverse and included Con A plus IL-2 [18,20], anti-CD3 [36], or Con A only [66,71,72].

The toxicity of a compound administered orally may be influenced by hepatic metabolism due to the "first pass" effect in the liver. Conceivably, this could account for a dissimilar profile of immunotoxicity after oral exposure. Increased liver mass observed after exposure to JP-8 led to investigations of cytochrome P450 (CYP450) induction. JP-8 did not induce CYP1A1 or promote downregulation of the aryl hydrocarbon receptor (AhR), suggesting that JP-8 may exert its toxicity via an AhR-independent mechanism [73]. However, it has been shown that hepatic CYP2B1, CYP2E1 and glutathione-*s*-transferase pi (GSTπ) are upregulated in response to oral exposure to JP-8 (2000 mg/kg/d for 1, 3, or 7d) [51]. Elevation of GST is corroborated in whole kidney and lung tissue by high-resolution two-dimensional electrophoresis studies following inhalation exposure to JP-8 [49,50]. GSTπ plays an important role in PAH-induced skin tumorigenesis and disruptions in this single enzyme can profoundly increase susceptibility to carcinogens [74,75]. Mice dermally exposed to gasoline demonstrate alterations in GST expression in hepatic and extrahepatic tissues [76]. CYP2E is shown to play a role in the metabolism of diethyl ether and other oxygenated components of gasoline [77]. Additional studies with JP-8 in this area may expand our understanding of systemic immunotoxicity attributed to the induction of metabolizing enzymes by the parent JP-8 mixture and its various metabolites.

Developmental Immunotoxicological Effects

Given the widespread use of JP-8 in the military and aviation industry, concern about occupational exposure of women of child-bearing age has emerged as this workforce continues to expand. Furthermore, accidental spills associated with pipelines or storage facilities can contaminate soil or water, posing unpredictable health risks to nearby residential areas. Taken together, these concerns provide adequate basis to investigate the developmental toxicity of JP-8.

The first studies to address developmental and reproductive toxicity with JP-8 were generally unremarkable. Cooper and Mattie [78] determined a no adverse effect level (NOAEL) of 500 mg/kg/d for dams and 1000 mg/kg/d for the offspring, based on significant changes in body weight, following oral administration of neat JP-8 over gestation days 6 to 15. No significant or dose-related changes occurred in a battery of tests for fetal malformations or reproductive parameters. Moreover, Schreiner and colleagues [79] evaluated the primary component of JP-8, hydrodesulfurized kerosene (HDS kerosene). In these studies, HDS kerosene did not cause apparent maternal, re-

productive, or developmental toxicity in rats following exposure to 494 mg/kg/d. This NOAEL is similar to that reported by Cooper and Mattie [78]; however, the route of administration differed in that HDS kerosene was applied dermally using a vehicle of mineral oil to reduce skin irritation.

More recently, the scope of developmental studies was expanded to include evaluation of immunotoxicity. Immunological parameters, host resistance, and thyroid hormones were evaluated in F1 mice exposed *in utero* to JP-8 [80]. C57BL/6 dams (mated with C3H/HeJ males) were gavaged daily on gestation days 6 to 15 with JP-8 (0, 1000, or 2000 mg/kg/d). At weaning (21d), pups had deficits in lymphocyte proliferative responses and alterations in thymic CD4-/CD8+ cells; however, these effects were no longer apparent in 8-week offspring. Natural killer cell activity, bone marrow cellularity and lymphocyte proliferative responses, complete blood counts, peritoneal and splenic cellularity, liver, kidney, and thymus weights, macrophage phagocytosis and nitric oxide production, splenic CD4/CD8 lymphocyte subpopulations, total T_3 serum levels, and host resistance to bacterial challenge (*Listeria monocytogenes*) were likewise within normal limits in adult offspring. In contrast, *in utero* exposure to JP-8 caused long lasting effects in humoral immunity, serum T_4 levels, and resistance to B16F10 tumor cell challenge. The latter effect is noteworthy given the central role that NK cells play in resistance to this tumor model. The IgM PFC was suppressed in adult offspring by 46% and 81% in the 1000 and 2000 mg/kg treatment groups, respectively. This is consistent with adult studies following oral or dermal exposure to JP-8 [66,71,72,73]. Furthermore, adult exposure studies demonstrated no increased susceptibility to tumor challenge [72], suggesting that fetal development is more vulnerable to the effects of JP-8.

CONCLUSIONS

During the early 1990s, JP-8 emerged as the universal fuel for aircraft, land-based vehicles, and heaters/air conditioning generators used during military deployments. This was concurrent with increased occupational exposure reports describing adverse health effects associated with the use of JP-8. Coupled with the emergence of Gulf War Illness, increased emphasis has been placed on determining potential health effects of JP-8 exposure.

Due to the fact that JP-8 contains hundreds of aliphatic and aromatic hydrocarbons, in addition to various performance additives, this complex mixture poses a serious challenge for risk assessment. Exposure assessment is complicated by the fact that JP-8 may be encountered as a vapor, aerosol, or liquid, and possibly as combustible products, and each physical state may contain different chemical entities. However, progress has been made in the identification of JP-8 components that may serve as reliable and predictable biomarkers of exposure, particularly for dermal exposures [12,35,81,82,83,84].

Studies have consistently identified the immune system as a target for JP-8. Due to variability in routes of exposure and chemical composition, it is likely that there are a number of pathways contributing to the development of immunotoxicity, including the generation of free radicals, release of platelet-activating factor, interference with immunological memory, or the alteration of neuropeptides and cytokines. With further

examination of these various mechanisms combined with additional host resistance models, a greater understanding of risk to infectious disease or cancer will be improved.

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14 Immune Modulation by TCDD and Related Polyhalogenated Aromatic Hydrocarbons

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INTRODUCTION

The immunotoxicity of the pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been studied for many years. Based on studies in mice, TCDD is one of the most potent immune suppressive chemicals known. Exposure to a single dose of TCDD in the low µg/kg (i.e., ppb) range impairs host resistance to infectious agents, and suppresses lymphocyte responses to a variety of antigens. Although the immunosuppressive action of TCDD has been studied for almost 30 years, many unanswered questions remain regarding the mechanisms by which the immune system is affected. It is clear that the immunotoxic process is initiated by the binding of TCDD to the aryl hydrocarbon receptor (AhR), a cytoplasmic binding protein that, together with AhR nuclear translocator

(ARNT/HIF-1β), functions as a ligand-activated transcription factor. However, the molecular consequences of AhR activation within hematopoietic cells have not yet been elucidated. Understanding the mechanisms of AhR-mediated immunotoxicity is important because the AhR binds many other polyhalogenated aromatic hydrocarbons (PHAH) that are present in the environment as contaminants, including other polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and polybrominated and polychlorinated biphenyls (PBBs and PCBs, respectively). In a larger sense, it is also possible that understanding the mechanisms of AhR-mediated immune suppression will open new avenues for drug development that may aid in the treatment of many human diseases, including autoimmunity, allergy, or transplant rejection.

Several comprehensive reviews of the immunotoxicity of TCDD have been published.[1–3] Therefore, the goal of this chapter is to highlight the most recent developments in the field, with particular attention given to studies that provide new insight into the possible cellular and molecular mechanisms of PHAH immunotoxicity. New data that address three less-studied aspects of TCDD immunotoxicity will also be reviewed, including altered innate responses, toxicity to the developing immune system, and deregulation of anamnestic immune responses.

ROLE OF THE AHR IN TCDD IMMUNOTOXICITY

Structure-activity studies and the use of mice congenic at the Ah locus have demonstrated that the immunotoxic effects of PHAH correlate directly with their binding affinity for the AhR.[4] More recently, AhR-null mice were shown to be highly resistant to TCDD-induced immunotoxicity, supporting an AhR-dependent suppression of adaptive immune responses by TCDD.[2,5,6] Interestingly, $AhR^{-/-}$ mice used in these studies mounted immune responses that were equivalent in magnitude and kinetics to the responses observed in $AhR^{+/+}$ mice.[6] This is a significant observation because the initial description of AhR-null mice indicated these mice had fewer B-lymphocytes in their secondary lymphoid organs[7] leading to considerable speculation that the adaptive immune system does not develop normally, and may not function properly in the absence of the AhR. Based on current studies, the presence of the AhR is not essential for the development of a functional immune system.

Although it is clear that the AhR mediates the immunotoxicity of TCDD, it is less clear what specific molecular events follow AhR activation and underlie deregulated immune function. In hepatocytes, activation of the AhR induces cytochrome P450s and other metabolic enzymes. While immune cells express the AhR,[8–12] cytochrome P450 enzymes are not highly expressed in hematopoietic cells, and induction of these enzymes has not been found to play a role in the immunotoxicity of TCDD. In fact, a recent study of CYP1A1-deficient mice revealed an insensitivity to most of the high-dose toxicity associated with exposure to TCDD, but immune suppression was still observed.[13] Therefore, additional genes are likely targets of the activated AhR-ARNT complex, and altered expression of these genes affects immune function. The core pentameric DNA binding sequence for activated AhR-ARNT (5’-GCGTG-3’) has been

found in the promoter region of many rodent and human cytokine genes,[14] and *in vivo* exposure to TCDD altered the expression of numerous immunoregulatory genes in the spleen, thymus and liver.[15] The functional status of these putative DRE and changes in gene expression at the protein level are beginning to be reported. Specific examples will be discussed in the appropriate context below.

Although AhR activation exerts direct control over the transcription of some genes, it may also affect the level of expression of immunoregulatory proteins whose genes do not contain DRE. Some of these changes are likely to be secondary to the primary DRE-dependent effect. However, the ligand activated AhR interacts with other proteins in cells that affect cellular function, including retinoblastoma protein and the p65 subunit of nuclear factor kappa B (NF-κB).[16–19] Since numerous immunoregulatory pathways are influenced by the activation or inactivation of NF-κB, AhR-NF-κB crosstalk may account for some of the changes caused by TCDD. There are also reports that immunophilin-like proteins associate with the AhR.[20–22] Since immunophilins are known targets for several potent immunosuppressive drugs, it is possible that AhR-dependent immunotoxicity involves modulation of immunophilin function. Further studies are necessary to elucidate the functionally-relevant non-DRE pathways of TCDD's immunotoxicity.

DEFECTS IN PRIMARY IMMUNE ORGANS

THYMUS

Thymic involution, consisting of the depletion of cortical lymphocytes, is one of the most widely studied immunotoxic effects of TCDD, and has been reported in all evaluated species.[23] In mice, thymic involution is an AhR-dependent process, and does not occur in TCDD-treated mice that lack AhR.[24] Current data suggest that TCDD mediates thymic atrophy by at least two distinct, AhR-dependent pathways. One pathway involves activation of the AhR in developing thymocytes, which leads to arrested cell cycle progression,[25,26] altered thymocyte maturation[27,28] and/or enhanced negative selection.[29,30] Over-expression of the anti-apoptotic gene *bcl-2* failed to inhibit TCDD-induced thymic atrophy,[31,32] while *lpr* and *gld* mice (which are defective in the expression of Fas and Fas ligand (FasL), respectively) are less sensitive to TCDD-induced thymic atrophy.[33,34] Based on this observation, the role of the Fas signaling pathway has been extensively investigated.[33–35] Several other genes associated with negative selection in the thymus were reported to be increased in thymus tissue from TCDD-treated mice.[29] In separate studies, expression of the adseverin gene in thymocytes has been implicated in TCDD's thymic toxicity. From studies using chimeric mice that expressed AhR only in nonhematopoietic tissue, adseverin gene expression was found to be dependent on the AhR in thymocytes, and independent of AhR expression in stromal tissues.[36] Likewise, TCDD-induced thymic atrophy required the presence of the AhR in hematopoietic cells, but not in the thymic stromal tissues[5]. Direct DRE-dependent effects in thymocytes are also supported by a recent study that used T-cell specific *lck*-conditional knock-out of

ARNT.[26,37] Mice that did not express ARNT in their T cells did not show thymic involution following TCDD exposure.

There is long-standing evidence that thymic stromal cells contribute to the thymic involution induced by TCDD.[25,38] A recent report by Camacho and colleagues[39] showed that TCDD treatment increased FasL expression in thymic stromal tissues in an AhR-dependent fashion. Further, *ex vivo* cell mixing experiments demonstrated that thymocyte apoptosis was not observed when they were co-cultured with stromal tissue from TCDD-treated AhR-deficient mice or FasL-deficient mice. The authors suggest that elevated FasL expression on thymic stromal cells may augment the death of Fas-expressing thymocytes in TCDD-treated mice. In separate studies, exposure to low concentrations of TCDD altered the expression of several adhesion molecules on human thymic epithelial cells *in vitro* and stimulated their terminal differentiation.[40] Also, using induction of CYP1A1 as an indicator of AhR activation, exposure to TCDD induced CYP1A1 in thymic epithelial cells and thymocytes.[41] Collectively, these more recent studies suggest that TCDD-induced thymic atrophy likely results from direct AhR-mediated changes in the developing thymocytes, but is probably exacerbated by additional direct effects on thymic epithelial cells.

Bone Marrow

TCDD causes bone marrow hypocellularity, with specific decreases in the total number of hematopoietic stem cells (HSC) and lymphocyte precursors.[42–46] Exposure to TCDD also diminishes mRNA levels of recombination activating gene-1 and terminal deoxynucleotidyl transferase in bone marrow cells.[47] The best characterized effect of TCDD on bone marrow is the impaired maturation of B cells. A single dose of TCDD causes a transient, time- and dose-dependent, and developmental stage-specific impairment in B cell maturation, with mature B cells affected first, followed by depletion of B cell progenitors.[45,46]

The AhR and ARNT are expressed in bone marrow stromal cells.[48,49] The expression of AhR and ARNT in HSC and lineage-specific precursor subpopulations has not been rigorously examined, but HSC from TCDD-treated mice were not able to reconstitute bone marrow of irradiated recipient mice.[50] These effects were not linked to changes in proliferation or cell death, and were not observed in AhR-deficient mice, suggesting a direct, AhR-mediated effect on HSC. In addition, using a co-culture system, Wyman and colleagues[51] showed that AhR-dependent events in bone marrow stromal cells contributed to suppressed differentiation of B220$^+$ Pro-B cells, suggesting that maturing B cells are not the only targets for TCDD-mediated suppression of B cell differentiation.

In summary, although numerous AhR-dependent changes in the bone marrow and thymus have been found in TCDD-treated mice, it appears that these effects are self-limiting in adult mice, as T and B cell numbers are not reduced in secondary lymphoid tissue except after exposure to high doses of TCDD or in the context of an adaptive immune response. More subtle effects, such as changes in the antigenic specificity of peripheral T and B lymphocyte populations, have not been documented.

DEFECTS IN ADAPTIVE IMMUNE FUNCTION

B LYMPHOCYTES

Mature B lymphocytes express AhR and ARNT,[10,12] and the ability of TCDD to suppress primary antibody responses has been known for a long time (reviewed in [4]). While the precise mechanisms resulting in suppression of T-dependent B cell responses *in vivo* have not been fully elucidated, it is clear that direct effects of TCDD on B cells plays a role. Studies using two B cell lines with differential AhR expression have linked suppression of LPS-induced IgM production to AhR activation, and have shown that AhR interactions within the 3'alpha immunoglobulin heavy chain enhancer directly influence antibody production.[52–54] TCDD also appears to directly modulate the expression of CD19 on human B cells, suggesting another mechanism by which TCDD down-modulates the responsiveness of mature B cells.[55]

Recent studies have identified potential target genes in B cells that may be deregulated by TCDD. These include TiPARP, a TCDD-inducible poly(ADP-ribose) polymerase,[56] Socs2, a negative regulator of cytokine signal transduction,[57] Pax5, a repressor of B cell differentiation,[58] $p27^{kip1}$, a regulator of cell survival and differentiation,[59] and AP-1, a transcription factor that is important for many aspects of B cell function.[60] Although many regulators of B cell activation and function are affected, TCDD does not appear to enhance apoptosis of mature B cells.[61]

Other studies have examined the effects of TCDD on antibody class switching, and demonstrate that TCDD reduces production of IL-2, IL-4, IL-5 and IL-6, which are all important regulators of class switching.[62] IL-5 production by T cells was especially sensitive to suppression by TCDD. In a separate study, the proliferation of germinal center B cells was suppressed in TCDD-treated mice.[61] However, it is unlikely that alterations in cytokine production by T cells are the sole cause of impaired class switching in TCDD-treated mice, since in some model systems in which exposure to TCDD suppresses IgG_{2a} production, altered levels of cytokines important for switching to IgG_{2a} (e.g., IFNγ) were unaffected.[63,64] Thus, exposure to TCDD likely influences antibody production and class switching by direct effects on B cell activation and via interactions between B and T lymphocytes.

In addition to suppressive effects on conventional B cells, constitutive activation of the AhR causes the loss of peritoneal B-1 cells.[65] B-1 cells, also called $CD5^+$ B cells, represent a small subset of B lymphocytes that plays an important role in innate immune responses to viral and bacterial infections. Consequently, the observation that constitutive AhR activation results in depletion of this specialized lineage suggests that TCDD may affect innate immunity to pathogens via as yet uncharacterized affects on B-1 cells.

T LYMPHOCYTES

The high degree of sensitivity of T cell-dependent immune responses to TCDD-induced suppression has led to the long-held hypothesis that T cells are important targets of

TCDD (reviewed in[1–3]). However, despite considerable effort, direct effects of TCDD on purified T cells *in vitro* have not been consistently demonstrated, and direct effects on T cells *in vivo* are difficult to prove. This issue was recently resolved by studies that compared the effects of TCDD on the graft versus host (GVH) response of T cells obtained from $AhR^{+/+}$ and $AhR^{-/-}$ C57Bl/6 (B6) mice.[66] Results showed that the CTL response of grafted $AhR^{-/-}$ T cells was resistant to TCDD-induced suppression, while under the same conditions, the CTL response of $AhR^{+/+}$ T cells was suppressed by > 90%. Furthermore, when $CD4^+$ and $CD8^+$ T cell subsets were isolated from $AhR^{+/+}$ and $AhR^{-/-}$ mice and then recombined prior to grafting, full suppression of the CTL was dependent on AhR expression in both T cell subsets. These results provide the first evidence for the requisite role of the AhR in T cells for TCDD-mediated suppression of T cell responses. Induction of CYP1A1 in purified T cells exposed to TCDD *in vitro* validates the presence of a functional AhR.[6,7]

While activated T cells are sensitive to TCDD, few, if any, significant changes have been reported in resting T cells following exposure to TCDD.[68–71] Recently, the activation-dependent effects of TCDD on T cells have been clearly demonstrated using T-cell receptor (TCR) transgenic mouse models in which the transgenic T cells are adoptively transferred into syngeneic hosts and then tracked by flow cytometry using transgene-specific markers.[64,72,73] Following antigen challenge, the response of the transgenic T cells can be tracked in parallel with the host T cells. For both $CD4^+$ and $CD8^+$ T cells, TCDD alters the response of antigen-activated T cells with little to no effect on the non-antigen specific T cells. In the following sections, findings of recent studies are summarized.

$CD4^+$ T cells

Several recent studies have examined changes in the activation, differentiation and survival of antigen-specific $CD4^+$ T cells following TCDD exposure. A common observation is that T cells in TCDD-treated mice appear to undergo a normal proliferative response during the first few days after antigenic stimulation, followed by a premature termination of the response. This is demonstrated by a significant decline in the number of antigen-specific $CD4^+$ T cells around day 4, prior to development of effector function.[72–74] Camacho and colleagues[74] suggested that the decline in T cell number following injection of the superantigen *Staphylococcus* enterotoxin A (SEA) was due to the activation of the Fas by TCDD leading to increased apoptosis of SEA-activated, but not resting, T cells. Funatake and colleagues,[73] on the other hand, found that TCDD reduced the number of Fas-expressing and Fas-deficient ovalbumin (OVA)-specific $CD4^+$ T cells to the same degree, excluding a role for Fas. Instead, TCDD appeared to induce a block in cell division on day 3 to 4, and this was followed by increased T cell death.

In order to ascertain the events leading up to the loss of activated $CD4^+$ T cells, changes in the activation phenotype of antigen-specific $CD4^+$ T cells were examined during the first 3 days after antigenic challenge.[72,73,75] Depending on the model system, several significant changes in T cell activation phenotype were observed following TCDD treatment, including decreased up-regulation of CD11a, enhanced down-regulation of CD62L, and increased expression of CD25.[73,75] At the same time, the activation-

dependent increases in CD4, CD44 and CD49d expression were not altered, indicating selective effects of TCDD on different parameters of T cell activation. Despite these changes in activation phenotype, the overall proliferation of antigen-specific $CD4^+$ T cells was not significantly altered by TCDD. Further analysis of the $CD4^+CD62L^{low}CD25^+$ subpopulation showed that these cells also expressed GITR and CTLA-4, a phenotype that has been associated with immunosuppressive $CD4^+CD25^+$ regulatory T (T_{reg}) cells.[75] When $CD4^+CD25^+$ cells were enriched from the spleen of TCDD-treated mice, they expressed functional characteristics associated with T_{reg} cells.[76] The development of an expanded $CD4^+CD25^+$ population in TCDD-treated mice was dependent on the presence of the AhR in the T cells, but not on the presence of pre-existing $CD4^+CD25^+$ cells. Taken together, these recent results suggest a novel role for the AhR in the generation of T_{reg} cells, and provide a new perspective on the cellular mechanisms that may underlie the profound immune suppression induced by exposure to TCDD.

$CD8^+$ T Cells

The effects of TCDD on the activation, differentiation and survival of $CD8^+$ T cells have been extensively investigated using two murine model systems, the allogeneic P815 tumor model[77,78] and the influenza A virus infection model.[64,79–82] The allo-MHC class I antigens expressed on the P815 tumors cells induce a potent CD4-dependent CD8-mediated CTL response. Similarly, primary influenza virus infection induces a CD4-independent CTL response that can be tracked using MHC class I-restricted tetramers and clonotypic anti-TCR antibodies to identify virus-specific $CD8^+$ T cells. In both models, TCDD exposure induces a strikingly similar dose-dependent suppression of the CTL response on days 9 to 10. The suppressed response is preceded by impaired $CD8^+$ T cell expansion, suppressed production of IL-2 and IFNγ, and reduced numbers of cells expressing an effector CTL phenotype. Time-course studies indicate that the number of influenza virus-specific $CD8^+$ T cells initially increase in TCDD-treated mice following infection but then abruptly decline on day 5.[79] Similarly, following P815 injection, $CD8^+$ cells in TCDD-treated mice begin to produce IL-2 and IFNγ, but production abruptly ends on day 6.[77] Taken together these results suggest that the initial activation of $CD8^+$ T cells is normal, but is then prematurely terminated in TCDD-treated mice, similar to that observed in $CD4^+$ T cells. The decline in the $CD8^+$ T cell response in TCDD-treated mice could not be explained by an increase in apoptosis in either model.[78,79] However, provision of exogenous IL-2 *in vivo* or *in vitro* partially restored the responsiveness of $CD8^+$ cells, suggesting that TCDD may induce a state of anergy.[78,79] In addition, when P815 tumor cells were transfected with CD86, the resulting CTL response was not suppressed by TCDD,[83] suggesting that provision of excess co-stimulation could rescue the $CD8^+$ T cells from TCDD-induced suppression. Interestingly, CD86-transfection of the P815 tumor cells resulted in the generation of a robust CTL response that was no longer dependent on $CD4^+$ T cells.[83] Thus, it is possible that the primary TCDD-induced defect in the CTL response occurs in the $CD4^+$ T cells resulting in the induction of anergy in the $CD8^+$ T cells. In fact, it is tempting to speculate that the early induction of $CD4^+CD25^+$ T_{reg} cells by TCDD contributes to suppression of the CTL response. This idea is consistent with the observation that TCDD exposure must occur within the first

3 days of the P815 response and within the first 4 days of influenza infection to be immunosuppressive ([77]and B.P. Lawrence, unpublished observations). Alternatively, these results are also consistent with a TCDD-induced defect in antigen presenting cells.

Antigen Presenting Cells

Several studies indicate that exposure to TCDD alters the function antigen presenting cells (APC). Prell and Kerkvliet[83] reported that *in vivo* TCDD exposure reduced the expression of CD86 on $B220^+$ and $Mac\text{-}1^+$ spleen cells during the allogeneic response to P815 tumor cells, suggesting that costimulation of T cells by APC may be defective in TCDD-treated mice. This hypothesis was further tested by Shepherd and colleagues [84] who evaluated the ability of exogenous anti-CD40 antibody to ameliorate the suppression of CTL activity caused by TCDD. However, even though anti-CD40 treatment increased expression of several costimulatory molecules (including CD86, MHC Class II, and CD54) on APC and resulted in high levels of circulating IL-12, CTL activity remained suppressed in TCDD-treated mice. Vorderstrasse and Kerkvliet[85] characterized the temporal and dose-related effects of TCDD on the expression of costimulatory molecules on dendritic cells (DC), and directly examined the ability of DC from TCDD-treated mice to activate T cells *in vitro*. Unexpectedly, TCDD increased rather than decreased the expression of several accessory molecules on DC, including MHC Class II, ICAM-1, CD40, and CD24. DC from TCDD-treated mice also produced more IL-12 and stimulated a higher T cell proliferative response in a mixed lymphocyte reaction. On the other hand, the number of DC recovered from spleens of TCDD-treated mice was significantly reduced within one week after TCDD treatment. It was postulated that this decline in DC number reflected the apoptosis of DC following their inappropriate activation by TCDD. Since the persistence of activated DC has been shown to influence the strength and duration of an immune response, a premature loss of DC in TCDD-treated mice could result in insufficient contact time with T cells to sustain their full activation and differentiation. Another possibility was that the inappropriate activation of DC by TCDD in the absence of antigen would cause the DC to lose their ability to phagocytose and process antigen for T cells. However TCDD did not affect the ability of DC to phagocytose latex beads, or to present KLH to KLH-specific T cells.[86] The processing and presentation of OVA to OVA peptide-specific transgenic T was also not suppressed by TCDD either *in vivo* or *in vitro.*[86]

Interestingly, changes in the activation phenotype of DC and the decline in DC numbers did not occur in $AhR^{-/-}$ mice, indicating an AhR-dependent response.[85] Ruby and colleagues [87] investigated the direct effects of TCDD on the maturation and survival of bone marrow derived DC *in vitro*. The results of these studies showed that TCDD directly enhanced TNF-α-induced DC maturation and enhanced Fas-mediated apoptosis. Based on microarray analysis of genes associated with apoptosis, TCDD increased the expression of several genes in purified DC, particularly FADD, DFF40, OX40L and caspase 9. TCDD also suppressed NF-κB signaling in DC in response to TNF-α or anti-CD40 stimulation,[19] suggesting that altered NF-κB signaling pathways may be an important mechanism for alteration of DC function following TCDD exposure.

Immunological Memory

The ability to respond more rapidly and robustly upon re-encounter with antigen is a unique feature of lymphocytes, and underlies the efficacy of vaccinations. Given the critical role immunological memory plays in human health and the fact that TCDD is a potent immune suppressant, it is surprising how few studies have focused on the effects of TCDD on immunological memory. This question was initially addressed about 25 years ago in two separate studies. Vecchi and colleagues[88] reported that the antibody response to injected sheep red blood cells (SRBC) was suppressed in mice that were given TCDD prior to both primary and recall challenge. In another study, mice that consumed animal feed spiked with TCDD for at least 5 weeks had a diminished secondary antibody responses to SRBC and tetanus toxin.[89] More recently, the effect of exposure to a single dose of TCDD on the immune response to secondary challenge with OVA was examined.[90] Seven days after re-challenge, levels of OVA-specific IgM and IgG_1 in plasma were modestly reduced, as was the proliferative response of splenocytes following *in vitro* re-stimulation with OVA. Similar to earlier studies, mitogen-induced proliferation in response to ConA and LPS were not affected. This study also examined the production of several T cell-derived cytokines. Treatment with TCDD at the time of the first OVA injection resulted in diminished production of IL-2, IL-4 and IL-5 and elevated levels of IFNγ after OVA re-challenge, providing the first clear evidence for effects of TCDD on the recall response.

A kinetic study of the memory response to viral infection showed that exposure to a single dose of TCDD prior to the primary infection diminished both antibody and cell-mediated responses.[91] Specifically, virus-specific IgG levels in lung lavage fluid and blood were substantially decreased in mice treated with TCDD prior to primary infection, and remained suppressed after re-infection. Class I MHC-restricted tetramers and phenotypic markers of T cell activation showed that a single dose of TCDD resulted in 50% fewer virus-specific memory $CD8^+$ cells in the peribronchiolar lymph nodes 60 days later, and upon re-infection, the CD8 T cell recall response was delayed.[91] Another interesting observation in this study was that administration of TCDD prior to re-infection (i.e., after immunological memory was established) had no effect on the recall antibody response. This is consistent with previous reports suggesting that the AhR-mediated events that suppress lymphocyte function occur very early during the activation of naïve lymphocytes, and once they have passed this window of sensitivity or have become memory cells, they are resistant to the suppressive effects of TCDD.[9,77,92]

DEFECTS IN INNATE IMMUNE FUNCTION

In contrast to adaptive immune function, fewer studies have examined effects of TCDD on the innate immune system. Furthermore, of the limited data available, much of it is inconsistent. For example, natural killer (NK) cell activity has been reported to be unaffected,[80,93] diminished,[94] and increased[95] following exposure to TCDD. Although a thorough assessment of AhR expression in all innate cell lineages has not been conducted, neutrophils and macrophages express the AhR,[8,96] and neutrophils are affected

by TCDD. In fact, one of the most consistently observed effects of TCDD on the innate immune system is an increase in the number of neutrophils at the site of antigen challenge.[82,97–100] While the mechanism by which excess neutrophils are recruited has not been elucidated, it is AhR-dependent and does not appear to involve the overproduction of soluble neutrophil chemoattractants, increased expression of adhesion molecules on neutrophils, or delayed apoptosis.[100] When examined *ex vivo*, the effects of TCDD on neutrophil functions have been variable, making it uncertain whether TCDD alters the function of neutrophils *in vivo*. For example, superoxide anion and hydrogen peroxide production, myeloperoxidase activity and β-glucuorindase release were unaffected in mice treated with TCDD.[96,100] In contrast, Choi and colleagues[97] reported that neutrophils elicited in response to P815 tumor cells produced more superoxide when stimulated *ex vivo* with PMA if obtained from TCDD-treated mice. Likewise, the cytolytic activity of neutrophils from TCDD-treated mice was either unaffected[96] or substantially reduced[97] compared to control neutrophils. Thus, while it is clear that AhR activation by TCDD affects neutrophil recruitment, the precise nature of this effect and the pathophysiological consequences require further study.

Effects of TCDD on macrophages have also been examined. When assessed *ex vivo*, macrophage functions such as tumor cell lysis, phagocytosis and oxidative burst were not suppressed by exposure to TCDD.[93,96,101] In other experimental systems, exposure to TCDD increases IL-1 and TNF production by macrophages.[99,102,103] However, the ability of TCDD to alter IL-1 and TNF production is likely organ- or stimulus-specific, because in the context of respiratory viral infection, exposure to TCDD had no effect on IL-1 or TNF-α levels.[80]

The effects of TCDD on other inflammatory cytokines and mediators of innate immunity have only been examined in a handful of studies, and at this point information is descriptive in nature. For example, the increase in IL-12 associated with antigen challenge is suppressed by TCDD in two different experimental systems,[82,84] but the consequences of this suppression remain uncertain. Although not extensively assessed, the infection-associated increase in type I IFN in the lung was unaffected mice treated with TCDD.[80] Likewise, other infection-associated soluble factors such as KC, IL-6, MIP-1α, were not enhanced by TCDD in lungs of influenza virus-infected mice.[100] In separate studies, exposure to TCDD had no effect on the levels of immunomodulatory arachidonic acid metabolites (e.g., prostaglandins and leukotrienes) or on arachidonic acid metabolizing enzymes.[104] The effects of TCDD on the recruitment or function of mast cells, basophils and eosinophils has not been examined, nor have studies been conducted to assess whether exposure to TCDD affects the expression or downstream signaling pathways controlled by pattern recognition receptors.

TOXICITY TO THE DEVELOPING IMMUNE SYSTEM

The sensitivity of the developing immune system to TCDD has been recognized for quite some time.[105,106] However, the period of time during immune ontogeny that is most sensitive to disruption by TCDD has not been identified, and differences in the endpoints examined, the dose of TCDD, and the time period of exposure to TCDD (continuous,

mid gestation, late gestation, perinatal, i.e., lactational) have made it difficult to compare findings among these studies.

Thymic atrophy has been observed following prenatal and perinatal exposure to TCDD.[27,107] In addition, *in utero* exposure to a single dose of TCDD, administered on gestational day (GD) 14, altered the proportions of thymocyte subpopulations in fetal and neonatal rats.[108] Developmental toxicity to the thymus has been observed in mice,[109] as well as in fetal thymic organ cultures,[25] but has not been observed in all studies,[110,111] suggesting that factors such as dose or timing may affect this endpoint. Additionally, alterations in thymocytes appear transient, as adult mice exposed to TCDD during development exhibit no thymic atrophy or changes in the proportion of thymocyte subpopulations, and skewing in the proportion of T cell subpopulations is not observed in peripheral T cells.[109–112]

In addition to effects on the thymus, persistent deficiencies in T cell-dependent function following developmental exposure to TCDD have been reported. Daily administration of TCDD to the dam between GD 6 and GD 14 resulted in offspring with diminished CTL activity against allogeneic tumor cells.[109] Suppression of delayed type hypersensitivity and contact hypersensitivity responses was observed in rats exposed *in utero* to a single dose of TCDD, administered on GD 14.[113,114] Cell mediated and humoral immune responses to viral infection were diminished in mice exposed to relatively low doses of TCDD throughout development.[111,115] This dosing paradigm was not overtly hematotoxic, as there were no defects in cell number or cellular composition of the bone marrow, thymus, spleen or lymph nodes. However, offspring from TCDD-treated dams exhibited excess numbers of neutrophils and higher IFNγ levels in their lungs after infection, suggesting that the effects of developmental exposure to TCDD are not exclusively immunosuppressive, but some functions are aberrantly up-regulated. In fact, female offspring that were exposed to TCDD via lactation had higher serum IFNγ levels following infection with *Listeria monocytogenes,*[116] and both male and female offspring demonstrated a decreased ability to fight infection with *Listeria monocytogenes*. Thus, developmental exposure to TCDD suppresses lymphocyte responses and enhances aspects of inflammation, with some effects persisting to 19 months of age.[106,108,113]

IS THE HUMAN IMMUNE SYSTEM AFFECTED BY DIOXIN?

In contrast to rodent data, the effects of PHAH on immune competence in humans have been difficult to measure, owing in some cases to the lack of documented exposure levels and in other cases to the low level of exposure (relative to rodent studies). The inherent population variance associated with studies using human subjects also impedes data interpretation. Nonetheless, several recent studies have reported small but statistically significant changes in various immune parameters in humans exposed to TCDD. For example, a recent assessment of Vietnam War veterans characterized immune system changes in relation to their operation in various areas of Vietnam.[117] Study subjects were classified into groups based on their Agent Orange exposure history and their current health status. The control subjects were healthy age-matched subjects with no Vietnam War military service. A significant increase in plasma IgE levels was found in

the veterans, compared to controls. *In vitro* assessment of peripheral blood cells showed significantly decreased plasma IgG_1 levels, decreased production of IFNγ, and increased production of IL-4 in cells from the veterans. No changes in the plasma levels of antibodies against double-stranded DNA or extractable nuclear antigens, both markers of autoimmune disease, were observed in veterans, nor were changes in the frequency or distribution of peripheral blood leukocyte subpopulations noted.

Compared to people in a noncontaminated area, plasma IgG levels were also significantly decreased in proportion to increasing plasma levels of TCDD in a cohort exposed in an industrial accident in Seveso, Italy.[118] There was no effect on IgM or IgA levels, or on complement levels; IgE was not measured. In separate studies, *in vitro* exposure to TCDD enhanced the spontaneous production of IgE by B cells isolated from atopic but not non-atopic individuals, but did not affect the levels of other isotypes.[119] Other recent studies have reported small changes in immune cells from individuals exposed occupationally to PHAH.[120,121] For example, compared to unexposed controls, a cohort of men exposed occupationally to TCDD had diminished IFNγ production in a recall response to tetanus toxin, while IFNγ production following polyclonal activation was unaffected.[120] This observation is consistent with mouse studies, in which antigen-specific responses are highly suppressed by TCDD, but mitogen-driven T cell responses are less susceptible to impairment.[83,88,122,123]

Further evidence that PHAH may indeed alter the human immune system stems from several epidemiological studies of children exposed *in utero* and lactationally to dioxins and PCBs. One study reported a correlation between increased PHAH levels in breast milk and cord blood and the incidence of otitis media in infants,[124] suggesting a possible impairment in host resistance to infection. A separate study noted a correlation between prenatal PHAH exposure and decreased monocyte and granulocyte numbers and alterations in T cell sub-populations,[125,126] as well as diminished vaccine efficacy and increased incidence of infection.[127] Decreased lung function and increased chest congestion have been associated with high perinatal dioxin exposure.[128,129] Similarly, a recent study of immunologic biomarkers in adolescents revealed a correlation between the level of PHAH exposure and respiratory complaints.[130] A study of children exposed to dioxin perinatally reported decreased allergy in relation to increasing dioxin exposure.[131] In addition, increased numbers of $CD4^+$ T cells and $CD45RA^+$ cells, a phenotype associated with naïve T cells, were noted. An increased percentage of naïve versus activated T cells is consistent with a generalized decrease in immune responsiveness associated with dioxin exposure. Collectively, these studies suggest that, similar to laboratory animals, exposure to PHAH likely leads to adverse immunological outcomes in humans, and the developing immune system may be more sensitive than the mature immune system to perturbation by TCDD and related compounds.

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15 Mechanisms by Which Ultraviolet Radiation, a Ubiquitous Environmental Toxin, Suppresses the Immune Response

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INTRODUCTION

Immunotoxicity is generally defined as "the study of adverse effects on the immune system resulting directly from environmental, occupational, or therapeutic exposure to chemicals, biological materials, and in certain instances, physiological factors, collectively referred to as agents. It encompasses immunosuppression, allergy, autoimmunity and inflammation."[1] Although drugs, volatile organic chemicals, arsenic, lead, and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin readily come to mind when considering agents that

induce immune toxicity, the immunotoxic agent that *all humans come into contact with on a daily basis* is the ultraviolet (UV) radiation (290 to 400 nm) found in sunlight. UV radiation is best known as the primary etiologic agent in the development of skin cancer and it is clear that the immune suppression induced by UV radiation is a major risk factor for skin cancer induction. In addition, UV exposure has been shown to suppress the immune response to microbial, fungal, and viral antigens. Because of the association between skin cancer induction and immune suppression, research efforts regarding the adverse effects of UV radiation has been the focus of dermatologists, cancer biologists and immunologists, but generally, not toxicologists. One can argue, however, that UV radiation is the oldest and most prevalent immunotoxin in our environment. Exposure to UV radiation induces immune suppression, as well as inflammation, and in some cases can contribute to allergic reactions, so it fits the definition of an immunotoxic agent. UV exposure suppresses the immune response in almost all vertebrate animal species tested so far, from fish to humans. The mechanisms involved appear to be very similar from species to species and doses of UV radiation that induce immune suppression in man compare well to those that suppress in experimental animal species. Immune suppression can be induced after chronic exposure, or after a single exposure to a relatively low dose (i.e., the amount required to cause distinct "redness" of Type 1 Caucasian skin, the minimal erythemal dose) of UV radiation. The focus of this chapter is to review the mechanisms by which this "granddaddy of all immunotoxins" suppresses the immune response.

PHOTOIMMUNE SUPPRESSION: BASIC PHENOMENA

Kripke and colleagues provided the first indication that UV radiation was immunosuppressive. In these experiments UV-induced skin tumors were removed and then transplanted onto normal age- and sex matched syngeneic recipient mice. Contrary to expectations, none of the transplanted tumors grew progressively when transplanted into normal immunocompetent recipient mice. Progressive tumor growth was only apparent when the tumors were transplanted into immunosuppressed recipient mice. This indicated that the skin cancers induced by UV radiation were highly antigenic (i.e., they were recognized and rejected by the immune system of the normal host, and would only grow progressively if the immune system of the host was compromised). While this observation explained why the tumors were rejected when transplanted into normal syngeneic hosts, it left unanswered the question of how these tumors developed in the first place. One way to explain these results was to propose that during chronic UV exposure the UV radiation was having two effects: skin cancer induction and induction of immune suppression. Experimental support for this hypothesis was provided by observing progressive tumor growth in mice exposed to a subcarcinogenic dose of UV radiation.[2] Furthermore, UV-induced immune suppression could be transferred from the mice exposed to a subcarcinogenic dose of UV to normal syngeneic mice by injecting the normal mice with CD3+, CD4+, CD8- suppressor T cells.[3,4]

In addition to tumor immunity, UV exposure was found to impair antigen presenting cell function, the generation of contact hypersensitivity (CHS) to chemical haptens and

the generation of delayed type hypersensitivity (DTH) to allogeneic histocompatibility, protein, and microbial antigens. Antibody production, allograft rejection, rejection of chemically induced regressor tumors, macrophage phagocytic/tumoricidal activity, proliferation of T cells to antigens or mitogens, as well as CTL generation were all normal in UV-irradiated mice (early work reviewed by Kripke[5]). Because CHS and DTH experiments are relatively short in duration (5 to 10 days) when compared to tumor immunity (6 to 8 weeks), can be suppressed by a single dose of UV radiation, and can be done with human volunteers, much of the work done to determine the mechanism(s) of UV-induced immunotoxicity employ these assays.

PHOTORECEPTORS IN THE SKIN

To induce immune suppression the electromagnetic energy of UV radiation must first be absorbed by an epidermal photoreceptor and then converted into a biologically recognizable signal. Three epidermal photoreceptors have been identified: DNA, urocanic acid (UCA) and membrane lipids.

DNA

DNA strongly absorbs UV radiation, especially mid-range UVB (290 to 320 nm) radiation. Two major DNA lesions are induced following UV exposure, pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts. Because the action spectrum (induction of a biological activity as a function of wavelength) for erythema closely matches the action spectrum for pyrimidine dimer formation, DNA is believed to be the chromophore for sunburn.[6] Pyrimidine dimer formation, or more properly, the failure to adequately repair dimers after solar irradiation is also the primary cause of sunlight-induced skin cancer formation.[7,8]

Data to support the idea that DNA is an epidermal chromophore for UV induced immune suppression was generated in a series of experiments in which liposomes containing the bacteriophage excision repair enzyme T4N5 were applied to the skin of UV-irradiated mice to repair pyrimidine dimer formation *in situ*. Electron microscopic examination of skin treated with gold-labeled-T4N5-containing liposomes documented the delivery of the repair enzyme into the nucleus and cytoplasm of epidermal keratinocytes and Langerhans cells.[9] Two effects were noted in mice exposed to UV and treated with T4N5-containing liposomes. First, the number of pyrimidine dimers in the skin was reduced. Second, immune suppression was blocked. Control liposomes containing a heat inactivated enzyme preparation were inert, and did not reverse the induction of immune suppression. The UV-induced suppression of both CHS and DTH was reversed by T4N5-containing liposomes. Applying the T4N5-containing liposomes to the skin of the UV-irradiated mice also blocked suppressor T cell induction.[10]

In humans, Stege and colleagues found that application of liposomes containing DNA-repair enzymes to the skin of UV-irradiated human volunteers immediately after UV exposure blocked the induction of immune suppression.[11] UV exposure also

suppresses human antigen presenting cell function.[12] UV exposure also triggers the release of immune regulatory cytokines by irradiated keratinocytes either *in vitro* or *in situ.*[13] Many of these cytokines and biological response modifiers have been shown to play a critical role in the induction of immune suppression (see below). Wolf and colleagues report that applying T4N5-containing liposomes to the skin of UV-irradiated human volunteers, blocked the production of the immunosuppressive cytokine, interleukin (IL)-10, at both the message and protein level.[14]

Urocanic Acid (UCA)

Urocanic acid (2-propanoic acid; 3-[1H-imidazol-4-yl] is located superficially in the stratum corneum. Metabolism of epidermal UCA does not occur *in situ* due to the absence of urocanase, resulting in the accumulation of UCA in the epidermis. Upon UV exposure, naturally occurring *trans*-UCA converts to the *cis*-isomer, in a dose dependent manner, until the photostationary state is reached, when equal quantities of *trans*- and *cis*-UCA are found in the skin.[15] Based on an analysis of the action spectrum for UV-induced immune suppression, and the fact that no immune suppression was observed in mice whose stratum corneum was previously removed by tape stripping, De Fabo and Noonan suggested that urocanic acid was the photoreceptor for UV-induced immune suppression.[16] Since the initial experiments many others have documented, the ability of *cis*-UCA to initiate immune suppression, documented its presence in the serum of UV-irradiated mice, and demonstrated that *cis*-UCA plays a role in UV-induced skin cancer induction. (For a more complete review of the role of *cis*-UCA in immune suppression see two excellent reviews by Norval and colleagues.[17,18])

Membrane Lipid Peroxidation and Free Radical Formation

UV-irradiation of cells perturbs cellular redox equilibrium, leading to free radical formation and membrane lipid peroxidation. Circumstantial evidence from studies in which anti-oxidant treatment blocks the UV-inducted impairment of antigen presenting cells function[19] abrogates the UV-induced suppression of CHS[20] and interferes with tolerance induction,[21]suggest that this stress response contributes to the induction of immune suppression. More direct evidence comes from studies in which immune suppression was activated by injecting oxidized phospholipids into mice. These oxidized lipids activate immune suppression by binding to the receptor for platelet activating factor[22] (see below).

How does UV-induced free radical formation activate immune suppression? Some have suggested that UV-induced cytokine production is involved. Because both DNA damage and oxidative stress can activate transcription of the cytokines that activate immune suppression,[23,24]one of the problems faced by investigators in the field was to divorce the effects of DNA damage from membrane oxidation. One approach was to look at the activation of transcription factors in UV-irradiated enucleated cells. Devary and colleagues[25] observed that both NF-κB and AP-1 were activated in enucleated

UV-irradiated cells. Using cell-free cytosolic keratinocyte extracts, Simon and colleagues[26] confirmed the role of membrane oxidation in NF-κB activation. Particularly important aspects of the experimental design employed by Simon and colleagues was the use of keratinocytes versus cells derived from a cervical cancer patient, and the use of biologically relevant UVB (290 to 320 nm) radiation versus UVC (200 to 290 nm) radiation, which is filtered out by the atmospheric ozone layer and does not reach the earth's surface. Overall, these data indicate that the activation of cytokine transcription, a step essential for the induction of immune suppression, can occur independently of UV-induced DNA damage and suggest that membrane lipid oxidation can serve as a UV photoreceptor.

What is not entirely clear is whether these three pathways (DNA damage, UCA isomerization and lipid oxidation) work together or separately, and what, if anything determines if one predominates over the other. Two reports indicate that UV-induced suppression of DTH was completely blocked by neutralizing antibodies to *cis*-UCA whereas, the suppression of CHS was less affected, suggesting that different photoreceptor pathways affect the regulation of different types of immune reactions[27,28]. Another report suggests that the viability of the antigen determines whether the pathway mediated by *cis*-UCA or DNA damage activates immune suppression[29]. The wavelengths involved may also play an important role; pyrimidine dimer formation and isomerization of *cis*-UCA are optimally induced by short to mid range UVB, whereas membrane oxidation is generally induced by longer wavelength UVA, which in some instances can be immunosuppressive.[30,31] Perhaps the more interesting question is: why did evolutionary pressures result in the development of three distinct photoreceptors for the activation of UV-induced immune suppression?

TRANSMISSION OF THE IMMUNE SUPPRESSIVE SIGNAL FROM THE SKIN TO THE IMMUNE SYSTEM

Unlike many environmental toxins, UV radiation is absorbed in the upper layers of the skin and does not penetrate into the underlying tissues and internal organs. Yet, UV exposure induces a systemic down-regulation of the immune response. That is to say, the immune response to an antigen applied at a non-irradiated site can be suppressed by prior UV exposure at distant site. Here again, multiple mechanisms have evolved to facilitate the transmission of the immune suppressive signal from the target organ, the skin, to the immune system.

MIGRATION OF UV "DAMAGED" ANTIGEN-PRESENTING CELLS

Langerhans cells form an epidermal dendritic cell network that functions to capture invading microorganisms, ingest them and then process their antigens into a form that can be recognized by lymph node T cells. During the migration from the skin to the draining lymph nodes, the Langerhans cell matures (upregulates costimulatory molecules, downregulates phagocytosis) and arrives at the lymph node ready to initiate a

productive immune response.[32]. UV exposure alters epidermal Langerhans cell function. UV-irradiation destroys the dendritic cell network and mice sensitized with contact allergens through UV-irradiated skin fail to generate a CHS reaction.[33] Not only was CHS suppressed in these mice, but subsequent re-sensitization with the same hapten at a distant non-irradiated site, failed to induce a secondary immune reaction (i.e., induction of immunological tolerance). Moreover, hapten specific suppressor T cells could be found in the lymphoid organs of mice sensitized through UV-irradiated skin.[34]

DNA acts as a photoreceptor for UV-induced suppression of antigen-presenting cell function. This was shown in a series of experiments in which UV damaged antigen presenting cells were isolated, and then the dimers were repaired *in vitro*.[35] This was accomplished by treating the UV-irradiated Ia-positive, FITC-positive dendritic cells with liposomes containing the pyrimidine dimer-specific photoreactivating enzyme isolated from *Anacystis nidulans*. This enzyme directly binds to the pyrimidine dimer, and in the presence of photoreactivating light (visible or long wave UVA), splits the dimer and restores the DNA to its original configuration. The liposomes were added to the UV-damaged Langerhans cells, and then the cells were exposed to photoreactivating light. The treated cells were then injected into mice, and the ability of the recipient mice to generate a CHS reaction was measured. Photoreactivation reduced the numbers of pyrimidine dimers present in the dendritic cells isolated from UV-irradiated mice and totally restored their antigen-presenting cell function. Treating the dendritic cells with photolyase-containing liposomes only, or treating the cells with photoreactivating light in the absence of liposomes failed to restore antigen-presenting cell function. Moreover, reversal of the sequence of events (i.e., photoreactivating light before liposome treatment, had no restorative effect). Because the photolyase-containing liposomes were added directly to the dendritic cells *in vitro*, in cultures devoid of keratinocytes, these data indicate that UV-induced DNA damage to epidermal Langerhans cells is sufficient to alter their function from a cell that immunizes to a cell that induces immune suppression.

A definitive role for *cis*-UCA in UV-induced suppression of antigen presenting cell function is still open to interpretation. Although the initial report indicated that *cis*-UCA could suppress antigen-presenting cell function in mice,[36] the effect of *cis*-UCA on human antigen presenting cell function are not as clear cut. When added to human peripheral blood mononuclear cells, *cis*-UCA failed to suppress mitogen-driven T cell proliferation, nor did *cis*-UCA suppress the generation of a mixed lymphocyte reaction.[37,38]. *Cis*-UCA did suppress the induction of the mixed epidermal cell lymphocyte reaction, but the effect was marginal (at best 20% to 30% immune suppression)[38] and these results were not reproducible.[39] Because keratinocyte-derived immunosuppressive cytokines, particularly IL-10 (see below), can suppress epidermal Langerhans cell antigen-presenting function, many have tested the hypothesis that *cis*-UCA induces cytokine production. The results have been uniformly negative; *cis*-UCA does not by itself activate keratinocyte to secret cytokines,[40,41] although one report indicated that *cis*-UCA acts in synergy with histamine to induce human keratinocytes to secrete prostaglandin E_2 (PGE_2),[42] which can modulate antigen presenting cell function.[43]

UV-Induced Cytokines and Biological Response Modifiers

A wide variety of biological response modifiers, bioactive lipids, and immune regulatory cytokines, including but not limited to platelet activating factor (PAF),[22] prostaglandins,[44] *cis*-UCA[16], histamine[45], calcitonin gene related peptide,[46] α-melanocyte-stimulating hormone[47], IL-10,[48] and IL-4[49,50] have been show to play a role in UV-induced immune suppression. Although the interplay between these various UV-induced cytokines is complex and not completely understood, data generated in my laboratory suggest that a cytokine cascade is activated that ultimately results in immune suppression.[5].

We believe that one of the earliest biochemical events leading to UV-induced immune suppression is the release of PAF by damaged keratinocytes. PAF activates a wide variety of cells, including platelets, monocytes, mast cells and polymorphonuclear leukocytes. PAF is secreted in response to oxidative stress and within minutes post-UV exposure.[52] Cells responsive to PAF express a seven trans-membrane G-coupled protein receptor. In addition to PAF, this receptor recognizes structural analogs of PAF, for example, oxidized phosphatidylcholine.[53] Binding of PAF or PAF-like molecules to its receptor stimulates a variety of downstream effects, including the activation of the MAP kinase pathway, activation of phospholipases, and the biosynthesis of a variety of cytokines and prostaglandins.[53]

Because PAF up-regulates the production of PGE_2[54] and because our previous work indicated that PGE_2 was a critical early step in the induction of immune suppression,[51] we tested the hypothesis that UV-induced PAF secretion activates cytokine production and initiates UV-induced immune suppression. Both UV radiation and PAF turned on the transcription of cyclooxygenase-2 (COX-2) and IL-10 promoter constructs. Treating keratinocytes with a selective PAF receptor antagonist prior to UV exposure suppressed the transcription of the COX-2 and IL-10 constructs. Further, as mentioned above, PAF-like lipids, such as UV-irradiated oxidized phosphatidylcholine (UV-PC) also activated COX-2 and IL-10 transcription. Injecting PAF and/or UV-PC into mice mimicked the effects of UV exposure and the suppressed the induction of DTH *in vivo*. Immune suppression, be it induced by PAF, oxidized phosphatidylcholine, or exposure to UV radiation, was blocked by a series of structurally unrelated selective PAF-receptor antagonists.[22]

As mentioned above, PAF and PAF-like molecules are rapidly synthesized by keratinocytes following UV exposure. We suggest that two mechanisms are involved. UV-induced free radical formation leads to membrane oxidation and the formation of oxidized phosphatidylcholine. The PAF-like molecules bind to PAF receptors in either a paracrine or autocrine fashion. This induces the release of arachidonic acid from the membrane, activates PLA_2 and promotes the synthesis of *bona fide* PAF.[55] The newly synthesized PAF then binds to PAF receptors, which upregulates the production of more PAF and downstream biological modifiers such as eicosanoids and cytokines. Ultimately this activates the cascade of events that leads to immune suppression.

The cellular target of UV-induced PAF is not clear. Keratinocytes express the PAF receptor and will secrete PGE_2 after PAF treatment.[54] Macrophages also express the PAF receptor, so it is possible that the inflammatory macrophages described by Cooper and

colleagues[56] are activated by PAF to secrete IL-10. Another interesting candidate is the dermal mast cell. Immune suppression cannot be induced in UV-irradiated mast cell deficient mice.[57] Mast cells are activated by PAF[58] and activated mast cells release many of the cytokine and biological response modifiers (IL-4, IL-10, PGE_2, histamine) that are involved in UV-induced immune suppression.[59] In addition, high dermal mast cell prevalence has been associated with the development of basal cell carcinoma in humans and chronic sun exposure increases mast cell numbers at the site of exposure.[60]

The ultimate target of UV-induced immune regulatory cytokines appears to be the dendritic cell. The underlying mechanism is suppression of dendritic cell IL-12 production. Dendritic cells isolated from the draining lymph nodes of UV-irradiated mice fail to secrete biologically active IL-12 when stimulated *in vitro*.[61] Because Th1 cells are dependent upon IL-12 for stimulation, and Th2 cells are not, these findings would explain the immune deviation observed when dendritic cells from UV-irradiated mice are used to present antigen.[62] Consistent with this hypothesis is the suppression of IL-12 secretion by IL-10[63] and the observation that monoclonal IL-10 reversed the UV-induced antigen presenting cell defect. Also consistent with this hypothesis is the observation that injecting recombinant IL-12 into UV-irradiated mice overcomes the induction of immune suppression[64] and immune tolerance.[65]

Besides the traditional role of IL-12 as a natural adjuvant that promotes Th1 cell function,[66] evidence exists indicating that IL-12 functions in nonconventional ways to reverse UV-induced immune suppression. As mentioned above, IL-10 is an essential cytokine necessary for UV-induced immune suppression. Interferon-gamma naturally counteracts the function of IL-10 and because IL-12 serves to up-regulate the production of IFN-γ, we tested the hypothesis that IL-12 was reversing immune suppression by promoting IFN-γ secretion *in vivo*. We found that the reversal of UV-induced immune suppression was independent of IFN-γ production in that immune suppression was still reversed when UV-irradiated mice were injected with IL-12 and neutralizing anti-IFN-γ monoclonal antibody. This prompted us to search for an alternative mechanism. Because of the importance of cytokines in UV-induced systemic immune suppression, we tested the hypothesis that IL-12 could be affecting cytokine secretion. To our surprise, we found that IL-12 reversed IL-10 secretion *in vivo*, in part by down-regulating transcription of the IL-10 gene.[67] These data indicate that IL-12 can suppress cytokine production, a novel role for IL-12 that has recently been reproduced by others.[68]

A second novel function for IL-12 discovered by photobiologists is its ability to induce DNA repair. Schwarz and colleagues observed that UV-induced, but not γ-irradiation-induced, apoptosis was suppressed by IL-12. As expected, UV exposure induced pyrimidine dimer formation in target cells. IL-12 was added immediately following UV exposure, and the cells were harvested 10 minutes or 3.5 hours later. At 10 minutes, there was no difference in dimer formation in cultures exposed to UV and treated with IL-12, or cells treated with UV alone. However, when the cultures were harvested 3.5 hours later, there was a substantial decrease in the numbers of dimers in the UV and IL-12-treated group. This observation suggested that IL-12 was promoting DNA excision repair. This was confirmed by three additional experiments: 1. Reversal of DNA damage as measured by a comet assay 2. Upregulation of nucleotide excision repair genes by IL-12. 3. Failure of IL-12 to reverse UV-induced damage in xeroderma

pigmentosium (XPA) knockout mice, which are deficient in nucleotide excision repair.[69] These data suggest that the IL-12-induced suppression of UV-induced IL-10 production[61] may be a function of its ability to repair pyrimidine dimer formation, analogous to what is found when T4N5-containing liposomes are applied to UV-irradiated skin.[23] This idea was confirmed recently. Schwarz and colleagues found that treating wild type mice with IL-12 prevented UV-induced immune suppression and the depletion of Langerhans cells from the skin of UV-irradiated mice. However, treating nucleotide excision repair XPA$^{-/-}$ mice with IL-12 did not restore immune function. This finding suggests that IL-12's ability to restore immune function in a UV-irradiated individual is dependent upon DNA repair. On the other hand, Schwarz and colleagues found that the ability of IL-12 to break UV-induced tolerance was independent of its ability to mediate DNA repair; IL-12 "broke" UV-induced tolerance in both wild type and XPA$^{-/-}$ mice. These findings indicate that IL-12 mediates its restorative effects on UV-induced immune suppression via two mechanisms. Its effect on Langerhans cell migration and overcoming the UV-induced suppression of CHS is mediated by inducing DNA repair; whereas the ability of IL-12 to break UV-induced tolerance occurs via a DNA repair independent mechanism.[70]

T "SUPPRESSOR" CELLS

CD4+CD25+ T Regulatory Cells

One consequence of hapten sensitization through UV-irradiated skin is the induction of T suppressor (T regulatory) cells.[34] Although many have reproduced the original finding, the concept of suppressor T cells is still associated with some degree of controversy. For many years, mainstream immunologists ridiculed the existence of suppressor T cells. Failure to identify the cells that transfer immune suppression, failure to clone these cells or identify cell surface markers unique to these cells, failure to clone putative restriction factors (i.e., I-J) contributed to the general demise of interest in suppressor T cells. Regardless, it is clear from adoptive transfer experiments that suppressor T cells reside in the lymphoid organs of UV-irradiated mice. Recent findings, however, especially regarding UV-induced suppressor T cells have shed new light on this old problem. An important observation was the finding that UV-induced suppressor T cells mediate their suppressive effects by releasing well-known and *bona fide* immune regulatory factors, particularly IL-10.[50] The second finding was that UV-induced suppressor T cells express well-recognized T cell activation markers. For example, CTLA-4 (CD152) is a marker expressed on activated T cells and one that has been shown to negatively regulate T cell function.[71] Schwarz and colleagues lost the ability to transfer UV-induced immune suppression when CTLA-4 positive T cells were depleted from the cell population injected into the recipient mice. Conversely, when CTLA-4-positive cells were enriched from the spleens of UV-irradiated mice, transferring as few as 5×10^5 into a normal recipient could transfer immune suppression. Injecting anti-CTLA-4 monoclonal antibody into recipient mice that received UV-induced suppressor T cells also blocked immune suppression. When the CTLA-4-positive cells were stimulated *in*

vitro, they secreted a variety of cytokines, including IFN-γ, transforming growth factor beta (TGF-β), IL-2, and IL-10, but only IL-10 monoclonal antibody neutralized the suppressive activity *in vivo*, indicating the mechanism by which the CTLA-4-positive suppressor cells abrogate CHS is by releasing IL-10.[72] Because these cells secrete IFN-γ and IL-10, but not IL-4 they cannot easily be designated as Th1 or Th2 cells, but more closely fit into a class of IL-10 secreting T regulatory cells known as Tr1 cells[73]. A very recent report demonstrated that these cells also play a role in suppressing UV-induced skin cancer induction.[74]

Another important cell surface receptor expressed by UV-induced T regulatory cells is CD25 (IL-2 receptor). The seminal discovery by Sakaguchi and colleagues that a naturally occurring, thymic derived, CD4+CD25+ T cell could regulate autoimmunity *in vivo*, provided a rebirth of suppressor T cell biology.[75] CD4+CD25+ T regulatory cells comprise 5 to 8% of total T cells, express CTLA-4 and glucocorticoid-induced TNF-like receptor on their surface. Foxp3, a member of the fork head winged helix family of transcription factors, is expressed by T regulatory cells and controls the function and development of these cells. CD4+CD25+ T regulatory cells suppress cell proliferation and inhibit IL-2 mRNA production in target cells. The cells have been shown to regulate autoimmune thyroiditis, diabetes mellitus, allograft rejection, tumor immunity, and the immune response to intracellular pathogens.[76] Recent studies suggest that the UV-induced T regulatory cells are CD4+,CD25+.[77]

NKT Cells

As mentioned above, the early studies of Kripke found that exposure to sub-carcinogenic doses of UV radiation suppresses the immune response of recipient mice and allows for the progressive growth of transplanted UV-induced regressor tumors.[2] Adoptive transfer of antigen-specific T lymphocytes[78]could transfer the immune suppression to normal syngeneic recipient mice.

Although the initial reports documenting the existence of suppressor T cells are over 20 years old, initial progress in determining the mechanism through which these cells actually blocked immune function was slow. An important advance in the field was the observation that UV-induced suppressor T cells mediate their immune suppressive effects by secreting IL-4 and IL-10.[50] Two types of CD4+ T cells are known to primarily secrete IL-4, Th2 cells and a unique subset of T cells known as Natural Killer T cells. Natural killer T (NKT) cells, unlike conventional T cells, co-express T cell surface markers (CD3, CD4, TCR αβ) and markers normally found on natural killer cells (DX5, NK 1.1, Fc-γ receptor, Ly 49a). Also unlike conventional T cells, NKT cells exhibit an activated phenotype ($CD44^{high}$, $CD62L^{low}$) and are found in very low frequencies in peripheral lymphoid organs.[79] Natural killer T cells are restricted by the non-polymorphic class I-like molecule, CD1 and while the majority of CD1-restricted NKT cells express the invariant Vα14Jα18 receptor,[80] populations of CD1 restricted NKT cells that express a different pattern of T cell receptor usage, albeit restricted, have been described.[81] Within hours of activation, NKT cells secrete high concentrations of immunoregulatory cytokines, including IL-4,[82] making them uniquely suited to function as immune regulatory cells.

We tested the hypothesis that the UV-induced suppressor T cell is a NKT cell. To do this we first isolated CD4+ cells from the spleens of UV-irradiated mice and then separated the conventional T cells from the NKT cells by selecting the CD4+, DX5+ cells. When stimulated with anti-CD3 monoclonal antibody, CD4+, DX5+ cells released large amounts of IL-4 within hours of stimulation. Transfer of CD4+, DX5+ T cells from normal mice, or injecting CD4+ DX5- cells from UV-irradiated mice did not suppress tumor rejection. Transfer of CD4+, DX5+ T cells from mice exposed to UV (single exposure) and immunized with *C. albicans* suppressed the induction of DTH, in an antigen-specific fashion. The UV-induced suppressor T cells were CD1 restricted. No immune suppression was observed in UV-irradiated CD1-deficient mice, nor did suppressor cells induced in wild type mice suppress the induction of DTH when transferred into $CD1^{-/-}$ mice. In addition, NKT cells from mice exposed to a sub-carcinogenic dose of UV radiation suppressed the rejection of a highly antigenic UV-induced skin tumor when transferred into recipient mice. Transfer of as few as 1 million CD3+, CD4+, DX5+ T cells from the spleens of chronically irradiated mice (UV tumor susceptible) into age-matched recipient mice suppressed the rejection of a UV-induced regressor tumor. These cells did not suppress the rejection of chemically induced regressor tumor, indicating that the cells only recognize antigens unique to UV-induced skin cancers. These data indicate that the UV-induced regulatory T cells that suppress DTH and tumor immunity belong to a unique class of T cells known as NKT cells.[83]

SUMMARY AND CONCLUSIONS

The phenomena and the mechanisms underlying UV-induced immune suppression have been of interest to dermatologists, immunologists and cancer biologists for over 25 years. Undoubtedly, the driving force behind these studies is the association between skin cancer formation and immune suppression. This is why dermatologists and immunologists are generally well versed with the phenomena of UV-induced immune suppression, but also why this subject is generally not well known by most toxicologists.

However, the lessons that are learned from studying the mechanism by which UV radiation induces immune suppression are valuable for immunotoxicologists. As an immunotoxin, UV radiation is unique, in that all of its energy is absorbed in the uppermost layers of the skin and very little penetrates through the epidermis. Yet, UV exposure induces a systemic downregulation of the immune response. Cell mediated immune reactions, particularly Th1 cell driven immune reactions, are especially susceptible to the immunosuppressive effects of UV radiation, yet T cells are not directly targeted by UV radiation *in vivo* because few T cells are found in normal skin. Rather, indirect mechanisms have evolved that contribute to immune suppression. Do other dermal immunotoxin employ similar mechanisms? For a number of years my laboratory has been studying the immune suppression that results after dermal exposure to jet fuels[84–86]. Much to our surprise, we have noted that the mechanisms involved are very similar to the immune suppression induced by UV radiation. We find preferential suppression of Th1-driven immune reactions such as DTH and CHS, we fail to see suppression of antibody production *in vivo*, and we note that cytokines such as PGE_2 and IL-10 are involved. Moreover, injecting jet fuel treated mice with recombinant IL-12 reverses

immune suppression. Perhaps more importantly, we find that we can block jet fuel-induced immune suppression by blocking PAF receptor binding *in vivo*. Furthermore, jet fuel treatment will induce keratinocytes to release PGE_2, and this effect can be blocked if we interfere with PAF-receptor binding. We also know that we can induce immune suppression by applying relatively small amounts of jet fuel to the skin over a period of time.[84] In light of the fact that the penetration of a compound through the skin is dependent in part upon local concentration and duration of exposure,[87] small repeated exposures may result in immune suppression with marginal penetration through the skin. This suggests that the effect of jet fuel on the skin maybe by itself sufficient to initiate a cascade of events that activates immune suppression.[88] Do other dermal immunotoxins behave in a similar manner? Where should we place our focus, on the skin or on the effect of the chemical directly on immune organs?

A prominent theme that becomes apparent when studying UV-induced immune suppression is redundancy. Why did nature provide for at least three photoreceptors for UV-induced immune suppression? Why are there multiple, and at times, overlapping mechanisms for immune suppression. Why have at least two different types of regulatory T cells evolved? More importantly, why does exposure to UV radiation, a common and daily event, induce immune suppression? We suggest that UV-induced immune suppression is a transient side effect of the processes used to repair UV-induced DNA damage and maintain genomic integrity. After genomic stress, a cell must progress through a series of checkpoints that determine whether that cell lives or dies. UV exposure promotes arrest at the G2/M checkpoint, which allows for DNA repair. If DNA repair is successful, the cell proceeds through the cell cycle, if not apoptosis and cell death results. MAP kinase p38 plays a critical role in the initiation of G2/M arrest after UV exposure.[89] At the same time MAP kinase p38 activates PLA_2 the first enzymatic step in PAF synthesis. One consequence of this process is the synthesis of PAF and the production of the down stream mediators (PGE_2, and IL-10) that induce immune suppression. The observation that reactive oxygen species, which are well known for their ability to damage DNA,[90] also induce immune suppression[20] and induce PAF synthesis,[91]is consistent with this hypothesis. Also consistent with this hypothesis are the recent findings reported by Böhm and colleagues.[92].UV exposure causes keratinocytes to release the neuropeptide α-melanocyte-stimulating hormone, which in turn cause the release of the immune suppressive cytokine IL-10.[93] Böhm and colleagues report that α-melanocyte-stimulating hormone reduces the number of pyrimidine dimers in UV-irradiated dermal fibroblasts and protects against apoptosis. These data are all consistent with the idea that immune suppression is a consequence of the cell's attempt to maintain genomic integrity.

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16 Immunotoxicology and Inflammatory Mechanisms of Arsenic

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INTRODUCTION

Environmental contamination by arsenic, from natural or anthropogenic sources, represents a global human health concern; millions of people worldwide are exposed to arsenic compounds through contaminated drinking water, occupational use of industrial arsenicals, inhalation of arsenic compounds released through mining, processing, and burning of ores and coal, pest control efforts, and use of certain pharmaceuticals and herbal remedies. As a naturally occurring element in the earth's crust, arsenic can be released into groundwater through geothermal activity and erosion. Drinking arsenic-contaminated water is the primary means of human exposure [1, 2].

This chapter presents specific information with regard to the effects of environmental and occupational exposure to arsenic on inflammatory processes, the immune system, and host defense. While the focus is on the *in vivo* and *in vitro* effects of arsenic on host immune responses (e.g., immunotoxicity and hypersensitivity) and their relationship to clinically observed manifestations of arsenic toxicity (e.g., inflammation and skin cancer), information on the potential mechanisms through which arsenic may exert its biological effects is also provided.

ARSENIC SPECIES OF PRIMARY BIOLOGICAL RELEVANCE

Humans can be exposed to several different chemical forms of arsenic and the adverse effects are, in part, a function of the species involved. Organic forms of arsenic (arsenobetaine and arsenocholine) are found primarily in fish and shellfish, and are considered to be essentially nontoxic. Arsine gas (AsH_3) is an N-type dopant source used in the production of gallium arsenide (GaAs) semiconductor devices. Due to its hemolytic effects, arsine is currently being investigated as a potential treatment for malaria [3]. Arsenic trioxide (As_2O_3), purified from a traditional Chinese medicine, is widely used to treat retinoic acid resistant acute promyelocytic leukemia. The ability of As_2O_3 to induce immune function changes, apoptosis, and differentiation has led to the investigation of this arsenical as a potential therapeutic agent for other malignancies. However, As_2O_3 inhibits many phosphatases and may disrupt multiple signaling pathways leading to both pro- and anti-oncogenic outcomes [4]. Long-term treatment with arsenic-containing drugs has been associated with development of Bowen's disease (intraepidermal squamous cell carcinoma), squamous cell carcinoma, and other cancers and skin disorders [5]. Most environmental arsenic contamination is in the form of inorganic trivalent arsenite (AsIII) and pentavalent arsenate (AsV), which undergo a series of reduction and methylation reactions in vivo and in vitro to form methylated metabolites. The proposed scheme is AsV → AsIII → methylarsonic acid (MAsV) → methylarsonous acid (MAsIII) → dimethylarsinic acid (DMAsV) → dimethylarsinous acid (DMAsIII) → trimethylarsine oxide (TMAsVO) [6]. Inorganic arsenic (iAs) is listed as a Group A human carcinogen [7]. Chronic exposure to iAs has been established as a key etiological component in the development of peripheral vascular disease and skin, lung, liver, bladder, and kidney cancer [1].

IMMUNOMODULATION BY ARSENICALS

HUMAN STUDIES

Ingested arsenic localizes to the skin [2, 7], where it may alter cutaneous immune responses. The delayed type hypersensitivity (DTH) response to 2,4-dinitrochlorobenzene (DNCB) was suppressed in Bowen's disease patients [8]. Langerhans cells (LC) in skin lesions and perilesioned skin from arsenic-induced Bowen's disease and carcinomas were reduced in number and were morphologically altered, having a notable loss of dendrites [9]. These data suggest that chronic exposure to arsenic in drinking water may

alter LC, inhibit antigen processing and presentation, and impair lymphocyte proliferation in response to cutaneous allergens.

Hormone effects may influence the immune response to arsenic [10]. *In vitro*, sodium arsenite ($NaAsO_2$) was more cytotoxic to CD3+ and CD4+ T cells from women than from men, while cytotoxic T-cells (Tc, CD8+) were not affected in either gender. Peripheral blood mononuclear cells (PBMC) from arsenic-induced Bowen's disease patients produced more IL-2 than controls [8]. Conversely, IL-2 secretion by PBMC from healthy donors exposed to arsenic in vitro was suppressed. Addition of recombinant IL-2 to the cultures reversed proliferative inhibition [11]. For the first 72 hours of culture the T-cell population was deficient in producing IL-2; at 72 hours recovery of both intracellular and secreted IL-2 was observed. During this time, a higher proportion of cells were in the G(O)/G(1) phases of the cell cycle than in the G(2)/M phases, suggesting that arsenic may block T-cell progression through the cell cycle by delaying production of IL-2 [12].

Arsenite induced abnormal monocyte differentiation in response to Macrophage Colony Stimulating Factor (MCSF) and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), and altered morphology, cell surface markers, and phagocytic activity of human macrophages [26]. Human PBMC stimulated with GM-CSF and incubated with As^{III} differentiate into small, CD14+, HLA-DR+ cells with membrane projections. These nonadhesive, abnormal macrophages induced greater allogeneic and autologous T-cell responses, and secreted more IL-1α and TNFα in response to lipopolysaccharide (LPS) stimulation compared to controls [13].

Inorganic arsenicals can modulate expression of certain pro-inflammatory cytokines and growth factors that are critical to maintaining homoeostasis and immune function. Sodium arsenite induced increased secretion of GM-CSF, TNFα, and TGFα in human primary keratinocytes [14], and TNFα and TNFR1 in PBMC in culture [15]. However, TNFα and IFNγ production by PBMC were decreased in Bowen's disease patients [8]. Gene expression analysis of skin from arsenic-induced Bowen's disease patients demonstrated upregulation of GM-CSF and TGFα in lesioned skin compared to non-lesioned skin [16]. Elevated levels of TGFα have been detected in the urine and serum of arsenic-exposed cancer patients and high-risk individuals who, subsequent to TGFα detection, developed cancer. The concentration of urinary TGFα among residents of six Bangladesh villages that have high arsenic concentrations in the water supply correlated with total urinary arsenic in individuals with skin lesions, but not in individuals without skin lesions, indicating that TGFα might be a useful biomarker for arsenic-induced skin disorders [17].

Experimental Animal Models

Host Resistance

Gallium arsenide altered host resistance in B6C3F1 mice, resulting in increased resistance to infection by *Streptococcus pneumoniae* and *Listeria monocytogenes* [18], but increased susceptibility to *Staphylococcus aureus* [19] and to tumor development by B16F10 melanoma cells [18]. However, inhalation exposure to As_2O_3 led to increased

pulmonary susceptibility to *Streptococcus* and *Klebsiella pneumoniae* in mice [20], while mice that were orally exposed to tetra-arsenic oxide were more resistant to B16F10 tumor formation [21]. The apparent differential responses in host resistance could be a function of the strain of the infectious agent used or the specific arsenical compound. Due to the ubiquitous nature of arsenic in the environment, many microbes, including *S. aureus* [22] and *L. monocytogenes* [23], have developed mechanisms of resistance, including an arsenate reductase and an arsenic efflux protein [22].

Humoral-Mediated Immunity

T-dependent antibody production in response to antigen is a complex process requiring functional interaction of T-cell, B-cell, and macrophage populations. Both the IgM and IgG antibody-forming cell (AFC) response to sheep red blood cells (SRBC) was suppressed in rodents following acute oral, intratracheal, or intraperitoneal exposure to GaAs [18, 24] and by subchronic oral exposure to $NaAsO_2$ [18]. *In vitro*, GaAs and $NaAsO_2$ induced a dose-dependent suppression of the primary antibody response in mouse splenocytes [25] and As_2O_3 suppressed IgM, but not IgG, secretion in baboon splenocytes [26]. Chelation of gallium and arsenic by chemical-specific chelating agents administered 1 hour prior to and for 24 hours following GaAs exposure demonstrated that only the arsenic component was responsible for the immunosuppressive effect both *in vitro* and *in vivo* [25]. Separation and reconstitution of macrophages, T-cells and B-cells indicated that the functional capability of all three populations was impaired. The AFC response of control splenocytes in culture was suppressed by supernatant from GaAs-exposed splenocyte cultures, suggesting that GaAs alters the secretion of soluble mediators. Addition of IL-2 to cell cultures resulted in a dose-dependant reversal of GaAs-induced AFC suppression following *in vivo* exposure, suggesting that IL-2 may be a primary target for GaAs toxicity in antibody production [18].

Antigen processing has been implicated as a potential target for arsenic toxicity. Although GaAs did not affect murine splenic B-cell numbers or cell-surface expression of MHC class II, *in vivo* GaAs exposed splenic B cells were less effective in stimulating antigen-specific CD4+ helper T-cell hybridomas with intact antigens to secrete IL-2, than were the corresponding controls [27]. However, the arsenic-treated B-cells competently presented peptide fragments of the antigens to T-cells and elicited a response, suggesting that arsenic impairs antigen processing but not antigen presentation. The proteolytic activity of thiol cathespsin, a protease involved in antigen processing, was reduced by GaAs, suggesting that diminished thiol protease activity may also play a role in GaAs-induced impairment of antigen processing [27].

Cell-Mediated Immune Responses

The antiproliferative effects of arsenic are well documented. *In vitro* both trivalent and pentavalent arsenicals inhibit murine [28] and bovine [18] phytohemaglutinin (PHA)-stimulated lymphoproliferation at concentrations of ≥ 3 μM. Lymph node cells from arsenic treated, FITC-sensitized mice displayed reduced lymphoproliferation in response to Con A, suggesting that the mechanism of antigen processing/presentation may be altered by arsenic exposure, inhibiting T-cell responsiveness [29]. However, *in vivo*

exposure to low levels of $NaAsO_2$ [29, 30] and high levels of $NaAsO_2$ or GaAs did not alter the proliferative response of splenocytes [18, 30] or lymph node cells [29] to T-cell mitogens in unsensitized mice and rats, indicating that arsenic is not acting via a general anti-proliferative mechanism. Exposure to iAs suppressed the DTH response to injected keyhole limpet hemocyanin [18], bovine serum albumin [24], and PHA [30].

Innate Immune Responses and Soluble Mediators of Immunity

Macrophages play an important role in hypersensitivity as antigen presenting cells (APC) and by secreting cytokines that activate T-helper cells [31–33]. *In vivo* $NaAsO_2$ exposure altered the morphology of, and suppressed phagocytosis, cell adhesion and chemotactic migration by mouse macrophages, indicating impaired cell function [19, 34]. Arsenic-exposed macrophages from ovalbumin-sensitized mice failed to stimulate either control or arsenic-exposed antigen-responsive lymphocytes. Arsenic-exposed lymphocytes were responsive to control macrophages, indicating that the compound may alter the mechanism of antigen presentation but does not significantly affect antigen recognition by lymphocytes [35]. In GaAs treated mice, macrophages at the exposure site exhibited an enhanced ability to activate antigen-specific T-cells, while macrophages distal to the exposure site were impaired [31, 32]. This difference may reflect the local inflammatory response versus a systemic effect. Inflammation, histological changes, and alterations in cytokine expression were also manifested at the exposure site, but not in other organs. Inflammation at the exposure site could induce production of cytokines that activate APC and modulate a number of immune functions [31–33].

As was demonstrated in B-cells [27], the effect of arsenic on macrophage stimulation of T-cells was antigen-specific. The response of splenic macrophages to intact antigens requiring extensive enzymatic processing was inhibited following *in vivo* GaAs exposure; however, macrophages were able to competently process and present peptide fragments, solubilized proteins and opsonized SRBC to appropriately primed T-cells. Phagocytosis of latex beads was unimpaired and effects on MHC-II expression were inconclusive, thus those factors do not account for the alteration in antigen presentation. Again, thiol, but not aspartyl, cathepsin activity was consistently correlated with altered efficiency of antigen presentation. Taken together, these data indicate that GaAs may influence early events in antigen processing, including enzymatic activity associated with specific phagocytosis processes, rather than antigen presentation [31, 32, 36]. Arsenic suppression of antigen processing/presentation is not specific to one APC type, but can affect Langerhans cells, B-cells and macrophages. Additionally, multiple antigen-processing pathways may be affected. Inhibited antigen processing and presentation impairs T-cell stimulation and function, which could lead to deficient humoral and cell-mediated immunity and systemic immune suppression [32, 36].

Keratinocytes maintain the biochemical and physical integrity of the skin and secrete a number of inflammatory cytokines and growth factors that regulate irritant and allergic responses [14]. Chronic exposure to arsenite in drinking water increased expression of GM-CSF, TGFα, EGFR and TNFα in the skin of Tg.AC mice [16]. Arsenic-dependent TNFα and IL-1α upregulation were observed in rat lung phagocytes [37] and cultured HEL30 murine keratinocytes [38], respectively. Arsenite and AsV induced TNFα expression in mouse macrophages, while DMA and TMA were

suppressive [39]. Gallium arsenide treatment altered the production of IL-2, IL-4, IL-5 and IL-6 by mouse splenocytes in a time- and exposure-dependent fashion, and induced suppression of TGFβ and upregulation of 17 chemokines and cytokines, including IL-1α, IL-1β, TNFα, IL-18, IL-6, TNFβ, IL-12, IL-10, MIP-1α, and MIP-1β, in resident murine peritoneal leukocytes. The predominant cytokine-producing cell population was macrophages, and to a lesser extent B-cells; no cytokine staining was detected in T-cells. These data suggest arsenic modulates myeloid and B-cell function and support the theory that arsenic may induce inflammation and suppress antigen processing/presentation to inhibit T-cell function [33].

Hypersensitivity Responses

Inorganic arsenic does not penetrate intact mammalian skin well in aqueous solutions or soil and, although it binds to the skin, arsenic residue can be effectively removed by washing with soap and water [40, 41]. Due to the negligible dermal penetration, contact exposure to arsenic-contaminated water, wood, or soil is not believed to cause a serious health risk [2]. Contact irritant dermatitis is characterized by the secretion of proinflammatory cytokines by cutaneous cells in response to chemical stimuli, resulting in recruitment and activation of neutrophils, erythema, edema, and possibly ulceration, folliculitis, and hyperpigmentation at the site of exposure [42]. Contact exposure to industrial arsenicals has been reported to produce dermal irritation, especially in smelters and miners [7]. Allergic contact dermatitis is a delayed-type hypersensitivity reaction during which epidermal LC recognize a foreign antigen, transport it to the draining lymph nodes, and present it to responsive T-lymphocytes, which undergo clonal proliferation. Sodium arsenite and sodium arsenate were found to be Grade I allergens (slight) in the guinea pig maximization test and induced dermatitis in 0.5% of the patients tested in a standard patch test [43].

Ingested arsenic is efficiently absorbed through the gastrointestinal tract (60–90%), and as described above, concentrates in keratin-rich tissues such as the skin [1, 2, 7]. As observed in humans, LC numbers and function were suppressed in Balb/c mice following drinking water exposure to $NaAsO_2$ [29, 44]. Both the induction and elicitation phases of dermal sensitization to 2,4-dinitrofluorobenzene (DNFB) were reduced following arsenic exposure, and the percentage of activated, antigen-bearing LC in the draining lymph nodes was significantly decreased following sensitization, suggesting that arsenic inhibits migration of mature LC to, and presentation of antigen in, the draining lymph nodes, which may result in reduced T-cell proliferation [29].

MECHANISMS OF ARSENIC-INDUCED INFLAMMATION AND TOXICITY

Proinflammatory Cytokines

The dermatotoxicity of iAs is well established. Epidemiological studies identify an increased incidence of keratinocytic tumors associated with iAs exposure, as well as

precancerous lesions [1]. Although multiple cell types and biological events contribute to the pathological effects of iAs in skin, keratinocytes, fibroblasts, and macrophages are thought to be the cells which significantly contribute to iAs-mediated dermatotoxicity. As described above, iAs stimulates the secretion of keratinocyte-derived growth factors such as GM-CSF, TGF-α, and proinflammatory cytokines such as TNF-α and IL-6 in primary human epidermal keratinocytes *in vitro* [14]. These studies also demonstrated that *c-myc* expression, a positive indicator of proliferation, was increased in keratinocytes treated with iAs at sub-micromolar concentrations. Fibroblast growth factor (FGF), as well as growth factor receptor expression and function, also have been demonstrated to be modulated by iAs [45, 46]. Together, it is suggested that altered cytokine and growth factor expression is related to the development of keratoses and associated inflammation following chronic arsenical exposure [16].

Cell proliferation, inflammation, apoptosis and differentiation are under the tight control of many of the growth factors and cytokines modulated by arsenic exposure. Inorganic arsenic stimulates *c-jun* and *c-fos* transcription and AP-1 DNA binding in human keratinocytes [47] and fibroblasts [48], and the increase in AP-1 activity is proposed to result in the chronic stimulation of keratinocyte-derived growth factors, increased cell proliferation, inflammation, and neoplasia [49]. The processes of programmed cell death, or apoptosis, contribute to carcinogenesis and inflammation as well, and are hypothesized to contribute to iAs dermatotoxicity as long-term iAs exposure causes generalized resistance to apoptosis in cultured human keratinocytes [50] and modulates the pro-apoptotic effects of other carcinogens [51]. Inorganic arsenic also induces keratinocyte apoptosis by the FAS/FAS ligand pathway, which correlates with alterations in nuclear factor-kappa B (NF-κB) and AP-1 activity [52], and alters the expression of several cell cycle regulatory proteins including p53 in human keratinocytes [53] and cyclin D1 expression in human fibroblasts [54].

Signal Transduction Targets

Cells recognize and respond to extracellular stimuli by using signaling cascades to coordinately regulate mitosis, apoptosis, and inflammation. One example of these cascades is the mitogen activated protein kinase (MAPKs) pathways, represented by extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), and p38 MAPKs [reviewed in 55]. MAPK pathways are involved in the inflammatory response where, for example, NF-κB antagonizes apoptosis induced by the inflammatory cytokine TNF-α via the JNK pathway [56], and 2,2'-azobis (2-amidinopropane) dihydrochloride, known to generate free radicals, stimulates an increase in the proinflammatory protein cyclooxygenase-2 (COX-2) by an increase in ERK, p38 and JNK phosphorylation [57]. Inorganic arsenic works through MAPK signaling (e.g., ERK, JNK, and p38) pathways to regulate various cell processes in part via the regulation of genes encoding *c-fos,* and *c-jun* transcription factors. The latter may play an important role in inflammatory processes that contribute to iAs-induced skin carcinogenesis. Working through MAPK signaling, iAs induces murine T lymphocyte apoptosis via the activation of JNK [58], and apoptosis in leukemia U937 cells that is dependent on activation of p38, inactivation

of ERK and the Ca^{2+}-dependent production of superoxide [59]. Finally, iAs has been shown to inhibit LPS-induced nitric oxide production in macrophages via effects on Raf-1, which is a component in the ERK MAPK pathway [60].

NF-κB is a ubiquitous transcription factor that is under the control of stress activated MAPKs and regulates inflammatory cytokine expression. Barchowsky and colleagues [61] showed that iAs activated NF-κB through an oxidative stress-mediated mechanism in endothelial cells. Subsequent studies suggested that activation of NF-κB by iAs was dependent on cell type and that epithelial-like cells appear to be highly sensitive to arsenic treatment [62]. Inorganic arsenic enhances AP-1 and NF-κB DNA binding and induces stress protein expression in precision-cut rat lung slices where a majority of the NF-κB is localized in the cytoplasm of type-II epithelial cells and alveolar macrophages [63]. Inorganic arsenic induces *in vitro* growth inhibition and stimulates apoptosis of malignant lymphocytes and myeloma cells via the suppression of the DNA-binding activity of NF-κB. This activity appears to be dependent upon intracellular glutathione (GSH) and hydrogen peroxide levels [64]. Inorganic arsenic has also been shown to stimulate apoptosis in peripheral blood T lymphocyte subsets by generating oxidative stress [65], and to induce polymorphonuclear leukocyte apoptosis via an increase in intracellular respiratory bursts [66].

Oxidative Stress and Reactive Oxygen Species (ROS)

The respiratory burst of infiltrating polymorphonuclear leukocytes and macrophages in inflamed skin produce high local levels of ROS. Superoxide anion, hydroxyl radicals, and hydrogen peroxides are ROS intermediates formed during oxidative metabolism and respiratory bursts, as well as following iAs exposure [67]. The mode by which intracellular ROS are generated by iAs is not completely understood but as iAs is oxidized within the cell into AsV, superoxide anions may be produced by the one-electron reduction of molecular oxygen [68]. There is also evidence to suggest that intracellular metabolism of iAs into DMAsIII is coupled with the production of superoxide anions and hydroxyl radicals [69]. *In vitro*, ROS have been shown to mediate iAs-related genotoxicity [70]. Clinically, the rate of 8-oxodG-positive DNA modifications, (a sensitive marker of oxidative damage), is significantly higher in iAs-associated human skin cancer than in iAs-unrelated human skin cancer [71].

Understanding the pathologic roles of oxidative stress and ROS may contribute to developing an integrated paradigm for cytokine expression, MAPK pathway modulation, and the generation of oxidative stress induced by iAs. ROS may be generated by mitochondria during respiration via distinct enzyme systems. Low levels of ROS contribute to cellular signaling and play an important role in normal cell proliferation; however, the constant activation of transcription factors (e.g., NF-κB and AP-1) elevates ROS levels during tumor progression [72]. Further, the ROS generated during cell division may inhibit phosphatases and, in particular, tyrosine phosphatases, facilitating the activation of associated receptor tyrosine kinases [73] that are some of the upstream activators in MAPK pathways. ERK-dependent cell division is, for example, superoxide dependent

[74]. ROS levels increase in cells exposed to stress agents [75], that promote apoptosis by stimulating ASK1, JNK and p38 [76], and p53 [77].

Intracellular and extracellular ROS activate tyrosine and serine-threonine kinases (i.e., the MAPK family members). Following TNF-α, TGF-β or EGF stimulation, intracellular ROS are generated which stimulate various signaling pathways [73]. Tyrosine kinase receptors (e.g., EGF, PDGF and TGF-α) may be activated by ROS directly via protein sulfhydryl group modifications, or inhibition of phosphotyrosine phosphatases (PTPases) and subsequent receptor activation. The latter is possible as PTPases contain a redox-sensitive cysteine at their active site [78], and oxidation of protein sulfhydryl groups results in the inactivation of PTPases.

The Relationship Between iAs, ROS, and Signaling

Recent studies offer a number of clues linking ROS and stress activated protein kinases (SAPKs; e.g., JNK and p38 MAPKs) to the mode of action of iAs. ASK1, an upstream regulator of SAPKs, is inhibited in non-stressed cells through its association with the redox regulator thioredoxin [79]. Increased ROS levels lead to the dissociation of this complex and thereby enable the activation of ASK1 and downstream SAPKs [80]. Interestingly, thioredoxin expression is increased by iAs in human fibroblasts [81] as is skin carcinogenesis in a keratinocyte-directed thioredoxin-1 transgenic mouse model [82]. A similar redox switch to thioredoxin has been documented for JNK: With ROS triggering the detachment of JNK associated glutathione-S-transferase-π(GSTpi) and thereby facilitating JNK activation [83]. JNK activation is seen with iAs treatment of human keratinocytes [84] and may be the result of the loss of negative inhibition via GSTpi due to elevated ROS levels. ROS-dependent activation of JNK may also involve downregulation of a JNK phosphatase [85]. Again, iAs has been demonstrated to modulate phosphatases that regulate JNK activity [86], possibly via increased cellular ROS. These points illustrate that ROS can stimulate specific signaling cascades (e.g., MAPKs) controlling cell function, some of which are targets of iAs.

SUMMARY

Arsenicals can modulate multiple components of the immune response in both laboratory rodents and isolated human cells. We have illustrated several of the relationships between factors thought to mediate arsenic-induced toxicity, such as cytokine and growth factor expression and the generation of ROS and alterations in MAPK signaling. These events affect each other and together play an important role in determining a cell's responsiveness to cell division, inflammatory events, and apoptotic signals. In combination they most likely work together in a "less than random" manner to contribute to iAs toxicity in primary and secondary lymphoid tissues as well as in immune cells associated with specific organ systems such as the skin and lung.

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17 Modulation of Inflammatory Gene Expression by Trichothecene Mycotoxins

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OVERVIEW

Inflammation is the normal host response to infection or injury that mediates immune elimination of pathogens and tissue repair. Inflammatory processes include increased production of cytokines, chemokines, nitric oxide, and eicosanoids by the innate immune system in conjunction with altered leukocyte homing, all of which greatly impact acquired immunity. Aberrant inflammatory responses evoke both acute injury such

as endotoxin shock and chronic diseases ranging from glomerulonephritis to cancer. Chronic inflammatory diseases impact millions of people in the United States annually and contribute extensively to morbidity, mortality, and health care costs.

Mycotoxins are low molecular weight secondary metabolites produced by fungi that are toxic to humans and animals.[1] Of the several hundreds of mycotoxins that have been identified to date, at least a dozen are believed to negatively impact public health. The chemical structures of these fungal toxins are as diverse as are their tissue targets. Many mycotoxins adversely affect immune function and this topic has been reviewed previously.[2] Although immunosuppression is frequently reported, recent studies highlight the ability of some mycotoxins, most notably the trichothecenes, to modulate inflammation. This chapter will discuss the mechanisms by which trichothecenes induce inflammatory gene expression and concurrent competing apoptotic pathways. In addition, the capacity of trichothecenes to interact with other inflammagenic stimuli will be highlighted.

TRICHOTHECENE-INDUCED INFLAMMATORY GENE EXPRESSION AND APOPTOSIS

Chemical Types and Occurrence

Trichothecenes are a diverse group of sesquiterpenoid metabolites that are produced by the fungi *Fusarium, Myrothecium, Cephalosporium, Verticimonosporium*, and *Stachybotrys,* which are found in food and the indoor environment. More than 180 trichothecenes have been identified, most of which can bind to eukaryotic ribosomes and inhibit protein translation.[3] The toxins are structurally divided with Types A, B, and D being most important in food and environment. The Type A trichothecenes include T-2 toxin that has been associated with human alimentary toxic aleukia, a disease resulting from consumption of *Fusarium*-infected grain in Russia during WWII.[4] There has been suspected use of this toxin as a chemical warfare agent.[5] Diacetoxyscirpenol (anguidine), another Type A compound, has been evaluated for its chemotherapeutic potential.[6] The Type B trichothecenes contain a keto group at carbon 8 of the parent epoxytrichothecene nucleus, and are detectable as contaminants of cereal grains such as wheat, barley and corn.[7] Of these, deoxynivalenol (DON) colloquially known as "vomitoxin" is most frequently encountered in cereal-based foods. DON contamination has been associated with increased *Fusarium* head blight in wheat and barley in North America resulting from cool wet spring weather conditions and the use of no till farming. Finally, the Type D trichothecenes contain a large macrocyclic ring fused to the epoxytrichothecene nucleus, and include the satratoxins, roridin, and verrucarins.[8] Type D trichothecenes are found in buildings that contain *Stachybotrys* mold contamination resulting from water or moisture damage.[9]

Adverse Health Effects and Cellular Targets

Based on trichothecenes' effects in experimental animals as well as their association with human and animal toxicoses, there is continued concern over the relationship

between exposure to these toxins and adverse health outcomes. Animal studies have revealed a complex spectrum of toxic effects. High dose acute exposure to trichothecenes causes "radiomimetic" responses that include diarrhea, vomiting, leukocytosis, and hemorrhage with extremely high doses causing shock-like syndrome that can result in death.[10] Chronic trichothecene exposure causes anorexia, reduced weight gain, diminished nutritional efficiency, neuroendocrine changes, leukocytopenia, and immune modulation.[11]

Understanding the molecular mode of action of trichothecene mycotoxins is critical for predicting potential deleterious human health outcomes. Although inhibition of gene translation is considered to be central to the effects of these toxins in cells, tissues and intact animals, many of the observed toxicities now appear to relate to rapid dysregulation of cell signaling and consequent alterations in expression of downstream genes that regulate inflammation and immune responses. Leukocytes, including macrophages, B cells and T cells, which represent the functional cell repertoire of the immune system, are exquisitely sensitive to trichothecenes.

Paradoxical Immunotoxic Effects

Ex vivo studies have revealed that trichothecenes can both inhibit and stimulate leukocyte function.[12] For example, trichothecenes are toxic to alveolar macrophages,[13] but drive differentiation of human myeloid leukemic cells.[14] Dose-dependent decreases or increases in B- and T-cell mitogen responses are observable in lymphocytes from animals exposed to T-2 toxin, DON, or various macrocyclic trichothecenes; these toxins similarly impair or enhance mitogen-induced lymphocyte proliferation *in vitro*.[12] Rank order of inhibitor potency in rodent and human lymphocyte proliferation assays is Type D > Type A group > Type B group and is dependent on degree of acylation as well as of uptake and metabolism.

Exposure to trichothecenes at levels that partially inhibit translation upregulates expression of many inflammatory and immune-related genes including macrophage, Th1 and Th2 cytokines as well as chemokines, cyclooxygenase 2 and inducible nitric oxide synthase.[15–18] Contrastingly, suppressive effects of trichothecenes on leukocyte function are intimately linked with the induction of apoptosis as has been demonstrated in macrophages, T cells and B cells both *in vivo* and *in vitro*.[19,20]

IgA dysregulation is a well-characterized immunopathological outcome that both illustrates the complexity of trichothecene effects and been a basis for mechanistic exploration. IgA production is markedly elevated in mice fed DON or the related trichothecene nivalenol and resultant immunopathologic effects occur that closely mimic a common type of human glomerulonephritis, IgA nephropathy (IgAN).[21] Relevant effects include elevations in serum IgA and IgE, circulating IgA immune complexes, kidney mesangial IgA deposits and hematuria as well as polyclonal activation of IgA secreting cells and polyreactive IgA autoantibody secretion. DON's capacity to drive polyclonal expansion of IgA-secreting cells in mice is mediated by increased cytokine production by macrophages[22] and T cells.[23] IL-6 induction is particularly critical for DON-induced IgAN based on *ex vivo* studies[23] and *in vivo* feeding studies with IL-6 deficient mice.[24] Prior upregulation of cyclooxygenase-2 (COX-2) and resultant produc-

tion of prostaglandin metabolites might also contribute to DON-induced IL-6 production *in vitro* and *in vivo.*[17]

Trichothecene immune modulation occurs across a spectrum whereby low doses stimulate inflammation and immune function but high doses evoke leukocyte apoptosis leading to immunosuppression. In some cases, these manifestations are concurrent. Elucidation of the mechanistic basis for changes in cell signaling and gene expression that occur over this spectrum will give insight into underlying molecular trichothecene effects, facilitate structure function studies, and ensure valid safety assessments.

Induction of Gene Transcription

Trichothecene-mediated elevations in cytokines, chemokines and other inflammation- and immune-related proteins are preceded by up-regulation of mRNAs for these genes.[12] Understanding how DON induces cyclooxygenase-2 (COX-2) illustrates the underlying mechanisms for such upregulation. COX-2 is a rate-limiting enzyme that catalyzes oxygenation of arachidonic acid to prostaglandin endoperoxides, which are subsequently converted enzymatically into prostaglandins and thromboxane A_2.[25] COX-2 resembles an early response gene product in that it is strongly induced by mitogenic and proinflammatory stimuli, and superinduced by protein synthesis inhibitors.[26,27] DON has been shown by our laboratory to induce COX-2 gene expression in the macrophage and the mouse as well as enhance *in vitro* production of prostaglandin E2 (PGE_2), a major metabolite of COX-2.[28,29]

Using COX-2 promoter-reporter constructs, Moon and Pestka[28] demonstrated that DON induces transcription in the macrophage. Interestingly, Type B trichothecenes at equitoxic levels (i.e., cause equivalent inhibition in cell viability assay) significantly promoted transcriptional activity of COX-2 gene in the constructed vector-transfected cells.[17] In contrast, representative Type A (diacetoxyscirpenol and acetyl T-2 toxin) and Type D (satratoxin F and roridin A) trichothecenes did not increase COX-2 promoter activity when present at equitoxic concentrations. As for COX-2, DON induces transactivation of TNF-α in macrophages[30] and IL-2 in T cells.[31] Effects of DON on transcription factors associated with inflammatory gene responses have been examined by electrophoretic mobility shift assay. NF-κB, AP-1 and C/EBP have binding sites in the promoters of proinflammatory cytokines. In the RAW 264.7 macrophage model, DON induces AP-1, C/EBP and NF-κB binding.[32] DON also increases NF-κB and AP-1 binding activity in EL-4 and primary T cells.[31,33] Taken together with reporter studies, increases in transcription factor binding in leukocytes are consistent with trichothecene-induced gene transcription.

Post-Transcriptional mRNA Stabilization

DON also enhances COX-2 mRNA stability.[28] Such modulation is usually explained by the presence of multiple copies of AUUUA motif in the 3'-untranslated region (3'-UTR) of COX-2 mRNA, since the AU-rich element (ARE) in the 3'-UTR of an mRNA targets a transcript for rapid degradation.[34] To test this possibility a constitutive SV40

promoter-luciferase reporter gene was ligated with three different 3'-UTR sections of the COX-2 gene. Cells expressing this reporter were treated with DON to analyze the effect of the toxin on stability of the reporter transcript via 3'-UTR.[17] DON enhanced the products of vectors containing the AU rich region, but had no significant effect on other reporters. Therefore, DON is likely to enhance COX-2 mRNA stability via AU-rich elements. All COX-2-inducing Type B trichothecenes showed significantly increased levels of reporter, which can be related to the stabilization of mRNA transcripts via 3'-UTR. In contrast, representative Type A and D trichothecenes had no discernable effect at equitoxic concentrations. The regulation of 3'-UTR including AU-rich repeats might thus be involved in mRNA stabilization by COX-2-inducing Type B trichothecenes.

DON affects mRNA stability of IL-6 and TNF-α similarly to that of COX-2.[30,35] DON also prolongs half-life of IL-2 mRNA in EL-4 cells in a concentration-dependent fashion.[36] These results suggest that DON-mediated cytokine mRNA upregulation in both macrophages and T cells is also explainable, in part, by enhanced mRNA stability.

Role of Mitogen-Activated Protein Kinases

What are the mechanisms by which trichothecenes exert their transcriptional and post-transcriptional effects? The 60S ribosomal subunit is a well-known molecular target of trichothecenes in leukocytes and other actively proliferating eukaryotic cells,[3] whereas attempts to demonstrate alternative receptors have not been successful.[37,38] Translational inhibitors that bind to ribosomes rapidly activate mitogen-activated protein kinases (MAPKs) and apoptosis via a mechanism termed the "ribotoxic stress response."[39,40]

MAPKs regulate cell growth, differentiation, and apoptosis[41] and are central transducers of the inflammatory response.[42] MAPK subfamilies include: (1) p44 and p42 MAPKs, also known as extracellular signal regulated protein kinase 1 and 2 (ERK1 and 2), (2) p54 and p46 c-Jun N-terminal kinase 1 and 2 (JNK 1/2), also referred to as stress-activated protein kinases (SAPK 1/2); and (3) p38 MAPK. We and others have demonstrated that trichothecenes activate JNK, ERK, and p38 *in vitro*[28,43,44] and *in vivo*[45] suggesting that the ribotoxic stress response might be central to transducing trichothecene toxicity. DON induces the phosphorylation of the MAPKs ERK 1⁄2, p38 and JNK 1⁄2 within 15 minutes in cloned macrophages, and the effects could last up to 4 hours.

Continuing with the theme of COX-2 regulation, ERK and p38, but not JNK, are involved in trichothecene-induced transactivation of this gene.[17,28] p38 also appears to mediate trichothecene-induced mRNA stability. DON-induced TNF-α expression is similarly regulated.[30]

Upstream Transducers MAPK Activation

Using chemical inhibitors of upstream kinases that potentially drive trichothecene-induced MAPK activation, two putative kinases have been identified: double-stranded RNA- (dsRNA)-activated protein kinase (PKR)[46] and hematopoietic cell kinase (Hck).[47]

PKR, a widely-expressed serine/theonine protein kinase, is activated by dsRNA, interferon, and other agents.[48] PKR was first described to inhibit translation via phos-

phorylation of eukaryotic initiation factor 2 α-subunit (eIF2α)—an evolutionarily conserved antiviral response. Besides eIF2α phosphorylation and auto-phosphorylation activities, PKR has wide serine-theonine kinase substrate specificity. Notably, this kinase integrates signals for ligand-activated stress-activated protein kinase pathways resulting in JNK and p38 stimulation.[48] PKR also mediates apoptosis induction by dsRNA, LPS, and TNF-α as well as induction of cytokines.

PKR is rapidly activated by DON in cloned macrophages (1 to 5 min) as evidenced by autophosphorylation and by phosphorylation of eIF2α.[46] This latter effect is concurrent with eIF2α degradation. Pretreatment of macrophages with inhibitors of PKR impairs MAPK phosphorylation according to the following rank order: JNK>p38>ERK. The capacity of DON to induce MAPK phosphorylation is markedly suppressed in macrophages containing an antisense PKR expression vector in a similar rank order. PKR suppression also inhibits induction of TNF-α and MIP-2 by DON.[49] Thus, PKR plays a critical upstream role in the ribotoxic stress response inducible by DON and other trichothecenes.

Hck, a member of the highly conserved Src-family of cytoplasmic protein tyrosine kinases, is expressed in myelomonocytic cell lineages.[50] This kinase transduces many extracellular signals that can impact cellular processes related to proliferation, differentiation and migration.[51] As expected for a signaling event upstream of MAPK activation, DON induced rapid phosphorylation of Hck (1 to 2.5 min).[47] Pretreatment of cells with Src-family-selective tyrosine kinase inhibitors blocks both DON-induced: (1) phosphorylation and activation of Hck, (2) MAPK activation, (3) phosphorylation of c-jun, ATF-2 and $p90^{Rsk}$ which are substrates of JNK, p38 or ERK, respectively,[49] and (4) downstream upregulation of TNF-α and MIP-2 gene expression. Thus, Hck activation is also likely to be another critical signaling event upstream of DON-induced MAPK phosphorylation and, ultimately, cytokine and chemokine upregulation.

Integrated In Vivo Model for Cytokine Gene Expression

DON induces proinflammatory cytokine mRNA expression in lymphoid tissue *in vivo* in a rapid (1 to 2 hr) and transient (4 to 8 hr) fashion, suggesting that MAPKs and upstream transcription factors that control gene transcription of these cytokines are activated prior to or within these time periods. As little as 1 mg/kg of DON transiently induces JNK and p38 phosphorylation over a 60 minute time period with peak effects being observed at 15 and 30 minutes, respectively, whereas, ERK remains phosphorylated between 15 to 120 minutes.[45] These data are consistent with the absorption and clearance of DON in the mouse which peaks at 30 minutes in most tissues.[52] DON modulates binding activities of transcription factors specific for potential regulatory motifs in cytokine promoters either concurrently (AP-1, C/EBP) (0.5 h) or subsequently (AP-1, CREB, NF-κB) (4-8 h).[45] The sequence and timing of these events are highly consistent with the kinetics of proinflammatory gene expression in the spleens of mice exposed to DON. This *in vivo* model should facilitate study of the interrelationships of MAPK phosphorylation, transcription factor activation and cytokine gene expression in animals exposed to trichothecenes and other toxic compounds.

Induction of Apoptosis

High doses of trichothecenes promote rapid onset of leukocyte apoptosis which likely contributes to immunosuppression. DON and other trichothecenes cause apoptosis *in vitro* in primary T-cells, B-cells and IgA^+ B cells[20] as well as HL-60,[53] U937 and RAW 264.7 cell lines[43] via caspase-mediated mechanisms.[54] These *in vitro* findings are relevant to the intact animal since *in vivo* administration of trichothecenes to rodents results in apoptosis in thymus, spleen and bone marrow.[55,56] Capacity of a trichothecene to induce apoptosis corresponds to ability to inhibit translation.[57]

Trichothecene-mediated cytotoxicity and apoptosis correlates closely with activation of p38, ERK and JNK implying contribution of these cascades to trichothecene-induced apoptosis.[43] Inhibitor studies indicate that p38 and ERK up-regulate and down-regulate trichothecene-induced apoptosis, respectively. At concentrations which partially inhibit translation, DON induces p38 and ERK1/2 phosphorylation within 15 minutes, in cloned murine macrophages, and these effects lasted up to 3 hours.[58] DON-exposed cells exhibited marked caspase 3-dependent DNA fragmentation after 6 hours, an effect that was suppressed and enhanced by the p38 and ERK inhibition, respectively. DON readily induces the phosphorylation and activation of p53 and this was dependent on p38. DON exposure also evoked BAX translocation and cytochrome C release. The p53 inhibitor PFT-α suppresses DON-induced phosphorylation of p53 as well as the p53 binding activity. Moreover, both PFT-α and p53 siRNA transfection abrogates DON-induced caspase-3 activity and subsequent DNA fragmentation. Concurrent with p53 activation, DON activates two anti-apoptotic survival pathways as evidenced by both ERK-dependent p90 Rsk and Akt activation. Taken together, the results indicate that DON initiates competing apoptotic (p53/Bax/Mitochondrial/Caspase-3) and survival (Akt/p90Rsk/Bad) pathways in the macrophage.

When upstream signaling transduction mechanisms contributing to DON-mediated apoptosis were investigated in cloned macrophages, specific inhibition of PKR and Hck additively suppressed DON-induced caspase-3 activity and apoptosis as well as p38, ERK, and JNK phosphorylation.[59] Inhibition of PKR and Hck also inhibits DON-induced binding and enzyme activity of p53.

Summary

Leukocytes are central targets of deoxynivalenol (DON) and other trichothecenes, which can be immunostimulatory or immunosuppressive depending on dose, exposure frequency and timing of functional immune assay (Figure 17.1). Whereas low dose trichothecene exposure transcriptionally and post-transcriptionally upregulates expression of cytokines, chemokines and other inflammatory genes with concurrent immune stimulation, high dose exposure driver leukocyte apoptosis and concomitant immune suppression. The MAPK p38 is an important transducer of downstream signaling events related to trichothecene-induced immune stimulation and apoptosis. Inhibitor and gene silencing studies suggest that PKR and Hck play upstream roles in DON-induced MAPK phosphorylation and subsequent induction of gene expression and apoptosis. Thus, p38

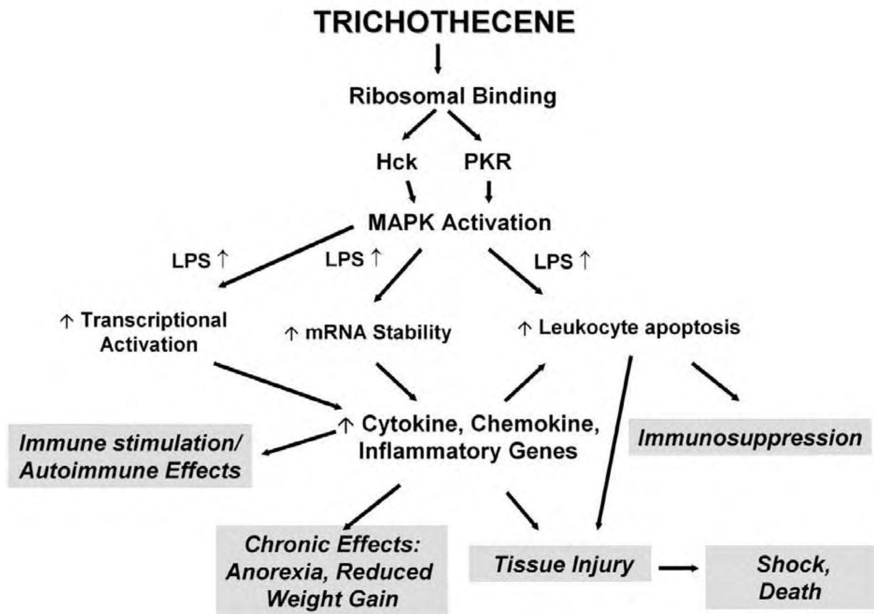

FIGURE 17.1 Mechanisms and sequelae associated with trichothecene mediated modulation of inflammatory gene expression. Trichothecenes can be immunostimulatory or immunosuppressive depending on dose, exposure frequency, and timing of functional immune assay. In mononuclear phagocytes, both PKR and Hck transduce a ribotoxic stress response to MAPKs that orchestrate downstream signaling events related to trichothecene-induced immune stimulation and apoptosis. Whereas low dose trichothecene exposure transcriptionally and post-transcriptionally upregulates expression of cytokines, chemokines, and other inflammatory genes with concurrent immune stimulation, high dose exposure drives leukocyte apoptosis and concomitant immune suppression. These events can be potentiated by a concurrent inflammatory stimulus such as LPS.

and ERK act as molecular rheostats in defining the ultimate response. When ERK and p38 are activated, inflammatory gene expression is favored. When only p38 is activated, an apoptotic response will likely ensue.

SUPERINDUCTION OF INFLAMMAGENIC RESPONSES BY TRICHOTHECENES

Trichothecene Potentiation of Lipopolysaccharide (LPS) Responses

LPS, the immunoactive component of endotoxin, is a constituent of the outer membrane of Gram-negative bacterial cell walls. LPS is found in the environment and has been extensively studied, both as a mediator of inflammation and as a major contributing factor to bacterial pathogenesis.[60] LPS exerts many of its effects through induction of pro-inflammatory cytokines, including IL-1β, TNF-α and IL-6.[61] An important feature

of LPS is its capacity to potentiate the toxicity of many toxins and toxicants encountered in food and the environment.[62] It is of particular concern that this potentiation can occur when LPS and chemical agents are presented to experimental animals at subtoxic doses.[63] Trichothecene immunotoxicity is synergistically enhanced by low dose LPS exposure in mice resulting pronounced thymic and splenic lymphocyte depletion.[64,65] Co-exposure to subtoxic doses of LPS and DON markedly induces apoptotic cell death in lymphoid tissues that include the thymus, Peyer's patches, bone marrow, and spleen of mouse.[66]

Mechanisms of Trichothecene Potentiation of Apoptosis

In mice, LPS and DON interact to elevate and prolong corticosterone levels which are critical for apoptosis induction, as evidenced by the ameliorating effects of the glucocorticoid receptor antagonist, RU 486.[29] LPS also potentiates induction of proinflammatory cytokines and cyclooxygenase-2 (COX-2) by DON, which might contribute to upregulation of corticosterone production and subsequent apoptosis in mice.[29,67] However, TNF-α or IL-6 deficiencies in mice do not attenuate the interactive effects of LPS and DON on corticosterone production or apoptosis.[29] Similar studies with pharmacologic inhibitors of COX-2 indicate that this enzyme is not critical in LPS plus DON -induced corticosterone production and lymphoid apoptosis.

IL-1β is another pro-inflammatory cytokine which is induced *in vivo* by LPS.[68,69] IL-1β mRNA is synergistically increased in LPS plus DON-treated mice.[67] IL-1β is a principal pro-inflammatory cytokine that is critical for innate immunity,[68] modulates immune and inflammatory responses[70] and is an important factor in inducing apoptosis *in vivo.*[71–75] IL-1β stimulates the hypothalamic-pituitary-adrenal (HPA) axis resulting in the release of adrenocorticotropic hormone (ACTH) and corticosterone.[76] IL-1β plays a central role in corticosterone induction and subsequent leukocyte apoptosis in mice co-exposed to LPS and DON. Co-exposure to LPS (0.1 mg/kg, i.p.) plus DON (12.5 mg/kg, p.o.) was found to significantly upregulate splenic IL-1β mRNA and IL-1β protein expression in B6C3F1 mice, as compared to treatment with vehicle or either of the toxins alone. Mice functionally deficient for the IL-1 receptor produced significantly less corticosterone upon co-exposure to LPS plus DON than did corresponding wild-type mice. Consistent with these findings, IL-1 receptor-deficient mice were refractory to induction of leukocyte apoptosis. Furthermore, i.p. injection of IL-1 receptor antagonist in wild type mice significantly inhibited LPS plus DON-induced increases in plasma corticosterone as well as apoptosis in thymus, Peyer's patches and bone marrow. To confirm IL-1β's capacity to induce apoptosis, mice were injected with the cytokine (500 ng/mouse, i.p.) three times at 2 hour intervals. Plasma corticosterone levels and thymus and Peyer's patch apoptosis in IL-1β-injected mice were significantly higher at 12 hour than control mice. Plasma adrenocorticotropic hormone (ACTH) levels in LPS plus DON-treated B6C3F1 mice did not correlate with the induction of plasma corticosterone or leukocyte apoptosis.

The aforementioned results indicate that IL-1β is an important mediator of LPS plus DON-induced corticosterone and subsequent leukocyte apoptosis and, furthermore, this cytokine possibly acts through an ACTH-independent mechanism. Thus,

LPS potentiates DON-induced corticosterone production by causing a prolonged elevation of systemic IL-1β. Furthermore, this corticosterone elevation is likely to drive subsequent induction of leukocyte apoptosis. The finding that IL-1R deficiency and IL-1ra treatment did not completely abrogate LPS plus DON-induced corticosterone production or apoptosis suggests that other redundant pathways might be in operation that don't involve IL-1β.

LPS Priming Potentiates and Prolongs DON-induced Proinflammatory Cytokine Responses

We further tested the hypothesis that LPS priming sensitizes a host to DON-induced proinflammatory cytokine induction and apoptosis.[77] In mice primed by intraperitoneal injection of LPS (1 mg/kg bw) and exposed orally 8 hours later with DON, the minimum toxin doses for inducing IL-1α, IL-1β, IL-6 and TNF-α serum proteins and splenic mRNAs were significantly lower than the DON doses required for vehicle-primed mice. LPS priming also decreased onset time and dramatically increased magnitude and duration of cytokine responses. LPS-primed mice maintained heightened sensitivity to DON for at least 24 hours with LPS priming doses as low as 50 μg/kg bw. DNA fragmentation analysis and flow cytometry also revealed that mice primed with LPS (1 mg/kg) 8 hours before exposure to DON (12.5 mg/kg) exhibited massive apoptotic thymocyte loss 12 hours later compared to mice exposed to DON or LPS alone. LPS priming decreased DON-induced p38 and ERK 1/2 phosphorylation suggesting that increased cytokine responses could not be explained by enhanced MAPK activation. Taken together, LPS exposure renders mice highly susceptible to DON induction of cytokine expression and this correlated with increased apoptosis in the thymus.

These data suggest that a wider window of xenobiotic susceptibility exists than previously expected from co-exposure studies for individuals exposed to LPS or possibly other inflammagenic agents (Figure 17.1). These results might take on even greater significance based on reports that inflammation increases susceptibility to lower doses of xenobiotic and might provide insight into the mechanism of various idiosyncratic adverse drug reactions.[63]

SUMMARY

Trichothecenes upregulate expression TNF-α and other inflammatory genes *in vivo* and *in vitro* via the ribotoxic stress response. This pathway can act in tandem with other inflammagenic stimuli such as LPS to greatly magnify the response. In the future, it will be important to: (1) understand the molecular linkage of PKR and Hck to binding by trichothecenes to ribosomes or as-yet unidentified receptor(s), (2) relate the magnitude and duration of trichothecene-induced activation of MAPKs to immune and apoptotic gene expression, (3) clarify the role of trichothecene structure on *in vitro* and *in vivo*, effects and (4) elucidate the cytochemical basis for interactions among MAPKs and upstream kinases as well as downstream mediators of trichothecene-induced gene expression and apoptosis. Additional research should focus on the effects of extended

mycotoxin exposure on chronic inflammation and innate immune responses to pathogens or gene products bearing pathogen-associated molecular patterns. Paradigms derived from such basic studies must ultimately be evaluated in the context of human leukocytes as a function of metabolism, genetic polymorphisms, nutrition, environmental chemical exposure, microbial infection, and ongoing inflammatory responses.

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Part IV

Immunotoxicity in the Lung

18 Host Defense and Immunotoxicology of the Lung

M. Ian Gilmour and Kymberly Gowdy

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INTRODUCTION

Respiratory allergies and infections are the most common form of illness in the United States and Europe and account for more missed school and work days than any other disease [1]. A substantial body of experimental work has clearly shown that airborne toxicants such as tobacco smoke, ozone, and other air pollutants can alter many aspects of the host defense network to either decrease resistance to infection, or exacerbate respiratory allergies and asthma [2]. Exposure to air toxicants can suppress a number of key host defenses including mucociliary clearance in the airways, pulmonary macrophage function, and development of specific immune responses such as IgG antibody production and cell mediated immunity. In contrast, immune stimulation in the form of increased T cell activity and IgE antibody formation has also has been shown to occur under some circumstances, resulting in increased incidence or severity of allergic lung disease.

These results continue to be confirmed in clinical, epidemiological, and experimental studies while basic research activities seek mechanistic explanations for these effects. In particular, increased understanding of dendritic and T regulatory cell function, as well as the discovery of additional classes of antimicrobial molecules and pattern recognition receptors (PRRs) has inspired a new wave of interest in host-pathogen and host-allergen interactions, and provided an interesting link between innate and adaptive immunity. As toxicology studies begin to investigate the functionality of these cells and molecules, a fuller understanding of disease susceptibility following pollutant exposure will emerge. The purpose of this chapter is to summarize new advances and understanding in pulmonary immunobiology, and to present recent information on how these processes are affected by inhaled pollutants.

IMMUNE FUNCTION AND IMMUNOSUPPRESSION IN THE LUNG

In a seminal review article, Green and colleagues [3] wrote that "despite the daily microbial assault that the respiratory tract experiences, the gas exchange area of the lung is maintained in a remarkably sterile condition by the combined antimicrobial activity of the mucociliary, phagocytic and immune systems." Much of this understanding was gained from experimental infections in rodents in which investigators reduced or eliminated specific defense components in order to demonstrate their anti-microbial value. Techniques involved monitoring the physical clearance of inhaled radio-labeled pathogens from the mouse lung and reduction in viable counts, as a means of comparing physical removal of the microbes to bactericidal activity.

Studies using isolated pulmonary macrophages from lung washes showed that exposure to various agents including ozone, nitrogen oxides, sulfur dioxide, metal compounds and tobacco smoke reduced the cells ability to ingest and/or kill bacteria through defects in bactericidal enzymes and impaired tumoricidal and anti-viral capability [4]. From this extensive body of work some air pollutants were described as immunotoxicants, because of their ability to suppress humoral and cellular host defenses and increase susceptibility to infection [5]. During the 1980s and 1990s, dose response relationships for these and other compounds were developed, and the mechanistic basis for effects studied through assessment of cellular function, antibody production after immunization, T cell phenotype changes, and host resistance assays.

At the same time that immunosuppression was being noted in a variety of different experimental systems, investigators were also reporting that air pollutant exposure, in tandem with antigen sensitization, could result in stimulation of IgE antibody and a subsequent increase in the number or severity of anaphylactic (allergic) type reactions (reviewed in [6]). These observations were first noted with O_3 and NO_2 and more recently after exposure to combustion particles such as oil fly ash and diesel emissions. While the mechanisms for these effects still need clarification, they likely lie at the level of antigen processing and presentation, at which stage the quality and quantity of specific immune responses is programmed. The following sections will describe the major components of the host defense network and how they may be compromised by air pollutant exposure, while the latter part of the chapter will review mechanisms for immunoenhancement and allergic adjuvancy.

Mucociliary Clearance Mechanisms

The first line of airway defense against inhaled airborne particles and microbial pathogens is mucociliary clearance. This process involves coordinated beating of ciliated cells propelling a complex layer of mucus and surfactant up the airways. Mucus, which exists in a biphasic layer, is secreted by goblet cells on the surface epithelium and the mucous and serous cells of the submucosal glands [7]. Four genes have been identified for airway epithelial mucins (MUC 1, MUC 2, MUC 4, and MUC5) [7]. Inhaled materials that land on the upper airways are propelled at a rate of 4 to 12 mm per minute up to the oropharynx where they are swallowed [8]. Particles that reach the lower airways and alveolar region of the lung are ingested by macrophages that then either migrate through interstitial pathways to the lymphatics, or are transported up to the mucociliary escalator.

Decreased mucociliary clearance has been observed in the lungs of smokers suffering from bronchitis [9]. In addition, exposure to other agents such as sulfuric acid slows ciliary beat [10], while exposure to relatively low concentrations of ozone (in monkeys and rats) causes exfoliation of the ciliary surface [11]. In general, respiratory infections as well as other forms of lung injury increase mucus production as a protective response. Despite this apparent benefit, overproduction of mucin impairs ciliary beating, resulting in mucus and trapped particles staying in the lungs [12]. High levels of mucus are also present in airway diseases such as bronchitis, chronic obstructive pulmonary disease (COPD) and asthma [13–15].

Antimicrobial Secretions

The respiratory tract is constantly bathed with a complex mix of surfactants and enzymes that maintains surface tension, facilitates gas exchange, and provides protection against reactive molecules and inhaled microbes. Some of the principal mediators, which are bactericidal or bacteriostatic, include lysozyme, complement components, collectins (surfactant proteins A and D), and the alpha and beta defensins (Table 18.1). Lysozyme is secreted by serous cells, macrophages and neutrophils, and has the ability to lyse and kill gram positive bacteria via enzymatic cleavage of peptidoglycan [16]. Exposure to ozone has been shown to reduce lung lining fluid levels of lysozyme in rats [17], while living in a highly polluted environment (New Delhi) has been associated with suppressed lysozyme production in the eyes [18].

The complement enzyme cascade is an important host defense mechanism in the lung, functioning to kill microbes through the classical and alternate pathways, augment phagocytosis by opsonizing bacterial membranes, and recruit additional phagocytes via chemotactic fragments C3a and C5a. Complement molecules are produced locally by macrophages, type II pneumocytes and fibroblasts [19], and inactivation with cobra venom factor, or through gene deletion, increases susceptibility to infection [20, 21]. Expression of the receptors for C3a and C5a is increased by exposure to numerous biological contaminants such as lipopolysaccharide (LPS; bacterial endotoxin) and allergens [22], while several environmental agents including ozone, diesel exhaust, cigarette smoke and other particulates also activate complement or increase the deposition

TABLE 18.1

Antimicrobial Defenses in Lung	Produced by	Target	Mechanism	Effects by Air Pollution
Lysosyme	Serous cells, neutrophils, macrophages	Gram positive bacteria	Lyse gram positive bacteria via cleavage of peptidoglycan	Decreased production after ozone exposure [121]
Complement (C3a, C5a)	Macrophages, type II pneumocytes and fibroblasts	Microbes	Opsinization by phagocytes and chemotaxis	Increased activity after exposure to LPS and allergens; ozone, diesel, cigarette [23, 28]
Defensins HD 1-6; HBD 1 and 2	Neutrophils (HD1-4); epithelial cells (HBD 1 and 2); macrophages	Bacteria, mycobacteria, fungi, and some viruses	Cidal activity and chemotaxis	Upregulated in the lungs of smokers [36]
Collectins	Alveolar type II cells and non-ciliated bronchiolar epithelial cells	Bacterial and fungal cell walls and viruses	Enhance phagocytosis of macrophages and neutrophils; activating cells	Cigarette smoke decreases the production of collectin [48]; ozone decreases function [49]

of C3 in the airway epithelium [23, 24]. Specifically, cigarette smoke stimulates the complement pathway by cleaving an internal thiol ester bond in C3 [25-27]. There are no reports showing a decrease in complement activity with air pollutant exposure, but rather an increase has been noted in a number of allergic systems, suggesting a relationship between air pollution-dependent increases in complement fragments and greater risk or severity of allergic airways disease [28].

Alpha and beta defensins are small molecular weight peptides which have cidal activity against gram positive and negative bacteria, mycobacteria, fungi, and some viruses [29]. In humans, six α defensins (HD 1-6) and two β defensins (HBD 1 and 2) have been characterized. Human α defensins 1-4 are synthesized by recruited neutrophils and released in response to stimuli such as proinflammatory cytokines or tissue injury [30]. HBD 1 and 2 genes are expressed in respiratory tract epithelial cells and HBD-2 is inducible by cytokines such as IL1-β [31, 32]. Activated alveolar macrophages (AM's) produce defensins which can also act as chemoattractants for dendritic cells and T lymphocytes, thus encouraging generation of specific immune responses [33]. Defensins are inhibited by high salt concentrations as is seen in cystic fibrosis patients, [34, 35] but presently there is no information on how exposure to lung toxicants affects defensin production and activity other than the fact they are upregulated in the lungs of smokers [36].

The pulmonary surfactant proteins A and D (SP-A and SP-D), termed collectins, are a family of carbohydrate binding proteins synthesized by alveolar type II cells and some non-ciliated bronchiolar epithelial cells [37]. Collectins are essentially opsonins that enhance phagocytic activity of macrophages and neutrophils [38, 39] and activate cells via ligation with the heat shock protein/collectin receptor CD91 [40] or through the

binding of Toll-like receptors [41]. Pulmonary collectins bind to various components of bacterial and fungal cell walls and viruses (LPS, lipoteichoic acids, lipoarabinomannans, polysaccharides, N-linked oligosaccharides in viral envelopes) through a carbohydrate recognition domain (CRD) [37]. SP-A-/- mice display delayed clearance of pathogens such as group B *streptococcus* (GBS) [42], *Haemophilus influenza* [43], RSV [44], *Pneumocystis carinii* [45], and *Pseudomonas aeruginosa* [46]. The defect in clearance can be attributed to fewer alveolar macrophages ingesting and clearing the invading bacteria. SP-D-/- mice also show decreased alveolar macrophage phagocytosis of bacterial and viral pathogens [43, 45]. In addition however, these animals develop alveolar proteinosis and dilated distal airways as a result of increased levels of tissue and macrophage-associated metalloproteinases, macrophage derived oxidants, and phospholipids [47]. In terms of immunotoxicity, it is clear that cigarette smoke decreases the production of SP-A and SP-D which may explain in part increased infections in smokers [48]. Ozone exposure also affects the function of SP-A by decreasing its ability to modulate proinflammatory cytokine production by monocytes/macrophages [49].

Other airway anti-microbial and anti-inflammatory soluble factors that protect the epithelium are fibronectin, lactoferrin and cathelicidin. All are secreted to help the epithelial surface eliminate inhaled materials. Fibronectin, which is a cell adhesive glycoprotein important in tissue injury and repair, is secreted by lung fibroblasts after a broad variety of stimuli, including nicotine [50]. Lactoferrin in airway epithelial cells increases after exposure to catalytically active metals present in some air pollutants. These proteins transport and store metals and compete for free iron, thus diminishing oxidative stress and damage to the lung epithelium. Cathelicidins have multiple functions in normal lung homeostasis in addition to their known anti-microbial and LPS-neutralizing effects [51].

Resident Cells

Some 40 cell types are found in the respiratory tract and virtually all have some form of host defense capability either through physical, regulatory, secretory, and phagocytic activities. The principal cells which keep the lung free of infection are the different types of epithelial and secretory cells, pulmonary macrophages, dendritic cells, neutrophils and lymphocytes, all of which interact in a complex and dynamic system. After exposure to an infectious or toxic agent, many of these cell types become activated (Figure 18.1) in order to respond and repair damaged tissue, and in some cases develop tolerance or immunity to a second insult.

Until recently, epithelial cells were considered to function solely as the ciliated barrier lining in the airways and as conduits for gas exchange at the air/blood interphase. As techniques have improved to isolate and culture these cells and measure their gene products, it has become clear that they have a key role in lung defense and repair. Epithelial cells secrete a number of anti-microbial compounds and immunoregulatory cytokines [52], and are also capable of ingesting and killing bacteria [53].

Epithelial cells differ in morphology and function depending on their location. The conducting airways have pseudostratified columnar (type II) epithelial cells which

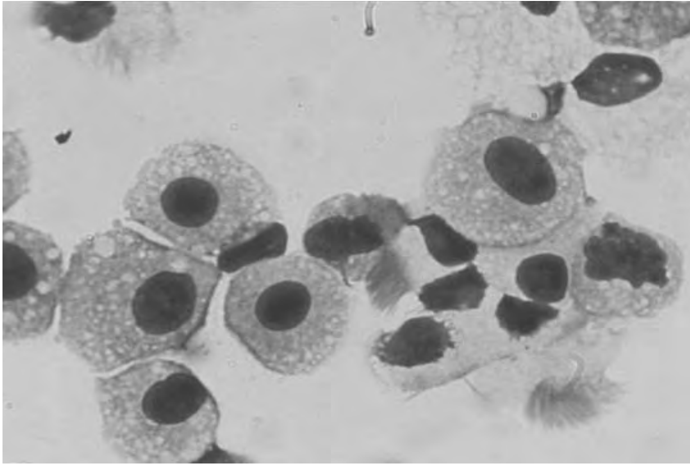

FIGURE 18.1 (See color insert following page 296) Pulmonary cells obtained from a rat lung wash 24 hours after infection with rat adapted influenza virus. The macrophages are heavily vacuolated indicating activation while ciliated epithelial cells are present indicating airway injury. Magnification × 1000, Diff Quick stain.

convert into cuboidal epithelial cells as the airways extend into branches. Embedded within the airway epithelium are secretory cells including goblet and clara cells. The epithelial lining in the conducting airways is an important barrier to external environmental stimuli, and when injured, has a variety of responses for rapid repair. Injury caused by inhaled pathogens or toxicants is characterized by a sloughing off of sheets of epithelial cells, leaving the underlying tissue more vulnerable to subsequent insult by those same or additional hazardous agents. Epithelial cells quickly proliferate and differentiate in order to maintain normal function. Chronic changes in the epithelial lining lead to limited airflow in conditions such as bronchitis, asthma and COPD [54].

The terminal and respiratory bronchioles (depending upon species) are enriched in secretory clara cells, whereas alveolar type I cells are the thin walled type I cells that facilitate gas exchange and are the primary producers of lung surfactant proteins and lipids. Clara cells are non-ciliated secretory epithelial cells in the airways that are distinct in morphology and release an anti-inflammatory molecule called the Clara cell 10 kD protein (CC10, CC16, or CCSP) [55]. Mice exposed to aged and diluted cigarette smoke and/or ozone have much less CC10 in the airways, suggesting a loss of protection against inhaled toxicants [56]. In another example, CC10 was also decreased in the lungs of mice exposed to diesel engine emissions (DEE) and then infected with *Pseudomonas aeruginosa* compared to control animals [57]. These reports indicate that air pollutants can alter clara cell function, resulting in compromised host defenses in the conducting airways.

Pulmonary macrophages are the principal phagocytes in the lung and function to clear both cellular debris and inhaled particulates, and to engulf and kill microbes. They are a diverse, relatively long lived and dynamic cell population, and differences in activity may reflect stages of maturity, level of stimulation or perhaps even specific cell sub-populations arising from different precursors. Macrophages are highly mobile

and able to mediate direct engulfment of particles through several pathways [58]. They bear numerous receptors on their surface including the mannose, glucan and scavenger receptors that bind to conserved molecules on the outer surface of microbes. In addition, macrophages express various types of complement and Fc receptors that facilitate uptake of opsonized material through the binding of C3b and specific antibodies respectively. During phagocytosis, the macrophages extend pseupodia around the receptor-ligand complex and engulf and internalize the bound microbe. The resulting internalized phagosome fuses with cytoplasmic lysozomal granules and undergoes a series of maturation steps; becoming increasingly acidic, generating toxic oxygen and nitrogen intermediates and hydrolases, leading to the enzymatic destruction of the foreign particle.

Macrophages are the best studied cell in the lung relating to immunotoxicity. For over two decades it has been appreciated that exposure to air pollutants including ozone, sulfuric acid, metals, and cigarette smoke components like acrolein suppress both uptake and killing of inhaled microbes (reviewed in [59]). These effects have been shown after ozone exposure in both experimental animals and human subjects, as well as during side by side *in vitro* exposures of cells from different species including humans [60]. Despite much research in this area, the actual mechanisms for these effects are not well understood. It is likely that some cells undergo direct membrane damage and loss of function, while others are influenced by the generation of immunosuppressive mediators such as $PGE_{2,}$ which reduces phagocytic activity [61]. Macrophages are also in themselves rich sources of prostaglandins, cytokines, and other regulatory molecules, and as such, play a key role in initiating and maintaining inflammatory responses and in eventual tissue repair and resolution of injury.

Macrophages express an array of scavenger receptors which enhance uptake of low density lipoproteins (LDL), bacteria and other inhaled particles [62, 63]. One molecule in particular, termed MARCO (MAcrophage Receptor with COllagenase structure), [64, 65] binds to gram positive and gram negative bacteria like the other scavenger receptors, but does not perform the normal duties of mopping up acetylated and oxidized LDL [66, 67]. In a recent study [63], MARCO deficient mice were used to examine the role of scavenger receptors in alveolar macrophages when faced with a pneumococcal infection and an acute exposure to TiO_2. The absence of MARCO impaired the ability of AM to clear the pneumococcal infection from the lungs, decreased opsonization of the environmental particles and enhanced pulmonary inflammation and cytokine release, indicating the importance of scavenger receptors in innate immune responses against inhaled air pathogens and pollutants.

Neutrophils are not present in large numbers in the normal healthy lung but infiltrate rapidly from the blood stream during inflammation and in response to infection and injury. Some of the principal chemo-attractive agents for neutrophils are complement components produced by macrophages, and pro-inflammatory cytokines such as the C-C and CxC chemokines, as well as the better known mediators like IL1β and TNF-α. Once recruited to the site of infection or injury, neutrophils readily engulf and deactivate microbes with a strong oxidative burst, involving the membrane NADPH oxidase complex [68]. A decreased neutrophil response, as is seen with alcohol exposure [69], leads to increased susceptibility to infection; however, defects in the function of these cells by air pollutants have not been reported [70].

Dendritic cells (DCs) are the principal antigen presentation cells in the respiratory tract and are pivotal in the generation of specific immune responses against pathogens as well as allergens. DCs originate from the bone marrow and migrate around the body before becoming resident in specific tissues such as the lung, and additional cells may be recruited during antigen exposure, infection, or injury [71]. Immature DCs are generally regarded as being phagocytic, but after antigen sampling this activity is markedly decreased, while specific surface markers including CD80, CD86, and MHC-II, are upregulated [72, 73]. The DCs then migrate to secondary lymphoid organs where they present antigen and co-stimulatory signals to circulating lymphocytes [74].

Four distinct human DC types have been identified: classic tissue DCs, Langerhans cells, monocyte-derived DC, and plasmacytoid DC [75]. The first three DCs are termed myeloid because of their origin and similarities of function. Plasmacytoid DCs on the other hand are classified based on a very immature phenotype, Toll Like Receptor (TLR) 7 and 9 expression, and vigorous release of type I interferon in response to viruses [76]. Myeloid DCs express high levels of CD11c, CD11b, and MHC II, whereas plasmacytoid DCs have low levels of CD11c and no CD11b. It has been hypothesized that myeloid and. lymphoid DCs have different roles in the immune response: myeloid DCs sample Ag in the periphery, and travel to secondary lymph nodes, whereas plasmacytoid DCs regulate immune responses and are involved in immunological tolerance [77, 78]**.** Chronic cigarette smoke exposure has been shown to decrease the number of DC (MHC II/CD11c^{high}) in the lungs, but not the lymph nodes, of mice compared to control animals [79]. Expression of the co-stimulatory molecule CD80 was also decreased, indicating a reduced capacity to present antigen. *In vitro* exposure of bone marrow derived DCs to nicotine has also been reported to inhibit cytokine production and T cell proliferation [80].

The respiratory tract is a rich source of intra- and sub-epithelial lymphocytes and has defined regions of bronchus associated lymphoid tissue (BALT) as well as numerous peripheral lymph nodes. T and B lymphocytes make up the effector arm of the specific immune response, generating cytotoxic and antibody mediated reactions which rid the lung of pathogens and prevent re-infection through the development of immunological memory. In addition to the classic helper (CD4) and cytotoxic (CD8) subsets, CD4 cells are also separated into T helper 1 and T helper 2 types depending on their cytokine profile and function [81]. CD4/CD25 T regulatory cells are the most recent cell type to be defined as a distinct sub-population and are thought to control the development of immunological tolerance through the production of IL-10 [82].

Ozone is the most studied inhaled pollutant that suppresses lymphocyte function in the lung (reviewed in [5]), whereas diesel exhaust has been reported to have an apparent adjuvant effect that biases T cell function towards an allergic phenotype [83]. In general, ozone exposure causes a decrease in T cell function, as measured by proliferative responses to mitogens and delayed type hypersensitivity reactions after immunizations [84]. These decrements have also been associated with increased susceptibility to infections such as *Listeria monocytogenes*, which requires T cell mediated immune responses in order to clear the pathogen [85]. The effect of ozone on antibody production is less clear. In some situations antibody levels are increased, while in others they are decreased depending upon the route of antigen administration, the exposure regimen and the class of antibody measured [86]. The mechanisms for these effects are

not understood although presumably altered T cell function changes communication between the afferent and efferent immune processes. It is known that skewed polarization between Th1 and Th2 immune responses drastically alters the quality and quantity of antibody responses as well as the potency and effectiveness of host defenses [87]. This is well demonstrated in the *Leishmania major* model in which polarization of Th1 or Th2 responses predicts survivability [88]. For example, BALB/c mice are more susceptible to protozoan infection despite eliciting a strong Th2 response, while C57BL/6 mice control and overcome the disease through the generation of a prominent Th1 response [89]. More recent studies using gene knock out mice have implicated the specific roles of IL-12 to control infection and IL-4 to exacerbate the disease, indicating that the cytokines ratios produced in the context of Th1/Th2 responses can profoundly influence the quality of immunity and disease protection [90].

While reported changes in specific immunity, as measured by T and B cell effector function, are commonplace, the mechanisms underlying these effects are poorly understood. From the ozone literature it is known that adrenalectomy can substantially mitigate ozone dependent T cell suppression [91], suggesting that endogenous corticosteroids and the stress response are involved. Several other possibilities exist, however. Exposure to immunotoxicants can change the mucosal milieu by causing inflammation and increased permeability of the epithelial barrier [92]. In this way antigen can penetrate more deeply or in greater amounts towards sub-epithelial immune tissue. At the level of accessory macrophages and dendritic cells, immunotoxicants may also affect uptake and processing of antigen or alter production of influential immunoregulatory cytokines such as IL12 [93].

NK cells are important effector non-T, non-B lymphocytes that recognize and bind to tumor and virus infected cells before mediating cytotoxicity through F porin production. In mice, immature (NK1.1$^+$Ly49$^-$) and mature NK cells (NK1.1$^+$Ly49$^+$) leave the bone marrow and travel to various lymphoid compartments including those in the lung [94]. Once resident in tissue, NK cells produce cytokines and chemokines (IFN-γ, TNF-α, CXCL8 (reviewed in [95]) and recognize target cells based on their absence of MHC class I or via receptors including NKG2D, Ly49s (mouse), and KARs (human) [94, 95]. This unique system of recognition allows NK cells to be one of the first lines of defense that is able to detect tumor cells and virus-infected cells. Exposure to ozone has been shown to decrease both pulmonary and systemic NK activity in animals [96] as well reduce circulating NK activity in humans [97], although the mechanisms for these effects are not known.

Pattern Recognition Receptors (TLRs)

Until the late 1990s, the term innate immunity referred to nonspecific host defenses such as bactericidal enzymes, macrophages, and other recruited phagocytes. This arm of the immune system was classified as not having memory responses or specificity to particular types and classes of pathogens. Toll-like receptors (TLRs) are conserved in both invertebrates and vertebrates. TLRs were first discovered in *Drosophila* as a genes important in embryonic development [98], and were later recognized as being important in resistance to bacteria and fungi [99, 100]. This conserved pathway was later identified

TABLE 18.2

Toll like receptor	Ligand	Deficiency
TLR1	Heterodimerizes with TLR2 to recognize triacyl lipopeptides.	n/a
TLR2	Microbial components such as triacyl lipopeptides (heterodimerize with TLR1), diacyl lipopolypeptides (heterodimerize with TLR6), peptidoglycan from gram positive bacteria, glycophosphatidylinositol (GPI) anchors from malaria-causing parasites, zymosan from fungi, and forms of LPS structurally distinct from those recognized by TLR4 [122].	TLR2 -/- decreased clearance of *Borrelia burgdorferi* lipoproteins; greater susceptibility to *S. aureas* [123]; *Streptococcus pneumoniae* [124]; and less resistant to *Mycobacterium tuberculosis* [125]
TLR3	Double stranded RNA; Poly (I:C); binds intracellularly [126]	TLR3 -/- mice had a decreased response to pol(I:C); reduced production of TNF, IL-6, IL-12, and resistance to the lethal effect of poly(I:C) when sensitized to D-galactosamine [126].
TLR4	LPS, F protein, heat shock protein (HSP) 60, HSP 70, RSV [122].	TLR4-/- and TLR4 lack of function mice (C3H/HeJ) have decreased clearance of *Haemophilus influenzae* [127], *Salmonella* [128], *Mycobacterium tuberculosis* [129], and RSV [130, 131]; and are more susceptible to *Candida albicans* infection [132].
TLR5	Flagellin (constituent of bacterial flagella).	Polymorphism in TLR5 gene introduces a premature stop codon (TLR5^{392STOP}) and is correlated with increased susceptibility to *Legionella pneumophila* [133], but not typhoid fever [134].
TLR6	Diacyl lipopolypeptides (heterodimerize with TLR2).	n/a
TLR7	Single stranded viral RNA and DNA.	IRAK-/- mice impair the TLR7 pathway and result in a deficient production of IFN α in serum when challenged with R-848 [[135]
TLR8	Genomic material of viruses	n/a
TLR9	CpG DNA, Single stranded viral RNA and DNA.	IRAK-/- mice impairs TLR9 pathway and plasmacytoid DC can not produce IFN α in serum when challenged with CpG ODN [135]
TLR10, 11, 12	TLR11- profilin from *T.gondi* [136]	n/a

in human and mice through DNA sequence homologies across various species. The TLRs recognize specific conserved microbial components termed pathogen associated molecular patterns (PAMPs) such as mannans from yeast cell walls, LPS from gram negative bacteria, lipoproteins and peptidoglycans from gram positive bacteria, and bacterial DNA as characterized by presence of CpG motifs.

The PRRs (pattern recognition receptors) recognize PAMPS, with two principal reactions: mediation of acute phagocytosis (internalization) and rapid cellular and tissue activation with subsequent stimulation of specific immune responses. TLR often work in tandem with other co-receptors such as CD14, which is a required accessory signal for LPS. TLRs provide intracellular signaling through the TIR (Toll/Interleukin-1 receptor) domain via the MyD88–IRAK-TRAF 6 cascade which upregulates transcription factors including NFκB. In addition to this process, the receptors also utilize additional signaling pathways such as TRIF and TRAM which stimulate the expression of type I interferons [101]. Table 18.2 shows the principal recognition components of the TLR family and consequences for susceptibility to infection in animals with defects in TLR genes. The TLR receptors are found on a number of cells types, including macrophages, dendritic cells and epithelial cells. TLR 1, 2 and 6 recognize gram positive bacteria and yeast through recognition of cell products including lipoteichoic acids. TLR 3 is usually located intracellularly and recognizes double stranded (viral) RNA. TLR-4, in association with CD-14, recognizes LPS on gram negative bacteria. TLR5 recognizes bacterial flagellin. TLR 7 and 8 recognize viral nucleic acids. TLR9 recognizes CpG motifs present on bacterial DNA. TLR 10, 11 and 12 have been identified but their function is not yet known. While these receptors are clearly important in the generation of protective immune responses, their modulation during immunotoxicologic events needs to be investigated. Inappropriate stimulation of TLRs in organs such as the lung has been implicated in the pathogenesis of diseases. One of the first studies to examine TLRs in lung inflammation [102, 103] reported that LPS resistant mice (C3H/HeJ) with a defect in TLR4 signaling had decreased pulmonary injury following ozone exposure, indicating a possible role of TLR4 in this effect.

Immunoenhancement and Adjuvancy in the Lung

As noted above, exposure to air pollutants in association with immunization with soluble protein antigens can result in stimulation of allergic immune responses through the promotion of T helper 2 cytokine responses and increased development of IgE antibody production (reviewed in [108]). This phenomenon was first described in mice [109] and in monkeys [110] exposed to ozone, but has since been demonstrated following exposure to NO_2 [111], and oil fly ash [112], and most frequently in association with diesel exhaust particles [113]. Animal experiments have since demonstrated that other types of particles, including ambient particulate matter (PM), carbon black particles (CB), and polystyrene particles (PSP), can act as immunologic adjuvants when administered with an antigen via intraperitoneal, intranasal, intratracheal, and inhalation routes of exposure [112, 114–119]. In most cases the particles alone cause inflammation but when administered during sensitization they also stimulate the development of allergic immune responses (in the form of increased IgE antibody and TH2 cytokines). Upon repeated challenge with antigen, these animals exhibit increased severity of allergic type disease (pulmonary eosinophils, airway hyperresponsiveness, increased mucus production, etc.) compared to control animals that received antigen exposure and vehicle control in the place of the pollutant.

The general thought is that these exposures cause some form of oxidative stress

resulting in redirecting or polarizing the immune response to a stronger Th2 phenotype [120]. Although the mechanisms by which this occurs are still unclear, it is plausible that the tissue injury leads to cellular activation and immune stimulation. It is known that oxidative stress from inhaled air pollutants results in epithelial cell damage, increased lung permeability, and the release of a broad spectrum of pro-inflammatory mediators. Epithelial damage and increased permeability enhances translocation of antigen to immunoreceptive sites in the submucosa, whereas increased cytokine output can profoundly affect inflammatory and immune cells both locally and in areas remote for the site of damage. In addition, altered dendritic and macrophage cell number and function may affect immune signaling during both initial antigen recognition and secondary clonal expansion.

FUTURE DIRECTIONS

A significant body of clinical observations and experimental research has unequivocally shown that exposure to ambient air pollutants and other toxicants can reduce host defenses and increase susceptibility to infection. There is also emerging evidence that certain exposures may result in greater incidence and severity of allergic lung disease and asthma. These problems are still a reality in many highly populated urban areas of the United States and are of even greater issue in developing countries where uncontrolled combustion emissions are increasing at alarming rates. Respiratory infections remain a primary cause of morbidity and mortality worldwide, and any kind of exposure that might increase susceptibility to these agents is of public health significance. This is particularly important as additional infectious agents emerge, either through antibiotic resistance or from genetic shifts in, for example, the influenza virus.

Similarly, respiratory allergies and asthma have increased dramatically over the past several decades and while these diseases have a complex etiology dependent on both genes and environment, it is clear that poor air quality is associated with worsening of symptoms. Atmospheric air pollution is a complex dynamic mix of gases, particles and vapors that arises from combustion processes as well as natural sources. Many of the components of urban smog (e.g., ozone and acrolein) are immunotoxic, although the long-term effects of chronic low concentration are largely unknown.

The explosion of information on innate immunity and its links with development of specific immune responses is setting the stage for a better understanding of host-pathogen interactions and potential therapies. We chose to focus on new advances in pulmonary immunobiology as a way of developing new ideas for future immunotoxicology studies. As more of these different host defenses and immunoregulatory processes are assessed during toxicity testing, it is likely that we will learn more about the mechanisms by which inhaled toxicants increase infectious and allergic lung disease. This, in turn, will provide more accurate information on the type and concentrations of pollutants that affect these processes, which ultimately could be used to for more targeted risk assessment in at risk populations.

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Part V

Developing Immunotoxicity

19 Immune System Ontogeny and Developmental Immunotoxicology

Ralph J. Smialowicz, Kathleen M. Brundage, and John B. Barnett

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INTRODUCTION

Animal testing for the identification and characterization of hazard(s), associated with exposure to toxic chemicals, is an accepted approach for identifying the potential risk to humans. The rodent, in particular the rat, has been the most commonly used species for routine toxicity evaluations. On the other hand, we know more about the immune system of the mouse[1] than any other mammal except humans. While most standardized toxicology testing occurs in adult animals, there are protocols which specifically evaluate developmental toxicity in the rat. These test paradigms include prenatal developmental, multi-generational reproductive, and developmental neurotoxicity evaluations.[2–7] Safety testing, in the developing rodent, provides important information which is relevant to the design, performance, and interpretation of developmental toxicity studies.

The rat has primarily been employed in developmental toxicity studies. There is abundant information available regarding breeding, husbandry and dosing of this rodent species. Additional advantages of using rats, rather than other rodents, in developmental toxicity testing include the following: rats are generally easy to breed; rat dams have large litters (i.e., depending on strain from 6-12 pups/litter); pups are generally more robust

and are relatively easy to cross foster; and rat pups can be gavaged with test chemicals as early as post natal day (PND) three [8]. Another important consideration in the use of the rat as a test species is that developmental immunotoxicity testing potentially can be evaluated by integrating immune function assessment with currently required multi-generational reproductive toxicity testing.[9,10]

It is generally accepted that the developing organism is more vulnerable than the adult to agent-induced organ-system toxicity. Since there is limited information on developmental immunotoxicity, safety information based solely on adult immunotoxicity data is unlikely to result in effective protection of the potentially most at-risk populations. There has been an increased interest in and emphasis on developmental toxicology, particularly as it relates to children's risk. This interest is reflected in documents, such as *Pesticides in the Diets of Infants and Children*[11] and *Research Needs on Age-Related Differences in Susceptibility to Chemical Toxicants,*[12] in which the immune system has been identified as an organ system of concern. In addition, two U.S. statutes require that the U.S. Environmental Protection Agency (EPA) perform "…separate assessments for infants and children…" for potential toxic susceptibility mandated by the Food Quality Protection Act [13] and the Safe Drinking Water Act [14]. Mammalian immune system ontology requires a series of carefully timed and coordinated developmental events during embryogenesis. Pluripotent hemolymphopoietic stem cells arise in the splanchnopleura and migrate to the fetal liver and spleen. This is followed by final residence of these stem cells in bone marrow and thymus. These stem cells are unique in that they are capable of self-renewal via proliferation and differentiation in to specific blood cell lineages (i.e., lymphocytes, monocytes, neutrophils, eosinophils, basophils, and erythrocytes).[15]

Defects in the development of the immune system due to heritable changes in the lymphoid elements have provided clinical and experimental examples of the devastating consequences of impaired immune system development.[16] The presence of functional defects, caused by exposure to certain chemical or physical agents during development, may range from life threatening suppression of vital components of the immune system to altered or poorly regulated responses that can be debilitating. Studies in experimental laboratory rodents indicate that exposure to immunosuppressants (e.g., chemicals, drugs, ionizing radiation) during immune system development can result in alterations of immune system function which may persist into adult life.[17]

A recent workshop[18] and forum[19] addressed issues of the appropriate immune function methods and rodent species for evaluating developmental immunotoxicity. In both meetings the rat was identified as the preferred animal model for developmental immunotoxicity testing. This chapter will review evidence which implicates certain chemicals and drugs as being developmental immunotoxicants in rodents.

HUMAN IMMUNE SYSTEM DEVELOPMENT

As with the development of other organ systems, the development of the immune system is a highly regulated process. Table 19.1 and Figure 19.1 provide a list of known events or markers that occur during immune system development. In humans, hematopoiesis begins at approximately 3–4 weeks of gestation with the development of blood

TABLE 19.1
Developmental Milestones of the Human and Mouse Immune System

Function		**Human (w)**		**Mouse (d)**
Development of hemagioblasts	—		—	<7[57]
Development of blood islands in the yolk sac		3-4[20]		7.5[60,65]
Yolk sac a primary site of hematopoiesis	—	4-6[21]	—	7.5[58,60,65,67]
Aorto-gonado-mesonephros (AGM) a primary site of hematopoiesis	—		—	7.5[60,65,67,72]
A major population of hematopoietic stem cells found associated with the ventral endothelium of the dorsal aorta	—	5[20]	—	
Fetal liver begins functioning as a hematopoiesis site	—	6[20]	—	10.5[60,72,73]
Appearance of γδ TCR T cells in fetal liver	—	6-8[30,31]	—	14[74]
Lymphatic vasculature starts	—	<6[29]	—	10.5[15]
Cells bearing rearranged Ig genes in fetal liver	—	8[28]	—	11[73]
CD45R+ cells found in fetal liver	—	10-12[25]	—	11[15]
Organogenesis of thymus begins	—	6[25,32]	—	—11[15]
Sites of lymphopoiesis develop	—	6-12[25,32]	—	10[67,68]
Secondary lymphoid organs begin to develop	—	7[28]	—	10.5[15]
Lymphoid and myeloid progenitors appear in fetal liver	—	7-8[25]	—	10[21,67,73,81]
GALT begins to develop	—	8[26,27]	—	
Lymph nodes start to appear	—	8-1228	—	10.515
Yolk sac begins to regress	—	10[21]	—	
Spleen develops	—	10-14[28]	—	13[76]
Tonsils develop	—	10-14[28]	—	
Peyer's patches develop	—	11-15[26,27]	—	15.5[15]
Appendix develops	—	11-15[26,27]	—	
B cell lymphopoiesis begins in bone marrow	—	12[25,28]	—	17[77]
B lymphocytes become detectable in blood	—	—12[25,28]	—	13[67]
CD4+ and CD8+ T cells begin to leave the rudimentary thymus	—	—13[28,34,35]	—	13[78,79]
CD4+ and CD8+ T cells detectable in spleen	—	14[34,35]	—	19[78,79]
Thymus development completed, i.e., medulla completely formed and normal progression of T cell development	—	15-16[25,33]	—	13[28]
CD4+ and CD8+ T cells detectable in cord blood (hu) or circulation (mo)	—	20[34,35]	—	19[79]
Bone marrow becomes the major site of hematopoiesis	—	22[28]	—	17.5[15,76]
Surface Ig+ lymphocytes in fetal liver	—		—	18[77]
Full complement of TCR-associated molecules in peripheral organs	—	23[28]	—	Early Postnatal[28]

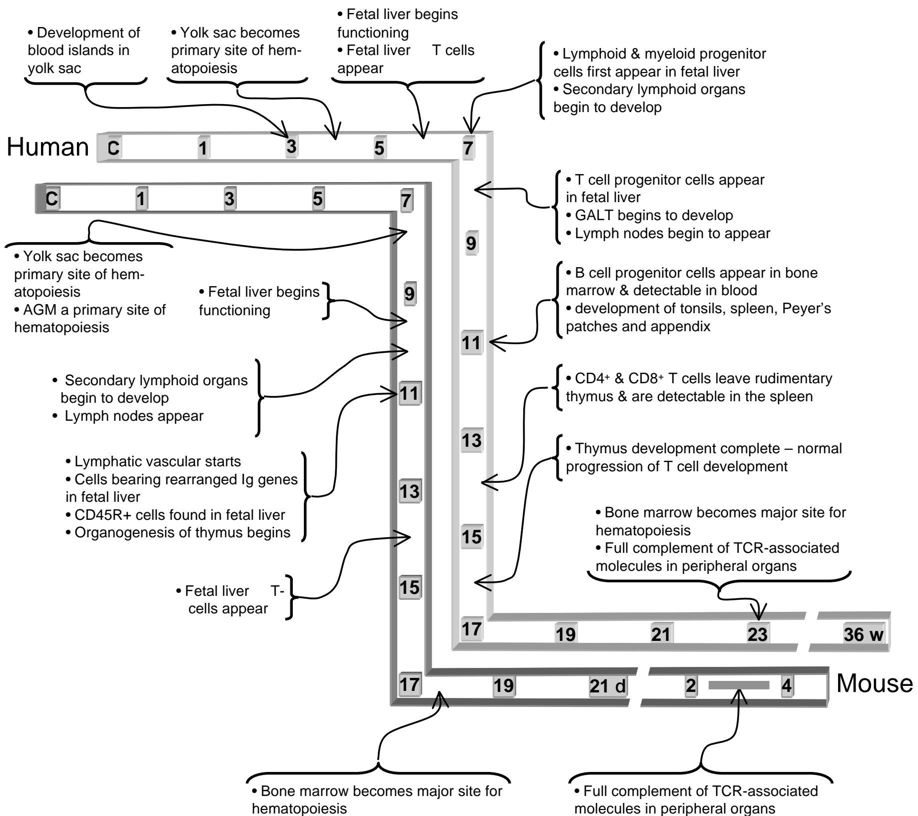

FIGURE 19.1 Human and Mouse Immune Ontogeny Timeline.

islands in the yolk sac.[20] From weeks 4–6 of gestation, the yolk sac is the primary site of hematopoiesis and remains so until 10 weeks of gestation.[21] Unlike the yolk sac of rodent embryos that remains active throughout gestation,[22, 23] the yolk sac in humans begins to regress at approximately 10 weeks of gestation.[21] Starting at approximately 5 weeks of gestation a major population of hematopoietic stem cells (HSCs) can be found associated with the ventral endothelium of the dorsal aorta.[20] It is in this area of the embryo that the fetal liver develops. The fetal liver becomes a primary site of hematopoiesis starting at approximately week 6 of gestation and continues as a primary site until approximately week 22 of gestation at which time the bone marrow becomes the major site for hematopoiesis. The most recent data suggest that the bone marrow is not seeded with HSCs from the fetal liver; rather a pluripotent $CD34^+CD38^-$ non-committed subpopulation develops independently in the bone marrow.[24]

Progenitor cells committed to develop into cells of either the lymphoid or myeloid lineage first appear in the fetal liver at approximately 7–8 weeks of gestation with sites of lymphopoiesis developing between 6–12 weeks of gestation. T cell progenitors migrate to the thymus from the fetal liver beginning at 7–8.5 weeks of gestation.[25] B cell lymphopoiesis begins in the bone marrow at approximately 12 weeks of gestation and around this same time B-lymphocytes are detectable in the blood. The gut-associated

lymphoid tissue (GALT) begins to develop at 8 weeks of gestation with the development of the lamina propria followed by the Peyer's patches and appendix at weeks 11 through 15.[26,27] Lymph nodes appear between 8–12 weeks of gestation followed by the tonsils and spleen at 10–14 weeks of gestation.[28,29]

T lymphopoiesis actually begins prior to the complete formation of the thymus. The fetal liver is the initial site of T cell lymphopoiesis starting at 6–8 weeks of gestation with the development of γ/δ T cell receptor (TCR)-expressing T lymphocytes.[30,31] By weeks 15–16 of gestation the thymic medulla is completely formed and the normal progression of thymocyte development occurs, including positive and negative T cell selection.[32,33] By 13 weeks of gestation $CD4^+$ and $CD8^+$ single positive T lymphocytes begin to leave the thymus and can be detected in the spleen and cord blood by weeks 14 and 20 of gestation, respectively.[34,35]

Table 19.1 summarizes the functionality of the human immune system during gestation. In newborns, the number of circulating B-lymphocytes is high and many cells have an immature phenotype. As the newborn ages, the total number of B-lymphocytes, in particular, those with an immature phenotype, start to decline to adult levels.[36,37] Some of the major properties of B-lymphocytes are absent or low at birth including isotype switching, secretion of immunoglobulins, and the presence of plasma cells in the bone marrow.[38,39] The lack of isotype switching is due primarily to the inability of newborn T lymphocytes to provide efficient help.[38,40] Antibody responses of newborns are lower than those of adults, particularly in response to non-protein T cell independent antigens (i.e., carbohydrates). This deficit remains past the first year of age.

The T lymphocyte population in newborns is also functionally immature (Table 19.1). At birth, the percent and number of $CD4^+$ and $CD8^+$ T lymphocytes are low, although the number increases to adult levels shortly after birth.[36,37] Unlike adults, newborns have a higher percent of γ/δ TCR-expressing T lymphocytes and over time (approximately 2 weeks) this percentage slowly decreases to adult levels.[34] Another major difference between newborn and adult T lymphocytes is the increased expression of surface markers normally associated with immature or naïve T lymphocytes (i.e., $CD45RO^-/RA^+$ [37,41,42]). Functionally, T lymphocytes of newborns are similar to adult T lymphocytes in that they respond to mitogens and in mixed lymphocyte reactions (MLRs). However, their ability to proliferate and secrete cytokines in response to antigen specific stimulation through their TCR is poor. Newborn T lymphocytes secrete less cytokines than adult T lymphocytes. [43,44,45,46,47] Interestingly, although T helper cell function is diminished in newborns, there is no difference in cytotoxic T lymphocyte function of newborns and adults.[48]

Cells of the myeloid lineage, including granulocytes, macrophages and eosinophils, have decreased function at birth[49,50] due in part to an increased number of granulocytes and macrophages expressing an immature phenotype.[49] The absolute number of granulocytes is also increased at birth compared to older children and adults. As part of this more immature phenotype, newborn granulocytes do not adhere to surfaces as strongly as adult granulocytes thus preventing them from migrating efficiently from the circulation to sites of inflammation.[50] In addition, neutrophils, macrophages, and eosinophils chemotaxis toward chemoattractants is decreased, probably due to lower expression of certain surface receptors such as the complement receptors.[50]

ANIMAL IMMUNE SYSTEM DEVELOPMENT

Many different animal species, including mice, rats, pigs, and nonhuman primates have been used as models to study the development of the immune system and the immunotoxic potential of chemicals.[51,52,53,54] The most commonly used animal models for studying the development of the immune system are rodents, with the immune system of the mouse being the most extensively studied. Although there are limited data on the effect of the same chemical agents on rats and mice,[55] the majority of the data that are available demonstrate that many chemicals have similar effects in both rats and mice.[55,56] Because of the many similarities between immune systems of rodents and humans, the use of rodents, be it mice or rats, is a reliable means of assessing the immunotoxic potential of chemicals. The short gestation period (approximately 21 days) and plethora of reagents available for studying the immune system of rodents make them an excellent model system to study the development of the immune system and its function.

As with humans, the development of the hematopoietic system in mice involves a series of progressions in which hematopoiesis begins in the yolk sac followed by the para-aortic splanchnopleura/aorta-gonad-mesonephros (PAS/AGM), the placenta, the fetal liver and finally the bone marrow (Table 19.1 and Figure 19.1). Development of the hematopoietic system in mice begins prior to day 7 of gestation with the development of hemangioblasts (progenitor cells that give rise to hematopoietic and vascular cells).[57] By day 7.5 of gestation the first signs of hematopoietic activity can be observed in the form of blood islands in the yolk sac.[58] Around the time of the development of hemangioblasts and prior to the formation of blood islands, genes important for hematopoiesis (GATA-1, GATA-2, tal-1 and rbtn2) are expressed.[59,60] Thus, hematopoietic commitment occurs early in gestation prior to the start of erythropoiesis and the development of what is often termed "primitive" erythrocytes.

Besides primitive erythrocytes, macrophages are the only other cell type of the hematopoietic lineage normally found in the yolk sac.[61] Little is known about these early or primitive macrophages except that the expression level of several genes important for macrophage function are altered compared to later stage and adult macrophages.[60,62,63] In addition, there is evidence to suggest that their differentiation pathways are dissimilar, lacking monocytic stages.[64] Overall, little is known about the yolk sac macrophages' developmental pathway and function in the mouse embryo.

The next site of hematopoiesis in the developing mouse embryo is the PAS/AGM, a specific region surrounding the heart. Starting at approximately day 8 of gestation the first HSCs capable of self-renewal and having the potential to differentiate into all of the different hematopoietic lineages in the PAS/AGM are detectible.[65,66] Although HSCs are detectable as early as day 8 of gestation it is not until day 10.5 that the HSCs present in the PAS/AGM develop the capacity to be transplanted into adult mice and completely reconstitute their hematopoietic systems.[66] The PAS/AGM continues to expand until day 11 of gestation at which time there is a sharp decline in its size and cell number, probably due to a migration of the HSC from the PAS/AGM to the fetal liver.[67,68]

Only recently has the mouse placenta been demonstrated to be a major hematopoietic organ. In the late 1970s, using major histocompatability haplotypes as markers, Melchers[69] demonstrated that the placenta of day 9 to day 12 mouse embryos harbored

B progenitor cells capable of differentiating into mature immunoglobulin secreting B cells.[69] Several laboratories have since demonstrated that beginning around day 8.5 of gestation, HSCs can be isolated from the fetal components of the placenta.[23] From day 11 to day 12 of gestation the number of HSCs in the placenta rapidly increases, exceeding the number found in the embryonic circulation and the AGM.[70,71] The frequency of more committed progenitors is less in the placenta than in the fetal liver.[23] Thus, the hematopoietic developmental profiles are quite different in the placenta and the fetal liver.

At approximately day 11 of gestation, the HSCs in the PAS/AGM start to migrate to and seed the fetal liver.[72] It has been proposed that the fetal liver, thymus and bone marrow are seeded by the same set of HSCs.[73] The fetal liver is the major site of hematopoiesis from approximately day 11 through day 18 of gestation.[74] The bone marrow does not become a site of hematopoiesis until late in gestation, approximately day 17.5 in mice, after mineralization of the long bones.[75,76] The bone marrow becomes the primary site of hematopoiesis at approximately day 18 of gestation and remains so throughout the life of the mouse. The spleen is a major site of hematopoiesis in mice starting at approximately day 13 of gestation. Unlike humans, myelopoiesis and erythropoiesis, but not lymphopoiesis, continues to occur in the mouse spleen after birth, albeit at a very low level.[76]

Based on Rag-1 expression, the first lymphoid progenitor cells are present at approximately day 9.5 in the PAS/AGM and the yolk sac.[67] Starting around day 10 of gestation, pre-B cell specific gene transcripts and rearrangements of D-J_H immunoglobulin gene segments are detectable in the PAS/AGM and liver. [67,68] At day 14 of gestation, B cell precursors are present in the fetal liver.[73] By day 15 of gestation, immunoglobulin heavy chain VDJ rearrangement is detectable in the fetal liver although over 80% of the rearranged VDJs do not make functional immunoglobulin heavy chains and the gene segment usage is limited.[67,77] A more diverse, polyclonal immunoglobulin heavy chain VDJ repertoire is detectable by day 17 of gestation in the liver and the percent of nonproductive rearrangements drops dramatically.[77] In the bone marrow and fetal liver lymphocytes are being generated.[77]

In the mouse, the thymus starts to develop on approximately day 10 of gestation and is colonized by migrating HSCs by day 11 of gestation.[78] On day 13 of gestation, double negative ($CD4^-CD8^-$) thymocytes appear in the thymus followed by double positive ($CD4^+CD8^+$) thymocytes on day 15.5 of gestation.[78] Molecular analysis has demonstrated that it is not until day 17 of gestation that thymocytes expressing rearranged TCRs appear.[79] $CD4^+$ and $CD8^+$ single positive functional thymocytes are detectable by approximately day 19 of gestation.[79] As in humans, the number of thymocytes is low at birth and continues to increase until approximately day 14 of age.[78,80] In mice, a steady state develops from 2 to 8 weeks of age when the size of the thymus and number of thymocytes decrease.[78, 80]

The first site of myelopoiesis[75] in the mouse embryo is the fetal liver, where the common myeloid progenitor, the megakaryocyte-erythrocyte-restricted progenitors and granulocyte-monocyte restricted progenitors are present. Myelopoiesis occurs in the fetal liver in the same manner as in adult bone marrow.[81] However, the proliferation capacity, colony forming activity and differentiation capacity is different between the fetal liver and adult bone marrow.[81]

It has been well documented that the neonatal immune systems of mice and humans are not the same as the adolescent/adult immune system in their ability to respond to antigenic stimulation and in the diversity of antibodies produced. Murine neonatal B cells do not make good, consistent antibody responses until 2 weeks of age even though the number and percent of B cells and T cells in their spleens remain relatively constant from 1 week to 2 weeks of age.[82, 83] In addition, neonatal B cells do not secrete as much antibody as adult B cells and signaling through the B cell receptor of neonatal B cells is not the same as in adult B cells.[84] In particular, phosphatidyl inositol is not hydrolysed in response to anti-immunoglobulin stimulation in neonatal B cells, although calcium is released from internal stores by an inositol 1,4,5-triphosphate-independent mechanism.[85] In addition, V_H gene family usage by the B cells in neonate mice is skewed to those V_H families closest to the J_H gene segments, unlike the adult B cell repertoire.[84,86] Data from Astori and colleagues[87] of the B cells.[87] The data suggest that further maturation of the B cell compartment is required after birth.

Neonatal T cells and macrophages also do not function like those of adults. In particular, macrophages, which function as antigen presenting cells, as well as secretors of chemoattractants and cytokines, secrete less proinflammatory cytokines and increased amounts of IL-10 in response to lipopolysaccharide (LPS) stimulation.[88] Neonatal T cells also require a stronger costimulatory signal than adult T cells in order produce a good response.[48,89] Without the additional costimulation, the T cells produce a dominant Th2 type response and a diminished Th1 type response.[48,84, 90] This skewed Th2 response is strongest if immunization occurs prior to 1 week of age and diminishes as the mouse ages.[84] This biased response in neonates may be related to the diminished capacity to secrete cytokines, in particular IL-12.[48, 84, 88, 89]

IMMUNE FUNCTION ASSAYS EMPLOYED IN DEVELOPMENTAL IMMUNOTOXICITY TESTING

Through the years many different immunological assays have been used to assess the effect of chemicals on the developing immune system. The lack of a set of standard immune assays to assess the developmental immunotoxicity of chemicals has made interpretation and validation difficult. In the late 1980s immunotoxicologists, as part of the National Toxicology Program, developed a tiered approach to assessing the immunotoxicity of chemicals in adult animals.[91] After the careful analysis of data from over 50 compounds the scientists determined which assays were the best predictors of immunotoxicity and changes in host immune defense responses.[92, 93]

More recently, the National Institute of Environmental Health Sciences and the National Institute for Occupational Safety and Health convened a workshop in which experts in the field of developmental immunotoxicology developed a tiered approach for assaying the developmental immunotoxicity of chemicals.[18] The recommended assays were separated into three groups: (1) an initial set of screening assays, (2) assays for validation of a correlation between the assay end point and functional outcomes in humans, and (3) assays for research development.[18] The initial screening assays included analysis of the primary antibody response to a T-dependent antigen, the delayed type

hypersensitivity response, complete blood count (CBC), and the weights of the thymus, spleen, and lymph nodes. For the analysis of antibody responses no specific antigen or age at the time of analysis was recommended. However, as discussed above, the immune system of embryos and neonates is not completely functional until approximately six weeks after birth. Thus, analysis of the primary antibody response is rarely performed prior to 6 weeks of age. A CBC has been demonstrated to be a sensitive measure of immune system development in neonates. A reduction in any immune cell population can have profound affects on the ability of the immune system to respond to a foreign pathogen.[93]

The immune function assays recommended in the second tier were chosen to assess the functional outcome of rodent exposure. The assays recommended included phenotypic analysis, macrophage function, and natural killer activity.[18].The assays in this group were chosen for several reasons. Phenotypic analysis can provide useful information on the loss or enrichment of a particular cell population as the result of chemical exposure. For example, several studies have demonstrated that chemicals such as benzo(a)pyrene and 2,37,8-tetra-chlorodibenzo-p-dioxin can induce profound changes to fetal liver and fetal thymus cell populations.[94, 95, 96] Due to the major role of macrophages in both the innate and adaptive immune response, their ability to secrete cytokines and to phagocytize and kill bacteria is a good way to assess immune system function. The surveillance function of natural killer cells and their importance in eliminating cells with abnormal phenotypes make assessing their function important. The final tier of assays involves assays for research development. In this tier, general and lineage specific assays were recommended.[18] In these assays the effect of chemical exposure on the hematopoietic process are assessed. Specifically, the effect of chemicals on the differentiation and proliferative capacity of lineage-specific and non-specific progenitor cells are measured. With these assays, the hematopoietic process can be assessed at all stages of development including embryonic, neonatal, and adult.

IMMUNE SUPPRESSION IN RODENTS EXPOSED TO IMMUNOTOXIC AGENTS

Immunotoxicity testing in rodents exposed to industrial and/or environmental chemicals, has been recognized as an important toxicological concern for over 25 years. Early immunotoxicity testing relied primarily on the mouse, due to the plethora of immune structure and function research performed by immunologists to better understand the human immune system. As such, the mouse has been the most employed rodent for immunotoxicity testing. Immune system function assays employed in screening for immunotoxicity were developed in adult mice. These same immune function assays have served to help identify toxicant induced immunosuppression in the rat.

A variety of compounds have been evaluated for developmental immunotoxicity. Early work with chlorinated hydrocarbon insecticides revealed that lactating women exposed to the insecticide chlordane, had residues of chlordane in their breast milk.[97] A study was performed in mice to determine if chlordane might alter the immune system of mouse pups exposed to chlordane via the dam's milk.[98] Female pups displayed

greater suppression of the contact hypersensitivity response (CHR) to oxazolone than did males. Other prenatal exposure studies revealed altered macrophage tissue necrosis factor (TNF) production[99] and altered macrophage tumoricidal activity.[100]

Prenatal exposure of mice to the fungicide hexachlorobenzene (HCB) resulted in suppression of the delayed type hypersensitivity (DTH) response to oxazolone. Similar to the DTH response, the *in vitro* mixed lymphocyte response (MLR) was also suppressed by *in utero* exposure.[101]

In utero exposure of mice to benzo(a)pyrene (B[*a*]P) resulted in suppression of the antibody response to SRBC, which persisted for up to 78 weeks in the offspring.[102] In a follow up study, injection of B[*a*]P from gestation day 11 to 17 resulted in suppressed antibody responses to SRBC, MLR and graft versus host responses (GvH). It was suggested that lesions caused by B[*a*]P in the developing immune system may predispose the host to the growth of neoplasms.[103]

Pregnant mice exposed to benzene from gestation day 12.5 to 19.5 resulted in the reduction of fetal liver pre-B and B cells. Responsiveness to the B cell mitogen LPS was decreased in spleen cell cultures. The results indicate that *in utero* exposure to benzene alters fetal lymphopoiesis that may be responsible for compromised immune responsiveness postnatally.[104]

Female mice given daily s.c. injections of diethylstilbestrol (DES) for the first 5 postnatal days after birth caused markedly reduced NK cell activity in adult offspring. No evidence of DES-induced cellular or humoral suppressors of NK cell killing was detected. While polyinosinic: polycytidylic acid (poly I:C) augmented the NK activity in control females, even very high doses of Poly I:C failed to increase the level of NK activity in neonatally DES-treated animals due to a loss of bone marrow NK precursors.[105] Female mice were injected with DES on PND 1 to 5. At 6 to 7 weeks old the cytotoxic activity against target cells in DES-treated females had only about one-half the cytotoxicity to YAC-1 cells compared to controls. The incidence of females developing methylcholanthrene-induced sarcomas, using a simple low-dose injection (10 or 20 μg), was higher among DES-injected animals than among controls. Taken together, the results indicate that female mice treated neonatally with DES have a functionally defective natural killer cell population, resulting in increased tumor susceptibility.[106]

Developmental immunotoxic effects of chronic pre- and postnatal exposure of rats to low levels of lead acetate in drinking water were examined.[107, 108] Thymus weights, IgM and IgG antibody responses, and lymphoproliferative responses to mitogens were all decreased. Blood lead level determinations in these offspring indicated that the lead levels observed were comparable to blood levels found in children in urban areas during the early 1970s.[109] Examination of the developmental immunotoxic effects of lead acetate in drinking water of male and female rat offspring, during pre-and/or post breeding, revealed suppression of the DTH response in female but not male offspring and decreased interferon.[110] Gender differences in immune function were observed in two follow up studies in which DTH responses and monocyte numbers were depressed in females but not males. The data indicate that differential gender-based immunotoxicity profiles exist following gestational exposure of rats to lead.[111, 112]

Certain organotins, such as di-*n*-octyltin dichloride (DOTC) and tributyltin oxide (TBTO), alter the structure and function of the thymus and consequently affect pri-

marily T cell-dependent immune function in rats. NK cell activity is also affected by organotins.[113] Direct gavage dosing of rat pups with DOTC, beginning at 3 days of age and continuing 3 times per week until 24 days of age for a total of ten doses, suppressed the lymphoproliferative response (LPR) to the T cell mitogens concanavalin A (ConA) and phytohemagglutinin (PHA) for up to 7 to 10 weeks of age.[114] Exposure to TBTO, employing the DOTC dosing regimen, resulted in suppression of the LPR to the T cell mitogens Con A, PHA, and pokeweed mitogen and the B cell mitogen *Salmonella typhimurium* which persisted until 10 weeks of age.[115]

One of the most studied developmental immunotoxicants is 2,4,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD). Exposure of experimental animals to TCDD results in a variety of toxic responses which differ in intensity among various species and strains.[116] Of the many organ/systems affected by TCDD one of the most sensitive is the immune system.[117] TCDD is a highly toxic compound that produces more severe effects when exposure occurs during development. Results of TCDD studies in experimental animals are of concern because humans potentially have their greatest intakes of TCDD and related polyhalogenated aromatic hydrocarbons during development. Breast-feeding infants have been estimated to consume 35–53 pg of TCDD toxic equivalents/kg body weight/day during the first year of life.[118] In comparison, various studies have calculated the adult intake to be in the range of 1 or 2 pg TCDD toxic equivalents/kg/day in Germany,[119, 120] the Netherlands,[121] and the United States.[122] In one study, a nursing infant was found to absorb 96% of the consumed TCDD, since nearly all of ingested TCDD is absorbed.[123] As in humans, transplacental and nursing represent the predominate sources of perinatal exposure to TCDD.[124, 125]

The immunotoxic effects of prenatal/neonatal exposure to TCDD in mouse and rat offspring results in immune system alterations. These include reduced weights and cellularity of thymus and spleen, increased susceptibility to transplanted tumor cells, increased allograph rejection time, and reduced bone marrow cellularity.[126] Exposure of the fetus to TCDD on GD 14 results in alterations in the lymphocyte stem cell population in the fetal and neonatal liver.[127] Following maternal treatment a significant reduction in the lymphocyte stem cell-specific enzyme terminal deoxynucleotidyl transferase (TdT) occurs. By GD14 TdT biosynthesis and TdT-specific mRNA is reduced by 50% in fetal liver lymphoid cells. An even greater reduction is observed in neonatal bone marrow, which persists to PND 18. The results suggest that during the perinatal period TCDD-induced thymic atrophy may be due to an effect on prothymocytes.[127]

Timed-pregnant rats were dosed with 1.0μg TCDD/kg on GD14. One day after birth, litters were cross-fostered to produce control, placental-only, lactational-only, and placental/lactational exposure groups. The DTH response to BSA was assessed in 5-month-old males. In these rats the severity of the suppression of the DTH response was related to the route of TCDD exposure (i.e., placental/lactational > lactational > placental), with suppression occurring only in the males receiving both placental and lactational exposure.[128] In order to determine the lowest maternal dose of TCDD required to suppress the DTH response in pups, dams were dosed with 0.1, 0.3, or 1.0μg TCDD/kg on GD14 and the DTH response to BSA was evaluated in 4- and 14-month-old pups. In the males, suppression was observed at a maternal dose of 0.1μg TCDD/kg at 14 months of age, while a maternal dose of 0.3μg TCDD/kg was required to cause suppression in

the 14-month-old females. Suppression of the DTH response to BSA was greater in both male and female offspring at 14 months of age than at 4 months of age.[129]

Pregnant rat dams were exposed to methoxychlor (MXC), an organochlorine pesticide which is more readily metabolized and excreted than is dichlorodiphenyltrichloroethane (DDT), and which is widely used for insect and larval control.[130] Dams were dosed by gavage with MXC from mid-gestation through the first week of birth, followed by direct dosing of pups from PND8 to PND42. This exposure regimen was employed in order to ensure pesticide exposure during the windows of developmental vulnerability for the immune system as well as the reproductive, and nervous systems. Of the immune system parameters examined only the PFC response to SRBCs was suppressed in 9-week-old males while females were not affected.

A second organochlorine pesticide, heptachlor (HEP), a chlorinated cyclodiene that was used primarily as an agricultural and domestic insecticide, was evaluated for its potential to suppress the immune system of rats. Rats were exposed to HEP pre- and postnatally, as described above for MXC. The IgM antibody response to SRBCs was suppressed at all doses in males but not females at 8 weeks of age. At 26 weeks of age, the IgG anti-SRBC response was suppressed in all of the HEP-exposed males, but not females.[131]

The chemicals examined for developmental immunotoxicity in the mouse and rat encompasses a range of classes that include metals, pesticides, drugs, and aromatic hydrocarbons. Suppression of immune function was observed in adult rodents exposed to each of these chemicals during immune system development. The duration of immune function suppression in rodents so exposed ranged from 3 weeks (i.e., DOTC and MXC) to 19 months (i.e., TCDD) after the last exposure to the chemical.

The choice of the period of chemical exposure during immune system development is dependent on the pharmacokinetics of the chemical relative to placental and lactational transport of the parent compound and/or its metabolites. Based on the review presented, for screening potential developmental immunotoxicants, it is recommended that dosing of the rat or mouse should occur during gestation and if possible the first week of lactation. Beginning at one week of age the pups should be dosed through 42 days of age as described by Chapin and colleagues[130] for rats. In this study a modified functional observational battery was used to assess neurobehavioral changes. Other offspring were evaluated for reproductive toxicity, while another group was evaluated for alteration in immune system function. This exposure paradigm could reasonably be employed to perform a comprehensive evaluation of the reproductive, neurobehavioral and immune system.

A review of the literature on chemical-induced immunosuppression in rats and mice, exposed during the pre- and/or postnatal period, was compared to exposure of adults. Five known immunosuppressants (i.e., TCDD, TBTO, DES, Pb, and diazepam) were reviewed. The data revealed that the developing immune system was more sensitive to chemical exposure than the mature immune system. Based on these evaluations, the authors concluded that it was "reasonable to assume that testing only in adults would not provide a sufficient level of sensitivity to define immunotoxicity in the neonate"[132]. In summary, this chapter provides compelling evidence that the developing, compared to the mature, immune system is more vulnerable to perturbation.

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20 Development of a Framework for Developmental Immunotoxicity (DIT) Testing

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INTRODUCTION AND DEFINITIONS

A basic tenet associated with most protocols devoted to an assessment of developmental toxicology is that children differ significantly from adults in their biological and/or physiological responses to environmental exposures or stressors.[1] In the context of immunocompetence, it is known that common infectious diseases occur more often, and are usually more severe in the very young when compared to adults. In most cases, initial contacts with the infectious agent will lead to immunity, and consequently, encounters with the same agent later in life will lead to less severe reactions. In some cases, age-related physical or physiological differences in tissues or organs are responsible for the increased susceptibility to infections. In addition, increased susceptibility can be due to the relative immaturity of the immune system in the very young that prevents the host from making an adequate response to microorganisms. Neonates are particularly susceptible to infectious agents that require adult-like production of antibodies and complement to mediate phagocytosis and bacteria killing. For children that survive with primary immunodeficiency diseases (i.e., primarily those with deficiencies only in

antibody production), there are increases in the frequency and severity of infections to both common and opportunistic infections. Children with less severe immunodeficiency usually have a higher incidence and severity of infections from common pathogens, such as upper respiratory infections or repeated inner ear infections, than the general age-matched population.

From a scientific perspective, the interest in developmental immunotoxicology (DIT) has been predicated around the possibility that the immune system may exhibit greater susceptibility to chemical perturbation during ontological development that may not be detected if immune function is only evaluated in adult animals. This greater susceptibility may be manifested as a qualitative difference, in the sense that a chemical could affect the developing immune system without affecting the adult immune system, or as a quantitative difference, in the sense that a chemical could affect the developing immune system at lower doses than the adult immune system, or as a temporal difference, in the sense that a chemical could produce a more persistent effect in younger animals than adults.

Any discussion of DIT should be centered on critical foundations in our understanding of the evolution of immunotoxicology as a scientific discipline, as a testing framework and as a regulatory focus. For the purposes of this chapter, "immunotoxicology" will be defined as the study of *adverse* effects on the immune system resulting from occupational, inadvertent, or therapeutic exposure to drugs, environmental chemicals, food additives or biological materials. In this context, immunotoxicology has already been accepted as an important component of safety assessment because screening for potential adverse effects on the immune system have become more commonplace.

In a multi-lab effort for the National Toxicology Program (NTP), under the primary direction of Dr. Michael Luster, a total of 51 chemicals were studied and included a variety of chemical classes, including catalysts, solvents, dyes, lubricants, pesticides, disinfectants, drugs, food additives, and natural products. It is important to emphasize that the chemicals selected for study by the NTP were nominated for testing because of a suspicion that they would target the immune system. The NTP studies compared a number of immune endpoints including several functional parameters, host resistance models, weights of select immune organs, leukocyte counts and differentials. These studies were conducted in young adult rodents, principally the mouse, and found that the enumeration of antibody producing cells (as in a T dependent antibody response (TDAR)), an assessment of lymphocyte subpopulations by cytofluorometric analysis and an assessment of natural killer activity were the most valuable in terms of predicting immunotoxicity.[3,4,5] None of these assays were 100% predictive alone and the studies by Luster and co-workers indicated that combinations of tests were needed. At the National Institute for Public Health and the Environment (RIVM) in The Netherlands, immunotoxicity studies were usually performed in the context of 28 day oral exposure studies, in which several doses were evaluated, including at least a high dose that induced overt toxicity, and a low dose that did not induce overt toxicity.[6] In these studies, assessing the pathology of lymphoid organs and tissues appeared to be a powerful indicator of immunotoxicity, although relying on histopathology was not always sufficient.[7]

The scientific discipline of immunotoxicology has evolved to the point where guidelines exist within many regulatory frameworks. For instance, Organization for

Economic Cooperation and Development (OECD) Guideline 407, for 28 day oral toxicity testing was updated in 1995 to include histopathological evaluation of the thymus and lymph nodes in addition to the histopathological evaluation of the spleen and peripheral blood differential leukocyte counts.[8] In the United States, the Environmental Protection Agency (EPA) published their immunotoxicity guidelines for pesticides and toxic chemicals in final form in 1999.[9] The EPA Immunotoxicity Test Guideline (Office of Prevention, Pesticides and Toxic Substances (OPPTS) 870-7800) requires the assessment of a functional parameter, the primary antibody response to a T-dependent antigen, such as sheep red blood cells (SRBC), in addition to weighing the spleen and thymus as the first tier of testing in young adult rodents. The EPA immunotoxicity guidelines also recommended two "optional" tests that could be triggered based on the outcome of the analysis of the TDAR on a case-by-case basis, although the specific criteria that will be used to trigger the "optional" tests are not identified in the guidance document.. In the field of human pharmaceuticals, the European Union released a revised Note for Guidance on Repeated Dose Toxicity in 2000, in which the use of a TDAR assay is required to complete the initial screening for immunotoxicity if other methods are not available.[10] More recently, the European Medicines Agency (EMEA) and the U.S. Food and Drug Administration (FDA) have also provided recommendations for the use of functional assays to assess the immune system of adult animals in their Guidance for Industry, Immunotoxicology Evaluation of Investigational New Drugs.[11] As the geographic areas differed in their approach, this issue was brought to the attention of the International Conference on Harmonization (ICH), and a final document has been agreed upon which is focused on providing recommendations on nonclinical testing for immunotoxicity induced by human pharmaceuticals.[12] The general principles that apply to this guideline are that all human pharmaceuticals should be evaluated for the potential to produce immunotoxicity. Methods used include standard toxicity studies (i.e., hematology, clinical chemistry, gross pathology, organ weights, and histopathology) and on the basis of a weight of evidence approach on certain triggers additional immunotoxicity studies (i.e., TDAR, immunophenotyping, natural killer cells assay, etc.) as appropriate. These triggers are pharmacological properties, intended patient population, structural similarity, disposition of the drug, or signs observed in clinical use.

HISTORICAL PERSPECTIVE ON DIT

Manifestations of chemical-induced DIT have been reviewed by Barnett[13] and by Van Loveren et al.[14] lending support to the speculation that the fetus and immunologically immature organisms are particularly sensitive to xenobiotics. Because the information is highlighted by Smialowicz and Barnett in another chapter in this volume,[15],this chapter will not go into detail on specific immunotoxicants. As evidenced in Table 20.1, there has been significant interest in DIT over the last decade. It is important to note that Table 20.1 is not intended to be an all-inclusive listing and merely serves to emphasize the recent level of activity in DIT on a global scale, with a definite focus on applications to a testing framework and to risk assessment.

Of the activities listed in Table 20.1, the International Life Sciences Institute (ILSI)

TABLE 20.1
Recent Activities in Developmental Immubotoxocology (DIT)

Description of Activity	Location	Date
1. ILSI RSI workshop: Research needs on age-related differences in susceptibility to chemical toxicants.	Washington, DC	June, 1996
2. EPA workshop: Critical windows of exposure for children's health.	Richmond, VA	Sept., 1999
3. SOT poster discussion session: Developmental immunotoxicity.	Philadelphia, PA	March, 2000
4. SOT workshop: Developing immune system: Sensitive target for perturbation by xenobiotics.	San Francisco, CA	March, 2001
5. ILSI HESI workshop: Developmental immunotoxicology and risk assessment.	Washington, DC	June, 2001
6. 10th Immunotoxicology Summerschool: Focus on developmental immunotoxicology.	Lyon, France	Oct., 2001
7. NIEHS/NIOSH workshop: Consensus workshop on methods to evaluate DIT.	Washington, DC	Oct., 2001
8. BgVV workshop: Children as a special subpopulation: Focus on immunotoxicology.	Berlin, Germany	Nov., 2001
9. SOT symposium: Children's health risk: What's so special about the developing immune system.	Salt Lake City, UT	March, 2003
10. ILSI HESI roundtable discussion on developmental immunotoxicology.	Washington, DC	May, 2003
11. Eurotox symposium on developmental immunotoxicology.	Florence, Italy	Sept., 2003
12. SRA symposium: State of the science in children's health research — DIT.	Baltimore, MD	Dec., 2003
13. 30th Toxicology Forum: Session on developmental immunotoxicology.	Aspen, CO	July, 2004
14. SOT sunset session: Developmental toxicology: Issues with including neurotox and immunotox assessments in reproductive toxicology studies.	New Orleans, LA	March, 2005
15. EPA Science Forum: DIT: A proposed testing framework	Washington, DC	May, 2005

Health and Environmental Sciences Institute (HESI) Workshop in June of 2001[16] and the National Institute of Environmental Health Sciences (NIEHS)/National Institute of Occupational Safety and Health (NIOSH) co-sponsored Workshop in October of 2001[17] were particularly effective in advancing our understanding of the design of a framework for DIT testing, perhaps because both workshops integrated developmental toxicologists into the discussion of DIT. Interestingly, there were clear differences in the approaches taken in these two workshops. The ILSI HESI workshop was "open" and attracted more than 75 participants from the United States and Europe. The program invested an entire day to a series of plenary lectures on comparative developmental immunology, including a perspective on the development of the human immune system,[18] on immunotoxicology models of immunosuppression, and on regulatory aspects of DIT.

The latter session included government perspectives from the EPA OPPTS, the EPA Office of Research and Development, the EPA Office of Children's Health Protection, the OECD, the EMEA and the Committee for Medicinal Products for Human Use (CHMP), in addition to industry perspectives reflecting both the chemical and pharmaceutical industries.[19] The NIEHS/NIOSH workshop was limited to 23 invited participants. A limited number of background scientific presentations were given, and the emphasis was on break-out group sessions in which a series of predetermined questions were raised and key points were captured by rapporteurs.[17]

In spite of differences in the organizational approaches, there was very good agreement between these two workshops on the key points important to development of a framework for DIT testing.[16,17,19] Both workshops concluded that the rat was the species of choice for screening for DIT potential, even though there are clear differences in the development of the immune systems of rodents and humans. This point was also the focus of a recent review,[20] as discussed below. Both workshops emphasized the importance of flexibility in experimental design, of the ability to assess the potential of DIT by an experimental design that encompassed all of the critical windows of development and of a test strategy that could be integrated into existing developmental toxicity protocols. It was noted at both workshops, as well as by others,[14] that it is important to ensure that exposure occurs during all of the critical developmental periods, and that both functional assays and more standard measurements of immunotoxicology (e.g., organ weights, pathology, hematology, and differential circulating leukocyte counts) be included. Finally, the participants in both workshops concluded that as of 2001, a specific design for a DIT protocol could not be determined, and identified a number of research data gaps.

APPROACHES TO DIT TESTING FRAMEWORK

It must be emphasized at the onset that there are still no validated or widely accepted methods for evaluating the effects of a chemical on the developing immune system. This section will consider some of the most appropriate approaches to identifying developmental immunotoxicants, in the context of recommendations and conclusions from many of the activities highlighted in Table 20.1.

One of the first points to consider in a DIT testing framework is the selection of an animal model. As noted above, the rat is the preferred species.[17,19] This conclusion was reached after summarizing the comparative biology of the developing immune systems in rodents,[21] dogs,[22] and humans[18], as well as the use of various species as models in DIT, including rats,[23,24] mice [25], pigs,[26] and nonhuman primates.[27,28] A recent review compared the anatomical and functional differences in the immune systems of the mouse, rat, dog, primate, and human[20] and their use as models for DIT testing. The review recognized that the database for interspecies comparison is incomplete, especially when comparisons are made to primates, and that we know the most about the developing immune systems of mice and humans. Even though it is generally assumed that immune ontogeny in the mouse and rat is similar, it is important to emphasize that a robust characterization of the key developmental milestones in the rat has heretofore

not been conducted. Furthermore, while immune system development in the rodent is delayed relative to the human, differential maturation does not obviate the choice of rodents as a test species, although it must be factored into the interpretation. Although dogs, like humans (and unlike rodents), are born with a functional immune system, the *in utero* development in dogs is delayed (i.e., only slightly advanced relative to rodents). A consideration of the dog as a model in DIT must recognize that there are also differences in placental structure between dogs and humans. As above, this observation doesn't obviate the choice of dogs as a test species, but it must be factored into the interpretation of results, especially as it relates to transplacental exposure. Other considerations in the selection of the dog as a model for assessing the potential of DIT include limitations in reagents and methods for use in this species, and the political sensitivity of using non-rodent species, such as the dog. Finally, the review concluded that organogenesis is the feature that most clearly distinguishes the developing and mature immune systems, and emphasized that immune organs (spleen, thymus, and bone marrow) are not typically assessed in developmental toxicity studies. In that regard, the review speculated that because chemical abnormalities in fetal organ development could be classified as teratogenicity, it might be more appropriate to consider some chemical-induced changes in the developing immune system as "immunoteratology" as opposed to DIT.

A second major point in a DIT framework is a consideration of exposure. As summarized in another chapter in this volume,[15] although researchers have exposed animals *in utero* to chemicals or drugs, the majority of studies, to date, have evaluated potential DIT based on the measurement of the immune status of the adult offspring.[13,29] This observation is not surprising, as most of the procedures that have been developed for assessing the effects of chemicals and drugs on the immune system have undergone extensive evaluation in adult rodents.[3,4,5,6,7] Interestingly, this observation is also not unique for immunotoxicology, as a review of the available literature in almost any area of health-related toxicology research indicated that an overwhelming proportion of previous testing had been directed toward exposure in adults, as opposed to offspring regardless of target organ.[29] However, as depicted in Figure 1A, the approach taken in this "conventional" DIT protocol is, in essence, an immunotoxicology "recovery" study [30] because of the length of time separating exposure (i.e., usually to pregnant dams over some portion of gestation) and the assessment of the impact on the developing immune system (i.e., usually in offspring when they become young adults). As such, this type of approach would determine if an effect was persistent, which, as noted above, is an important potential manifestation of a chemical's effect on the developing immune system that must be considered. However, it has been emphasized that both persistent and transient effects are important to consider in a DIT testing framework.[16,19,30] The ultimate consequences of the recommendation to consider transient effects was the conclusion that exposure should continue until the impact on the immune system is assessed and the recognition that protocols other than the conventional one depicted in Figure 20.1A need to be characterized.

In this regard, an alternative protocol (Figure 20.1B) was formulated in which pregnant dams would be exposed throughout gestation and lactation, and in which the immune tests were conducted in rat pups or weanlings.[30] A critical question in this scheme is whether methods validated in young adult rodents would be effective in assessing the

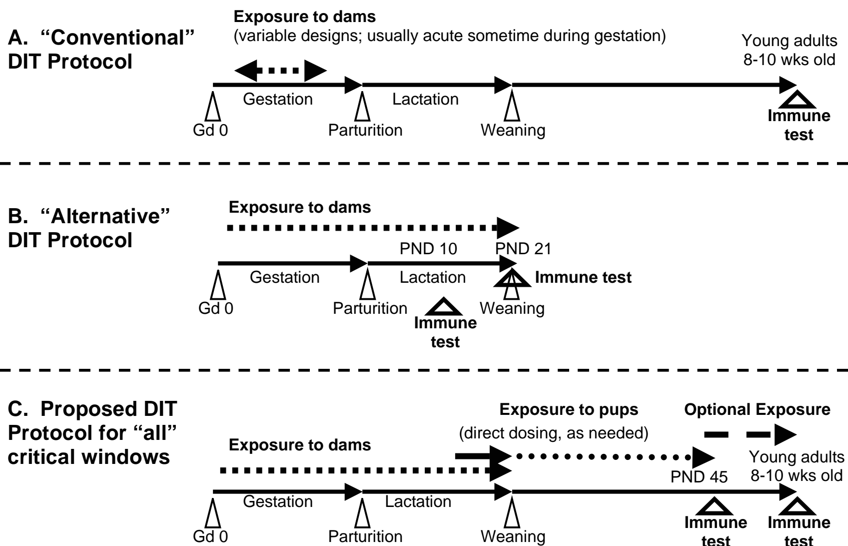

FIGURE 20.1 Comparison of various approaches to DIT.

status of the immune system of immature animals, as depicted in the alternative DIT protocol (Figure 20.1B). Results of a study by Ladics and colleagues[31] suggested that it may not be possible to measure a TDAR in rat pups due to the immature status of their immune cells, an observation which was consistent with a phenotypic analysis of lymphocyte subsets in pups. While the phenotypic analysis in rat weanlings demonstrated cell numbers in the various lymphocyte subsets that were comparable to adults, the magnitude of the TDAR was less than the historical range observed in adults, although still of sufficient magnitude to be measurable.[31] As such, these results suggested that the measurement of an immune test in rats on postnatal day (PND) 10 (Figure 20.1B) should be limited to nonfunctional parameters, including histopathology, and that it must be recognized that the measurement of an immune test in rats on PND 21 (Figure 20.1B) could include either histopathology or a functional parameter but that the latter would be suboptimal. There is general agreement that the TDAR with SRBC reaches magnitude similar to adult levels in rats at around PND 45.[32]

As noted above, the consensus recommendation was to assess the potential of DIT by an exposure design that encompassed all of the critical windows of development.[16,17,19,30] This proposed protocol, as depicted in Figure 20.1C, illustrates that the pregnant dams would be exposed throughout gestation and lactation, that pups would begin direct exposure at weaning (or sooner via direct dosing, as depicted in Figure 20.1C by the solid line, and as discussed further below), and that the immune test would be conducted in young adult rats at or around PND 45 (or even later in life if additional optional exposures are integrated). There is little doubt that the immune test can be successfully conducted as proposed in Figure 20.1C, but a significant question that does arise is how to assure exposure of the young offspring to the compound. As noted above, the general consensus is that pups in DIT studies should be exposed throughout

the entire treatment protocol, a consideration which is quite consistent with the goals of immunotoxicology guideline studies conducted in adult animals. While exposure cannot be specifically controlled *in utero* (i.e., placental transfer is a function of the chemical being tested), offspring may be exposed to compounds either via lactational exposure or through direct oral dosing. The decision to direct dose pups may depend on the compound. If data indicate that lactational transfer does not occur with a given compound, then direct dosing may be necessary to assure exposure throughout the treatment protocol. It is generally recognized that it is possible to directly dose rat pups beginning at PND 7, although some investigators have reported successful oral dosing as early PND 4. In designing direct exposure studies, several important issues must be considered, including the age at which direct dosing may be commenced, and the need for, and value of determining a compound's plasma levels in pups and the dose to be given. One potential problem of dose selection for direct dosing is that *in utero* or lactational exposure practically guarantees that a lower dose will be delivered to the pups if the same dose is delivered to the dams and to the directly-dosed newborn. Dose selection will be influenced by an understanding of the maximum tolerated dose in pups and the absorption, distribution, metabolism, and elimination characteristics of the compound in young animals. The latter may be impacted by the developmental status of the gastrointestinal tract and general metabolic processes,[1] and/or potential target organ systems. It is recognized that the determination of plasma levels in rat pups is technically challenging and no consensus was reached on whether the acquisition of toxicokinetic endpoints in pups was critical.[17,19,30] More recently, it was concluded that pup exposure should be understood, that careful consideration should be given to determining the need to directly administer test substance to pre-weaning pups, and that preliminary range-finding toxicokinetic data may be necessary.[33]. However, it was also noted that while it is relatively straightforward to verify exposure qualitatively, it is much more difficult to provide a robust quantitative measurement of exposure in offspring during gestation and lactation because there are no generally accepted standardized procedures.

Another consensus recommendation was that, when possible, methods to assess DIT should be included in existing developmental and reproductive toxicology protocols.[17,19,30] The feasibility of this approach has been previously demonstrated in studies described by a number of investigators.[34,35,36] While it could be argued that DIT methods could also be integrated into the EPA Developmental Neurotoxicity Protocol (OPPTS 870-6300[37]) the consensus was that this approach would not be technically feasible due to the large number of animals already required to conduct that study.[7,19,30]

Even though it was emphasized at the outset of this section that there are currently no validated or widely accepted methods for evaluating the effects of a chemical on the developing immune system, the final point to be considered in the context of a DIT testing framework is the current thinking for the integration of specific methods. Due to the extensive evaluation and validation of immune assays/endpoints that has already occurred in adult rodents,[3,4,5] the importance of capitalizing on assays already validated in adult rodents for assessing DIT has been emphasized.[17,19,30] To this end, and consistent with the current EPA,[9] EMEA,[10] FDA,[11] and ICH [12] immunotoxicity testing guidelines, it was agreed that the TDAR was ready to be included in a DIT protocol,

as long as the caveats identified above[31,32] were considered. Several other assays have been considered for inclusion in a DIT, including macrophage function, complement analysis, surface marker analysis (i.e., phenotypic analysis of immune cells by flow cytometry) and the use of general and lineage-specific stem cell assays.[17] However, it was concluded that the utility of these tests in a DIT protocol would require further investigation.[17] As noted above, host resistance models were a critical component of the NTP studies;[3,4,5] but they were considered to be less appropriate for a DIT screen,[19] because they may cover only part of the function if the immune system, and are usually laborious and expensive.

A group of 30 immunotoxicology experts from the United States and Europe, representing government, industry ,and academia, met in May of 2003 in a roundtable discussion to reach consensus regarding the most appropriate methods to assess DIT for hazard identification.[38] Among the questions they considered was the role that pathology can play in DIT testing. Interestingly, neither EPA nor FDA currently requires histopathology of immune organs in guideline reproductive studies. Roundtable participants agreed that immunopathology was an endpoint that could easily be incorporated into existing developmental and reproductive toxicology protocols. Participants suggested that if immunotoxic effects were observed in adult subchronic studies, then histopathology of immune organs should be included in the protocol of a reproductive toxicology study. However, some participants questioned whether routine histopathology would be sensitive enough to detect all potential immunotoxic effects, particularly in the developing immune system, and suggested that more sensitive methods, which assess immune function, may need to be utilized. For example, histopathology of immune organs failed to detect the developmental immunotoxicity of lead, whereas immune functional assays did detect lead induced alterations to the developing immune system.[39] Others felt that immunopathology would be sensitive enough to detect immunotoxic effects, but that more defined methods involving target tissues, specific immune markers, grading, and image-analysis may need to be identified.

REGULATORY PERSPECTIVE ON DIT

From a regulatory perspective, the interest in DIT has been predicated around the possibility that the developing immune system may exhibit greater susceptibility to chemical perturbation than young adults, and that guideline studies conducted exclusively in young adult animals would not detect this greater susceptibility. For example, U.S. and EU authorities have recently adapted their pharmaceutical legislation to include requirements for pharmaceuticals for children. In the context of this legislation, both the FDA and the EMEA are working on guidance documents on testing in juvenile animals to define when this testing is appropriate and needed. The FDA released their final guidance document in August, 2005.[40] With these guidance documents in place, the thinking process on DIT should now begin to result in clear recommendations. Without any doubt, the integration of parameters reflecting the immune system, to date, has been quite minimal. For example as noted above, immune organs are still not routinely included as potential target organs in most developmental toxicity protocols.

While immunotoxicology has evolved to the point where testing guidelines exist within many regulatory frameworks, the discussion to incorporate appropriate experimental approaches and assays available to assess DIT is still in its infancy, in spite of an impressive level of activity (Table 20.1). Regulatory agency concerns about potential risks to the developing immune system from exposures to pharmaceutical or environmental chemicals could lead to the decision to require a DIT study for a specific test substance. The 2002 FDA guidance on immunotoxicity evaluation suggests that DIT should be performed using rats in standard reproductive studies, rather than needing a stand-alone study, [11]an approach which is completely consistent with the recommendations highlighted above. Moreover, while the EPA currently has not published an official guideline/protocol for DIT testing, the Agency has also consistently encouraged combination endpoint studies where it makes sense to do so.[38]

The FDA juvenile toxicity guidance document recognizes that there are different endpoints in the development of the immune system[40] and that these responses mature at different rates. For example cell development largely occurs before birth, although antibody synthesis in response to antigen challenge, as measured by the TDAR, does not reach adult levels until PND 41-56, and NK cell activity is mature at PND 21. These different developmental time frames raise the question as to whether there is a single test that covers the different aspects of the immune system, and accounts for this difference in the state of development.

One of the driving forces in the nonclinical regulatory field is that compounds should be tested in animals in order to avoid potential adverse effects that may be expressed as clinical disease. For example, it is known that many of the immunosuppressive drugs that are used during pregnancy can cross the placental barrier and enter the fetal immune system. A number of criteria have been identified as possible "triggers" that might contribute to the determination that a DIT study was needed.[38] *Class-specific triggers*: A number of chemicals have been identified as having immunotoxic potential. For structural analogs of known immunotoxicants, confirmation of immunotoxic potential, in some cases including DIT potential, could be deemed necessary. The current state of the science provides an incomplete understanding of the correlation between chemical structures and immunotoxic potential. *Findings from animal toxicology studies*: Studies are generally conducted in adult rodent or nonrodent species, and include screening level assessments of various endpoints across multiple organ systems, following different durations of exposure via the route that is expected to be most relevant to humans. A typical toxicology data base data will also include standard studies that assess effects of prenatal and/or postnatal administration of the material to laboratory animals. Treatment-related perturbations of immune system structure or function could be identified as adverse clinical findings (e.g., susceptibility to disease); alterations in immune system functional endpoints (e.g., TDAR) in adult animals; alterations in organ weight, macroscopic findings, or histopathology of spleen, thymus, or lymph nodes; alterations in differential white cell counts or serum immunoglobulin levels; or cellular alterations that are nonspecific indicators of an effect on immune response, for example, increased numbers of macrophages in lung tissue or an increased incidence of inflammatory dermal lesions. *Intended patient population*: If a drug is intended to be used in a patient population in which the majority is immunocompromised by the disease state or concurrent therapy (e.g. to prevent perinatal transmission of HIV infection),

assessment of potential immunotoxicity in a developmental toxicology study should be considered. Moreover, the labeled indication for a drug often only represent a subset of the real world use, and the potential for off-label use should be taken into consideration in determining the need for DIT studies. *Potential neonatal exposure*: Another issue 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.

The DIT protocol recommended by previous workshops[16,17,19,30] and depicted in Figure 20.1C has been extensively considered in the context of a testing framework, and two important conclusions were reached.[38] The first conclusion was that when a compound has been tested in a standard adult immunotoxicity study and found to be an immunosuppressant, testing using the proposed DIT protocol may be redundant. In this instance, the consensus was that carrying exposure out to PND 45 (or beyond) prior to functional evaluation would likely yield the same result as previously seen in the adult. Thus, compounds that are immunotoxic in adults are likely to be immunotoxic to the developing immune system and assessing the relative sensitivity of the juvenile immune system may require functional assessment at an earlier age than currently proposed. The potential issues involved in addressing this approach were already considered above in the context of the alternative DIT protocol (Figure 20.1B). The second conclusion was that if a study was conducted which assessed all of the critical windows of exposure (i.e., the proposed DIT protocol depicted in Figure 20.1C) and that study was negative, there would be no need to conduct further immunotoxicity testing solely in adult animals.[38] In this instance, carrying exposure out to PND 45 (or beyond) in the proposed DIT protocol prior to functional evaluation would likely identify a similar response as would be seen if a standard immunotoxicity study were conducted in adult animals only.

An important point to be considered in the context of a regulatory perspective on DIT testing is whether the inclusion of a TDAR, immune organ weights, and immunopathology in a testing framework would be adequate for assessing DIT. Although data are limited, there are some examples of developmental immunotoxicants that affect only cell mediated immunity (CMI, e.g., the delayed-type hypersensitivity response) including lead[39,41,42] and TCDD.[43] It is important to emphasize, however, that the preferential alteration of CMI vs. humoral immunity in the developing immune system was clearly identified as an important data gap needing further investigation.[19,30] As such, the inclusion of an endpoint reflecting the status of CMI in a DIT testing framework will be considered in the next section on data gaps.

A final point is related to the way developmental studies are carried out in humans using immunization with vaccines such as Hepatitis B.[44] In just the last five years, more attention has been devoted to the testing of vaccines in animals, as described in the EU guidance on nonclinical testing of vaccines.[45] Testing for efficacy and toxicity has been described by the World Health Organization (WHO).[46] However, certain pediatric vaccines (e.g., to respiratory syncytial virus) are being reformulated without a thorough understanding of efficacy and other new vaccines are being developed. Evaluation of these products, particularly those that include new adjuvants, should address whether the immature immune system is more sensitive or less sensitive than that of the adult, and whether the administration of adjuvants early in life is always beneficial.

DATA GAPS AND FUTURE DIRECTIONS FOR ASSESSING DIT

As emphasized above, two important workshops held in 2001 identified a number of data gaps and research needs to advance the state-of-the-science for assessing the potential for DIT.[16,17,19,30] Moreover, participants in a 2003 roundtable discussion emphasized that a number of important research efforts are currently underway in the areas of DIT, examining immune system responses following developmental exposures to known immunotoxicants.[38]

Some methodological aspects of standardized DIT testing need to be further developed. For example, there is no question that pharmacokinetic data could be useful in establishing dose levels for the study, although there is no consensus on the design of pharmacokinetic studies.[33] Concerns about ensuring exposure to 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. Most important, the manner in which various mandated developmental studies can be combined needs to be examined, in order to reduce the number of animals used in testing.[33]

Another question is whether an endpoint reflecting the status of CMI should be included in any DIT protocol.[17,19] For the measurement of CMI, roundtable participants suggested that a validated DTH or T-cell responses to anti-CD3 be evaluated.[38] The DTH assay is considered by the NTP as part of the Tier II test panel.[3] Although reports indicate that the delayed-type hypersensitivity (DTH) response can be assessed in weanling rats.[41] roundtable participants agreed that data are lacking as to whether cell-mediated immune (CMI) assessments in younger animals are feasible.[38] Ultimately, the characterization of a "validated" endpoint which measures CMI, and the determination of whether such an endpoint should be an essential part of a DIT framework remain critical research needs.

It is known that immunotoxicity represents a continuum[47] with immunosuppression leading to increased susceptibility to infections and neoplasms at one end of the continuum, and inappropriate immunostimulation leading to hypersensitivity and autoimmunity at the other end of the continuum. While current methods applied to adult animals focus on immunosuppression and hypersensitivity (i.e., mostly in the context of contact and respiratory sensitization), there is no assessment model for the induction of autoimmunity, or immune system over-stimulation. There is no doubt that the emphasis in most, if not all of the activity in DIT (Table 20.1) has focused on immunosuppression. Even though the models to study hypersensitivity have been well-characterized in adults and provide the basis for regulatory guidelines, these models have heretofore not been studied in young animals. It is important to note that the models used to assess contact sensitization are in fact measuring a DTH. The inclusion of an assessment of CMI response in a DIT testing framework has been recognized as desirable, yet, as noted above, there is some uncertainty regarding the status of validation for this methodology.

Further research is needed to more effectively assess risks to developing humans. For pharmaceuticals, more information is needed to determine whether an effect on a biomarker from an adult study can be applied to set safe levels in juveniles; information on biomarker expression in juveniles is lacking. It is also not known whether juvenile humans are more sensitive to immune system perturbations than adults, although ani-

mal data have clearly identified age-related sensitivities for some chemicals (e.g., lead, TCDD), as summarized in another chapter in this volume.[15] 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 response and the role of stress. Evaluations of the public health implications of immune system suppression will further elucidate the benefit of more fully characterizing immunotoxic risks.

While immunotoxicology screening tests have undergone a series of validation exercises and that immunosuppression can lead to an increased incidence and/or severity to infectious and neoplastic diseases, interpreting results from immunological tests in experimental animal studies or even epidemiological studies conducted in adults or 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. As noted at the onset of this chapter, "immunotoxicology" was defined as the study of adverse effects on the immune system resulting from occupational, inadvertent, or therapeutic exposure to drugs, environmental chemicals, food additives and, in some instances, biological materials, with an emphasis on "adverse." 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. This may require, for example, development of a model to equate moderate changes in leukocyte counts, CD4 cell numbers and/or immunoglobulin levels, tests that can be readily performed in children, to potential changes in the incidence or severity of infectious diseases. 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 certain estimates or assumptions to be applied to account for differences in the integrity of the host's anatomical and functional barriers and the overall immunocompetence of an individual that is affected by genetics, age of the child, gender, use of certain medications, nutritional status, and environment.

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Part VI

Wildlife Immunotoxicology

21 Invertebrate Immunotoxicology

Tamara S. Galloway and Arthur J. Goven

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INTRODUCTION

It has been hypothesized that many compounds released into the environment as a consequence of human activity may be capable of altering immune function in exposed human and wildlife populations, leading to permanent alterations in the structure and regulation of the immune system and to increases in disease incidence. This hypothesis is supported by a growing body of evidence from laboratory studies in which it has been shown that individual chemicals, including aromatic hydrocarbons, heavy metals, organotins, oxidant air pollutants, organophosphates, organohalogens, solvents, and particulates, may adversely affect different components of the immune system.[1,2] In humans, a number of studies have examined links between accidental or environmental exposure to immunotoxic contaminants and apparent increases in disease incidence, and similar links have been suggested for certain wildlife populations. Recent attention has focused on marine mammals inhabiting the heavily polluted coastal waters of North-Western Europe.[3] An outbreak of phocid distemper virus-1 that occurred in the late 1980s in Harbor seals (*Phoca vitulina*) was instrumental in suggesting human activity as a factor in the dynamics of the disease, as high levels of organochlorine compounds were found in the tissues of seals that had died. Subsequent studies have confirmed and extended these findings, providing evidence for potential mechanisms of toxicity[4]

(chapter 24 of this volume) and highlighting the extremely high body burdens of persistent organic pollutants that can accumulate in meat eating species such as *Orcinus orca,* the Pacific killer whale.

Very little is known about how exposure to immunotoxic compounds and the habitat degradation that occurs in polluted environments affects the health and survival of the invertebrate phyla. This is a serious omission, not only because invertebrates account for over 95% of all animal species, but also because they frequently occupy key ecological positions. For example, the huge decline in populations of the herbivorous sea urchin, *Diadema antillarum,* which occurred in the Caribbean basin in the 1980s following an outbreak of an as-yet unidentified infection, caused a major ecological impact on the coral reef systems in the area.[5] Susceptibility of invertebrates to disease may also be of huge economic importance. The intensive aquaculture of bivalves and crustaceans is frequently prone to devastating outbreaks of viral and bacterial disease, such as the hypodermal and hematopoietic virus outbreaks that have crippled shrimp farming activities in many parts of the world.[6] Management of these situations is hampered by a lack of knowledge of the impact of anthropogenic factors on the subtle balance that exists between infection and immunity in the invertebrate phyla.

A final compelling reason for studying immunotoxicity in the invertebrates is that certain invertebrate species represent powerful model systems that are increasingly amenable to manipulation by molecular genetics.[7] The complete genome has now been sequenced for several invertebrates, including the fruitfly *Drosophila,* the nematode *Caenorhabditis,* the tunicate *Ciona,* and the mosquito *Anopheles*. These new sequence data have made it possible to develop a more extensive understanding of defense genes, their homologies and lineages and is starting to reveal more about the selective pressures and ecological circumstances that have driven the development of different immune strategies and regulatory systems.

In this chapter, an update of a previous review,[8] some of the general features of invertebrate immune systems and their regulation and function are given. A brief review of laboratory and field studies of the effects of immunotoxic compounds in selected invertebrate species is provided and compared with the existing knowledge of vertebrate responses. One of the main problems in wildlife toxicity is that frequently, the organisms for which the most detailed mechanistic information is available, such as the fruitfly, are not always the most ecologically relevant in polluted environments. The concluding section discusses this problem, and outlines some of the recent technological developments that may make it easier to study the responses of ecologically and commercially important animals, including corals, sea urchins, and lobsters. Recent interest in marine pollution and its consequences has focused much recent research on marine invertebrate species, which consequently feature prominently in the remainder of this chapter.

INVERTEBRATE IMMUNITY

The Invertebrate Phyla

In ecotoxicology testing, the invertebrate phyla are often omitted, or a single species such as the midge *Chironomus riparius* or the common mussel *Mytilus edulis* may be

included as a surrogate for other species and phyla. Yet the invertebrates constitute a vast collection of diverse organisms spread among around 30 major animal lineages and separated, since the last common ancestor of the protostomes, deuterosomes, ecdysozoans, and lophotrochozoans, by well over 500 million years of evolution.[9] It is no wonder, then, that the invertebrate phyla includes such diversity of biochemical and physiological function, morphology and habitat preference, each of which contributes towards differences in vulnerability to different toxicants at different stages of the lifecycle. Invertebrates encompass colonial or solitary lifestyles, benthic or pelagic habitats, may be short or extremely long lived (Figure 21.1). Many invertebrates, such as sea anemones, corals, flatworms, and protozoans, form mutualistic associations with photosynthetic microbes, such as the symbiotic dinoflagellate algae (zooxanthellae) that inhabit certain reef building corals, raising interesting question about the control of immunological tolerance in these species.[10] There are some excellent and detailed reviews of invertebrate immunity and the many, often varied defense strategies that have evolved to allow species to occupy specific niches and habitats.[10,11] What follows is designed to provide a brief overview of invertebrate immune function, sufficient to understand the different ways in which environmental contaminants may erode disease resistance in invertebrate animals, leaving them vulnerable to infectious diseases they might otherwise have been able to resist.

Origins of Adaptive Immunity

The main function of the immune system in all species is to provide protection against infectious agents and tumors and to differentiate self from non-self. A functioning

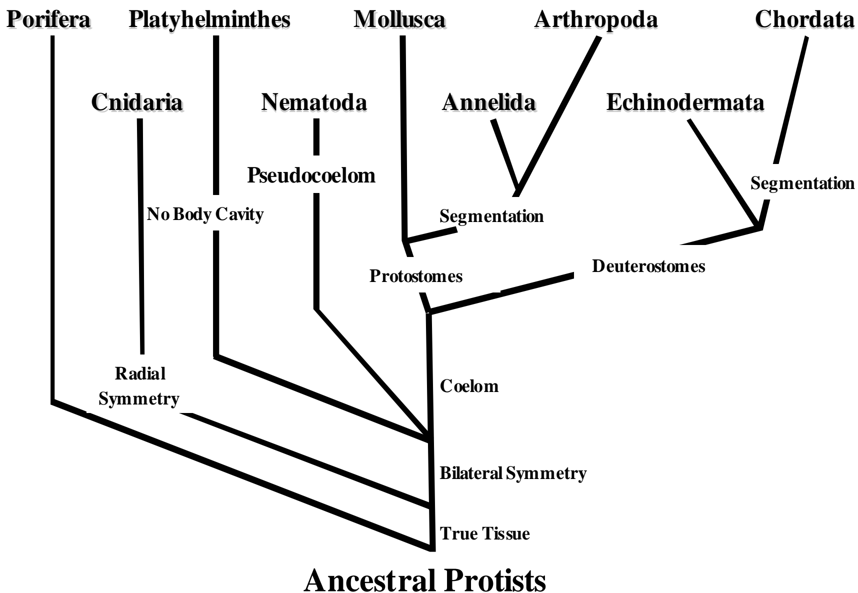

Figure 21.1 A simplified phylogenetic tree of the invertebrate phyla.

immune system must therefore contain two basic components, a system of recognition and one of response. Immune functions may be innate (comprising germ-line encoded pathogen recognition systems with no memory) or adaptive (capable of responding somatically and of retaining memory).

The adaptive immune system of higher vertebrates has evolved under strong selective pressure. It is a lymphocyte based system capable of anticipating and responding specifically to a huge variety of antigenic structures through somatic rearrangement and clonal expansion. Characteristic components of adaptive immunity include primary and secondary lymphoid tissue, lymphocytes, antigen receptors (both Ig, and T cell receptor, TCR, MHC I and II, recombination activation genes, (RAG), somatic hypermutation, and gene conversion. Adaptive immunity is believed to have evolved from the invasion of an ancestral Ig superfamily variable gene by a transposable element containing recombination activation genes (RAG 1 and 2).[12] The identity of this ancestral gene is not known, although several candidate variable genes of uncertain function have emerged from sequencing studies in the early and pre-vertebrates (lampreys, tunicates, echinoderms).[13] This fundamental event, which coincided with the appearance of the ancestral jawed vertebrates, has been crucial for the development of a functionally complete combinatorial immune system, as all of the antigen receptor loci rearrange using the same RAG- dependent mechanism.[14]

Despite intensive efforts over many years, no trace of the vertebrate adaptive immune system, with its vast, randomly-generated diversity of antigen receptors, has been positively identified beyond the cartilaginous fish.[14] This has led to the use of terms such as "ante-antibody immunity" (Ezekowitz, quoted in[7]) to describe the ancient, innate defense mechanisms found in the invertebrates. The survival and extraordinary success of the invertebrate phyla suggests that these innate mechanisms: the anatomical barriers, the pattern recognition receptors (PRRs), anti-microbial peptides (AMPs), phagocytic cells, synthesis of reactive oxygen species, RNA interference (RNAi), and melanisation and complement pathways (outlined in more detail below) are extremely effective (Table 21.1). Many components of this basic system are highly similar, suggesting that they have a common origin and have been conserved across millions of years of evolution; for example the Toll-like receptors that sense foreign microbial products, have been found in all multicellular organisms examined to date.[15,16] Other innate factors show much greater variability, suggesting specific adaptations to the life history characteristics and habitat preference of the organism.

Evidence is also starting to accumulate for the existence, in lower vertebrates and invertebrates, of different evolutionary strategies for the generation of immune receptor diversity. Recent reports have described multiple gene copies and enhanced somatic recombination, independent of RAG, capable of generating diverse immune receptors,[17,18] and even of memory[19,20] in response to pathogen exposure. The pulmonate gastropod, *Biomphalaria glabrata,* is an intermediate host for the human parasite, *Schistosoma mansoni,* and its immune responses have consequently been intensively studied. *B. glabrata* produces a diverse superfamily of hemolymph proteins with lectin like properties, called fibrinogen-related proteins or FREPs, consisting of an Ig superfamily domain (Igsf) and a fibrinogen domain. Zhang and colleagues[17] demonstrated somatic diversification

TABLE 21.1
A Simplified List of the Invertebrate Phyla, Summarizing Some of the Common Immune Molecules and Mechanisms that have been Reported for at Least One Representative Species

Phylum	Species example	Immune molecules and mechanisms
Porifera	Sponges	Lectins, PO
Cnidaria	Corals	PO, complement, programmed cell death
Nematoda	Roundworms	Lectins, PO, AMPs, programmed cell death
Annelida	Polychaete worms, Earthworms	Lectins, PO, NK cells, haemolysins
Mollusca	Gastropods, Bivalves	Lectins, PO, AMPs, molluscan defense molecules, fibrinogen related proteins, cytokines
Crustacea	Arthropods, Crustaceans, Insects	Lectins, PO, Leucine rich repeats, Toll, Tol interleukin receptors, AMPs, complement
Echinodermata	Starfish, Sea urchins	Lectins, PO, leucine rich repeats, complement, scavenger receptors, Tol interleukin receptors, cytokines
Urochordata	Sea squirts	Lectins, PO, leucine rich repeats, complement, cytokines, NK cells

PO = phenoloxidase, AMPs = antimicrobial peptides, NK cells = natural killer cells. Adapted from Flajnik and Du Pasquier.14

of a FREP Igsf variable gene region in response to pathogenic challenge at a considerably higher level than for control genes, suggesting the existence of a mechanism for somatically diversifying these immune defense molecules. At around the same time, a new type of variable lymphocyte receptor composed of highly diverse leucine-rich repeats (LRRs) was reported in lampreys.[18] Individual lymphocytes expressed uniquely rearranged receptors, apparently formed by the insertion of different LRR segments. The underlying processes by which these two separate events occur (gene conversion, somatic hypermutation or alternative splicing) have not so far been identified, although their existence illustrates the strong selective pressure across the phyla to develop a system of diversification in the face of a constantly changing pathogenic challenge.

Evidence for memory in invertebrate immunity has come from studies of the copepod *Macrocyclops albidus* and its defense against a natural parasite, the tapeworm *Schistocephalus solidus*.[19] When infection rates were measured for *M. albidus* exposed to parasites with varied antigenic features, the results revealed that the defense systems of the copepods reacted more efficiently to previously encountered antigenically similar parasites. As argued elegantly by Kurtz,[21] these results support an enhancement of immunity that is also specific, consistent with a form of invertebrate immunological memory. This suggestion has implications, not just for our current understanding of invertebrate immunity, but also for the ways in which it may be impacted by natural and anthropogenic stress.

OVERVIEW OF INVERTEBRATE IMMUNE FUNCTION

The first line of defense against pathogens is some form of physico-chemical barrier, such as the exoskeleton of coelenterates, molluscs, arthropods, and echinoderms or the mucous layer of many coelenterates, annelids, and molluscs. The nematode worm *C. elegans* exhibits a coordinated behavioral reaction in response to bacteria, including an attraction to nutritious and avoidance of pathogenic strains.[22] If anatomical defense are breached, pathogens will encounter reactive blood cells, (hemocytes or coelomocytes) and an array of non-specific and inducible cytotoxic molecules. The developmental origins and inter-relationships between the various types of circulating hemocytes are often uncertain, but they perform essential immune functions of self/non-self-recognition, phagocytosis, encapsulation, melanisation, and the release of antimicrobial and lytic agents in most species.[23]

Phagocytosis and encapsulation, in which pathogens are sequestered within several layers of cells, are the predominant cellular immune responses. Genetic and molecular studies have shown that bacterial products are recognized[24] and transduced by host cells[25,26] by similar pattern recognition receptors and signal transduction pathways in both vertebrates and invertebrates.

Once activated, phagocytic cells exhibit both oxidant-dependent and oxygen-independent cytotoxic humoral responses including the synthesis of lysozyme, proteolytic and hydrolytic enzymes, antimicrobial peptides, and free radical species.[27] Reactive oxygen and nitrogen species are generated by the respiratory burst enzymes and by nitric oxide synthase, in response to bacterial challenge. Free radical generation may be augmented during melanogenesis[27, 28] and through the action of other enzyme systems and there may be wide variation in the intrinsic phagocytic and radical-generating activity of hemocytes, even between seemingly closely similar species.[29]

The synthesis of constitutive or inducible antimicrobial peptides (AMPs) has been reported in all invertebrate species so far studied and over 1000 AMPs have been isolated and characterized at the level of their primary structure.[30] These may be gene-encoded, or may result from the processing of larger precursor molecules; for example, AMPs derived from the C terminus of hemocyanin have been implicated in immune defense in crustaceans, including the shrimp, *Penaeus vanname* and the crayfish *Pacifastacus leniusculus.*[31] All natural AMPs share the ability to adopt an amphipathic structure that is believed to be essential to their anti-microbial activity, although they show extreme diversity in their structure, tissue distribution and expression. For example, in *Drosophila*, seven different AMPs are synthesized in the fat body and secreted into the blood following infection,[32] whereas in many other invertebrates, the primary site of synthesis appears to be the circulating hemocytes which express AMPs constitutively.

Communication and integration of the invertebrate immune response incorporates many functions analogous to vertebrate regulation, although the components involved may not always be homologous to their vertebrate counterparts. The prophenoloxidase-activating system controls the deposition of melanin around damaged tissues or intruding objects and a large number of pattern recognition molecules that may mediate the activation process have been identified, mainly in insects and crustaceans.[28] This fundamental process is tightly regulated by a cascade of serine proteases and inhibi-

tors, in a cascade analogous to the vertebrate complement cascade.[33,34] Under certain conditions, arthropod hemocyanin may show PO activity,[35] and although these results are still to be conclusively confirmed, they raise interesting questions about the possible joint regulation of oxygen carrying and wound healing functions.

Several putative functional analogues of vertebrate inflammatory cytokines have been reported in invertebrate species, including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-like molecules in annelids, molluscs, insects, echinoderms, and protochordates.[36] These and other regulatory cytokine-like products are typically produced by invertebrate phagocytic hemocytes. Gene homology studies tentatively support the existence of both a cytokine network and cytokine receptors, although the lack of conclusive homology has led to the suggestion that vertebrate and invertebrate cytokines evolved convergently, each exhibiting a crucial, functionally conserved lectin-like recognition activity.[37]

Overall then, a picture emerges of a central battery of innate immune functions in invertebrates of remarkable efficiency that has been augmented and adapted by different species to reflect the specific ecological circumstances and immunological pressures to which they are exposed (Table 21.1). The vast expansion in sequence information has allowed a huge step in our fundamental understanding of invertebrate immune functions, their regulation and evolutionary lineages, yet there are still gaps in our knowledge. One of these relates to how invertebrates recognize and eliminate viruses. Many important invertebrate viruses exist, including the arboroviruses that can infect both invertebrate vectors and their vertebrate hosts. Viral epizootics can cause huge commercial impact, particularly in aquaculture, where diseases such as White spot syndrome virus can devastate shrimp farming activities, yet the nature of invertebrate antiviral mechanisms remains obscure. Invertebrates lack antibodies, cytotoxic T cells and interferons, the classic vertebrate responses to viral infection. Natural Killer (NK) cell-type activity has tentatively been described in some species[38] and a C-type lectin with marginal sequence similarity to CD94 suggests the presence of NK like cells in the urochordate *Ciona.*[39] RNA silencing (RNAi), a sequence-specific response to double-stranded RNA that then responds to any RNA sharing homology with the initial recognition sequence, is thought to represent a genomic defense mechanism to protect against viruses and transposons.[40] Many invertebrate viruses have single stranded RNA genomes, suggesting that RNAi may be an effective intracellular antiviral mechanism.[41] A full description of exactly how invertebrates prevent viral entry into cells and identify and kill virally infected cells remains a challenge.

EVIDENCE FOR IMMUNOTOXICITY IN INVERTEBRATES

IMMUNOTOXIC CHEMICALS AND THEIR EFFECTS

The vast majority of information regarding the immunotoxic effects of different chemicals has been derived from laboratory studies of vertebrate responses in which the mechanism of action is evaluated and the subsequent effects on different immune parameters and susceptibility to infection is determined. It is much harder to extrapolate

from these carefully controlled studies to predict the likely consequences of environmental exposures in genetically and morphologically diverse invertebrates existing as different life forms and inhabiting widely disparate environments. Table 21.2 lists some environmental pollutants suspected of causing immunotoxicity in invertebrate species, listed according to chemical group. A complete chemical classification of immunotoxic compounds is complicated by their structural diversity and by the fact that certain chemicals and their metabolites exhibit multiple mechanisms of toxicity. Most invertebrate studies have concentrated on adult life forms, largely because the lack of knowledge of the ontogeny of immune responses makes the study of developmental changes in invertebrates extremely challenging. This is a drawback, as different life forms may encounter and respond quite differently to pathogenic pressures. Hatchling earthworms, *Eisenia andre*i, were roughly three times more sensitive to the toxic effects of mercury, had a lower proportion of phagocytically active cells, and a lower absolute phagocytic index than adults.[42] Mercury is a potent immuno-stimulant or -suppressant, depending on dose. While adult worms responded hormetically to low mercury exposures with a burst of phagocytic activity, hatchling worms were unable to mount such a response.[42] In mice, prenatal exposure to low doses of mercury induced cellular immune dysfunction that persisted into adulthood[43] and it would be interesting to explore whether a similar depression of coelomocyte function persisted into adulthood in earthworms.

Impairment of the immune system resulting from alterations during early development, following hatching or metamorphosis, may cause subtle, life-long changes in immune function that are not expressed until the animal reaches adulthood. Both immune activation and nutritional modulation during early life stages in the field cricket, *Gryllus campestris*, caused a long-term modulation of antibacterial activity and of

TABLE 21.2
Examples of Chemicals Suspected of Causing Immunotoxicity in Invertebrates, Listed According to Chemical Group

Chemical class
Heavy metals, metalloids
e.g., lead, cadmium, mercury
Aromatic hydrocarbons
PAHs, benzene, toluene, hexachlorobenzene
Halogenated hydrocarbons
Pesticides, TCDD, PCBs, PBBs, methoxychlor
Pesticides
Chlorfenvinphos, malathion, parathion, aldicarb, atrazine, carbaryl, chlordane, dieldrin, endosulphan, heptachlor, DDT, DDE
Particulates
synthetic nanoparticles, plastic debris
Organometals
Organotins, TBTO, DBTO, TPT
Complex mixtures
Sewage effluent, Contaminated sediments, Pulp mill effluents
Natural products
Cyanobacterial toxins, Estrogens, phytoestrogens

Abbreviations: PAH = polyaromatic hydrocarbon, TCDD = Tetrachlorodibenzo-p-dioxin, PCB = polychlorinated biphenyl, PBB = polybrominated biphenyl, TBTO = tributyltin oxide, DBTO = dibutyltin oxide, TPT = triphenyltin.

prophenoloxidase concentration that persisted in adults.[44] Developmental immunotoxicity might speculatively have less of an impact in r-selective species, whose survival strategy in adapting to hostile environments relies on a short life span combined with numerous offspring, than on a long-lived species living under a K selective, stable regime.

Species-specific differences in vulnerability may result from altered uptake, metabolism or detoxification pathways, a prime example being the organophosphorous (OP) pesticides. Susceptibility to the cholinesterase-inhibiting neurotoxicity of OP pesticides depends on the balance between bioactivation, catalyzed by monooxygenases, and detoxification, involving esterases and other enzyme pathways, and many invertebrate species are highly vulnerable. This species-specific vulnerability appears also to extend to the immunotoxic effects of OPs. Rickwood and Galloway[45] reported the hemocyte phagocytic activity and spontaneous cytotoxicity responses of the mussel *Mytilus edulis* to be extremely sensitive to the OP chlorfenvinphos at environmentally relevant concentrations. A similar sensitivity in the phagocytic response to the effects of malathion was reported in the American lobster, *Homarus americanus.*[46] The immunotoxic effects of OP pesticides are believed to be mediated in part by an inhibition of serine hydrolases (e.g., NK cell granzyme activity) and cell-surface esterases.[47]

Conversely, the immunotoxic and carcinogenic effects of PCBs have been largely overlooked in invertebrates because they lack the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor that is believed to mediate many of the toxicological effects of PCBs in mammals. Recent evidence highlighting apparent AHR homologues in a wide range of invertebrates, including nematodes, insects and gastropods, helps to explain why PCBs are still able to exert immune modulating effects in diverse species.[48,49] This topic is discussed further in subsequent sections. Although the mechanism of toxicity of a contaminant may be the same, the net effect may differ, even in closely similar species. Inhibition of hemocyte functions by polyaromatic hydrocarbons (PAHs) has been attributed largely to PAH-induced modification of hemocyte membranes rather than to a specific receptor-mediated event. Laboratory exposure of three marine bivalve species to the PAH phenanthrene caused quite different levels of immune modulation in the mussel *M. edulis* and the edible cockle *Cerastoderma edule*, while the razorshell *Ensis siliqua* showed no evidence of immunomodulation at all under identical conditions, although difficulties in the animal husbandry of this species may have been a contributing factor.[50] Flow cytometry was used by Sauve and colleagues,[51] to evaluate the sensitivity of different earthworm species to the immunotoxic effects of heavy metals including Ag, As, Cd, Hg, Ni, and Pb. Species specific sensitivity varied by a factor of up to 10, although mercury was consistently the most immunotoxic element.

Mechanisms of Immunotoxicity

The fundamental processes of immunity (pattern recognition, intracellular signaling, phagocytosis, cytotoxicity, clotting) remain largely similar and equally vulnerable to the effects of immunotoxicants whatever the invertebrate species. It could be argued that the constitutive nature of most invertebrate defense leaves them better equipped to

resist the effects of toxicants that may act through the impairment of metabolism or of immunological memory. The persistent environmental contaminant and AHR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one example of a toxicant that in vertebrates diminishes the secondary antibody and memory CD8 T cell response to viral infection,[52] an outcome likely to have less significance in invertebrates. The widespread and persistent nature of aromatic hydrocarbon contaminants such as TCDD and PCBs and their high toxicity means they are contaminants of high concern, particularly in marine ecosystems. The PCBs include 209 possible congeners, differing in their chlorine substitution of the biphenyl ring. Those with chlorine substituted in both *para* and *meta* positions can adopt a dioxin-like coplanar configuration and these dioxin-like PCBs are believed to exert their toxicity by activating the AHR in a similar manner to TCDD.

Studies of PCB immunotoxicity in oligochaetes and polychaetes using the commercial mixtures typically discharged into the environment[53] have confirmed a general decrease in viability, altered cytology and reduced phagocytic ability, similar to PCB-induced reductions in innate immune functions described in vertebrates. Studies on human neutrophils have however demonstrated that individual PCB congeners can cause quite different effects on innate immune responses, depending on their structure, prompting similar studies of individual congeners in invertebrate models. Coteur and colleagues[49] studied the impact of four coplanar PCB congeners on the production of reactive oxygen species (ROS) by the immunocytes of the sea urchin *Paracentrotus lividus* and confirmed a stimulation of ROS production similar to the increased respiratory burst reported in human polymorphonuclear cells,[54] presumably due to activation of phospholipase and protein kinase activities.

Detailed studies of the signal transduction pathways involved in hemocyte activation have been undertaken in mussel hemocytes.[25] Hemocyte treatment *in vitro* with noncoplanar PCB congeners resulted in a time-dependent increase in the phosphorylation state of the stress-activated p38 mitogen-activated protein kinase (MAPK) pathway, previously shown to be crucial for hemocyte antibacterial responses. Ortho-substituted PCBs were also able to induce tyrosine phosphorylation of STAT5, a member of the STAT (Signal Transducers and Activators of Transcription) family of transcription factors involved in vertebrates in cytokine-mediated gene expression.[55] These studies highlight the similarities in mechanism that underpin the immunosuppressive action of noncoplanar PCBs that may act independent of the AHR receptor. Once in the environment, PCBs are slowly biotransformed to yield higher proportions of the noncoplanar congeners, with implications for the long-term immunotoxic potential of PCB contaminated habitats.

Another widespread and highly toxic environmental contaminant is tributyltin (TBT), used extensively as an antifoulant boat paint until recent legislation restricted its use. TBT is hydrophobic and associates with organic matter and paint particles in muddy sediments, where it may have a very long half-life of up to 20 years, representing a persistent reservoir that poses a long-term risk to benthic organisms.[56] The immunotoxic action of TBT has been linked to the inhibition of Ca^{2+}-ATPase activity, causing massive efflux of Ca^{2+} from intracellular stores. Subsequent changes in Ca^{2+} homeostasis cause cytoskeleton disorganization, leaving phagocytes unable to move towards and ingest foreign particles. This mechanism may also be responsible for some of the ad-

ditional toxic effects of TBT. Hagger and colleagues[57] proposed the disruption of Ca^{2+} homeostasis as a potential explanation of the multiple genotoxic effects of TBT noted in the mussel *M. edulis* exposed to very low concentrations of TBT. Extreme increases in intracellular Ca^{2+} can trigger a cascade of events leading to apoptotic or necrotic cell death, including the activation of Ca^{2+}-induced endonucleases, leading to DNA damage and fragmentation. The induction of tumors in invertebrates following pollutant exposure is rare, but Kurelec[58] has described a genotoxic disease syndrome in molluscs, in which DNA damage is accompanied by impaired enzyme function, enhanced protein turnover, impaired immune function, decreased fecundity and faster ageing. On this basis, altered immune function might provide an indication of additional, underlying toxic effects. This illustrates the complex task of deconvoluting the exact mechanisms by which animals are impacted in polluted environments.

Immunotoxicity and Its Environmental Impact

Laboratory studies have so far provided the basis for linking chemical exposure to modulation of the invertebrate immune system, but the only way to verify the ecological significance of these findings is by conducting field studies. If the disease resistance of invertebrate species occupying key ecological niches is impaired by environmental contamination, disproportionate impacts may result through alterations to ecosystem function. Part of the problem is in knowing exactly what to measure in conducting surveys, given the multifactorial nature of many diseases and the lack of baseline data and epidemiological information on what constitutes a "normal" level of disease in a given invertebrate population. In addition, epizootics may occur for many reasons, with altered environmental conditions, either due to climate change or human activity, a key factor in influencing disease transmission. Gross changes to habitats through dredging, land reclamation, introduction of novel hosts or infected stock from elsewhere can alter the pattern of established and emerging disease. It has been suggested, for example, that mass mortalities in bivalve populations are largely the result of the movement of infected stock during aquaculture.[3] Climate variability has the potential to induce major changes in health and productivity, leaving different populations more susceptible to disease. This is particularly evident in corals, which respond to higher temperatures (such as those resulting from the El Nino Southern Oscillation) by bleaching, a process in which symbiotic photosynthetic algae are expelled. A link between bacterial infection and the pathogenesis of bleaching has been strengthened by the finding that in at least one coral, *Oculina patagonica*, the infectivity and virulence of the bacterium *Vibrio shiloi* that frequently infects it is extremely temperature-dependent.[59]

In view of these complexities, environmental studies that seek to verify proposed cause-effect relationships between contamination and response need to be carefully designed to avoid bias and misunderstanding. Most environmental assessments adopt a multi-tiered approach to testing, in which combinations of biological responses (biomarkers) are measured in tissue samples, body fluids or at the whole organism level to indicate exposure to or adverse effects of contamination.[8]. Auffret and colleagues[60] surveyed Pacific oysters from the Atlantic coast of Brittany after the *Erika* oil spill between

2001 and 2003 and observed major changes in immune competence at the most heavily oil-impacted site. Their multiparametric analysis included identification of structural and functional changes and evidence of pathological damage. The level of variation in many of the parameters they measured highlighted the inherent variability likely in organisms in genetically, phenotypically and geographically diverse populations.

A deployment study design was used[61] to determine the impact of contaminated marine sediments in the Baie des Anges, Quebec, on the bivalves, *Mya arenaria* and *Mactromeris polynima.* The sediments contained high level of organic contaminants, including PCBs and PAHs. After 12 weeks, a significant inhibition of phagocytosis activity was seen in both species, although physiological parameters such as mantle protein, malondialdehyde levels, total protein and glycogen levels in the digestive gland were not affected. The suppression of phagocytosis correlated strongly with the bioaccumulation of PCBs in the tissues, providing strong evidence of a causal association. Marine molluscs were again the species of choice for a field survey of New Bedford Harbor, Massachusetts, a Superfund site on the U.S. EPA National Priority List due to a historical legacy of contamination with heavy metals, PCBs and PAHs.[62] Suites of biomarkers combined with chemical analysis were used to establish a relationship between contamination and toxicity in the ribbed mussel, *Geukensia demmissa.* Multivariate statistical analysis revealed that as contaminant concentrations increased, there was a decrease in immune function (hemocyte viability, spontaneous cytotoxicity response), an increase in genotoxic damage (micronucleus formation) and a decline in the physiological condition (heart rate, condition index) of the mussels. Interestingly, a restricted access zone had been in operation around the estuary shores after laboratory studies had confirmed that contaminated sediments from the area were likely to cause immunosuppressive effects in humans and an increased cancer risk.

A multibiomarker field study of Southampton Water, England confirmed the potential for PAH-contaminated sediments to induce immune modulation in exposed animals. A range of invertebrates was included in the analysis, including filter feeders, grazers and omnivores, to determine the sensitivity to contamination of organisms occupying different trophic levels.[63] Multidimensional scaling identified the shore crab *Carcinus maenas* to be the more sensitive to contamination than the edible cockle, *Cerastoderma edule* or the periwinkle, *Littorina littorea.* Significant decreases in phagocytosis activity were attributed to the presence, at the most contaminated boatyard site, of sedimentary TBT, PAHs, surfactants, and oils.

In terrestrial environments, earthworms and insects, including the ant *Formica*[64] and the damselfly *Calopteryx virgo*[65] have proven tractable sentinel species for studying the immunological effects of pollution and of altered environments. Massicotte and colleagues[66] showed that cement kiln dusts caused highly significant impacts on cell viability and phagocytic activity in the coelomocytes of the earthworm *Lumbricus terrestris* exposed through the soil. When the testing was repeated using an *in vitro* exposure of extruded coelomocytes, the high level of variability from day to day made the detection of significant change impossible, highlighting the difficulties that can be encountered with different cell types and model systems.

Another problem in analyzing the effects of contamination in the field is the complex, fluctuating mixture of pollutants that typically exist in impacted environments.

Mixture toxicity is notoriously complex. For example, the interactive effects of three common agricultural chemicals, lindane, methyl parathion and atrazine, were found to be variably additive or synergistic, depending on the nature of the mixture, but also on the test species (waterfleas, algae or bacteria[67]). The toxicity of atrazine has proved to be a contentious issue.[68] The immunosuppressive action of atrazine has been clearly demonstrated in diverse species from rats[69] to the pond snail, *Lymnaea stagnalis*,[70]altering immunocyte density and distribution and inhibiting phagocytic activity. Numerous papers suggest that atrazine is an endocrine disruptor, causing detrimental effects to wildlife at extremely low doses.[71] However, problems have arisen in incorporating much of this respected, peer-reviewed toxicological data into government regulatory policy because it has been argued that the methods used have not received any official validation.[68] This highlights a central problem in researching the ecotoxicological problems of pollution in diverse wildlife species, for which validated methods may be unavailable.

BIOMARKERS AND EMERGING TECHNIQUES

The multibiomarker studies described in the previous section have been developed with reference to the tiered testing systems adopted by regulatory bodies to assess the toxic effects of substances on the integrated functioning of the vertebrate immune system and its components.[47] Following this model, Tier 1 tests involve a general screen of immune function, Tier 2 tests consider functional parameters and a more comprehensive evaluation of the mechanisms involved, specific immune function tests and host resistance challenge assays. Table 21.3 lists some examples of biomarkers of immunotoxicity that have been developed and applied to different invertebrate species. Some of these are comparable to vertebrate testing assay, others, such as the IMCOMP-P, which detects the pore-forming activity of antimicrobial peptides using fluorescence probes, are designed specifically for invertebrates, in this case, corals.[72]

The focus of ecotoxicology research is to limit the impact of environmental contamination and habitat degradation on population dynamics, community structure and ecosystems processes. This approach recognizes the importance of relating measurements of individual parameters to the overall function of the organism and to its reproductive success, growth, and viability.[73] However, a lack of fundamental understanding of the activation and regulation of key factors has hindered the interpretation of invertebrate immunotoxicity studies, as it's not always clear whether a particular parameter might be expected to increase, decrease or stay the same under particular conditions. An alteration in the activity of the phenoloxidase pathway might be interpreted to be a specific toxicological response, but in the absence of adequate information to the contrary, might also be caused by the presence of certain pathogens or changes in regulatory factors associated with life stage. Adult edible crabs, *Cancer pagurus*, affected by shell disease syndrome and found to have intrahemocoelic bacterial infections, had few measurable changes in immune parameters,[74] highlighting the constitutive nature of the measured defense. Modulation of invertebrate immune parameters may remain localized to the site of infection or injury and alterations may be missed if only whole body or hemolymph responses are considered. A comparison of uninfected oysters, *Ostrea edulis*, with

TABLE 21.3
A Comparison with Vertebrate Testing Systems (US EPA Subdivision M: Assessment Guidelines) with Biomarkers of Immunotoxicity That Have Been Developed and Applied to Different Invertebrate Species

Test area	Higher Vertebrate Measurement	Suggested Invertebrate Biomarker
Tier 1		
Immunopathology	Hematology (blood cell count), weight of spleen, thymus and liver, histology of spleen, thymus, and lymph nodes	Histopathology, cell viability, lysosomal membrane stability
Humoral immunity	Plaque forming assay	Plaque forming assays
Cell-mediated immunity	Mitogen-induced T-cell proliferation, NK cell activity	Spontaneous cytotoxicity assays
Tier 2		
Immunopathology	Differential B and T cell count	Differential cell count, apoptosis
Humoral immunity	Plaque forming cell assay	Rosette formation, AMP pore-forming activity
Cell-mediated immunity	T-cell cytotoxicity, delayed type hypersensitivity	
Non-specific immunity	Peritoneal macrophage count and phagocytic activity	Phagocytosis
Resistance assays, general condition	Implantation of syngeneic tumors, bacterial, viral, and parasitic infection	Occurrence of sentinel disease (infection, neoplasia), growth indices, respiration rates, heart rates

those naturally infected with *Bonamia ostrea* showed the main response in the infected oysters to be a change in hemocyte type distribution and a localized increase in tissue infiltrating hemocytes, with no other gross changes evident.[75] In Manila clams, *Ruditapes philippinarum* affected by Brown Ring disease, caused by the bacterium *Vibrio tapetis*, changes in hemocytes and defense factors were concentrated in the extrapallial fluids located between the mantle and the shell, highlighting the importance of these fluids in the defense process of the animals.[76]

The potential application of genomics to wildlife and non-model organisms has the promise of providing a more detailed insight into their immunological status and an understanding of their responses to each other and to the environment and may go some way to addressing these issues. Corporeau and Auffret[77] used flow cytometry incorporating *in situ* hybridization with oligonucleotide probes to quantify an increase in the expression of heat shock protein C70 and metallothionein genes in immunocompetent cells of the European flat oyster, *Ostrea edulis* in response to stress-inducing conditions. A problem remains that the most popular invertebrate species used in ecotoxicology, the freshwater crustacean *Chironomus riparus,* the amphipod *Daphnia magna*, and the common mussel *Mytilus edulis*, have yet to be sequenced as genomic models and

there is a current lack of DNA sequence resources for ecotoxicologists to access. The NSF Tree of life Programme aims to sequence the entire genome of 62 chosen species across the phyla, providing a framework for understanding the evolutionary relationships and environmental interactions of a wider range of different species and includes several species of interest to ecotoxicological testing, including the water flea *Daphnia pulex*, marine polychaete *Platynereis dunerilii*, the mud snail *Ilyanassa obsolete,* and the sponge *Callyspongia diffusa*. This has the potential to add to knowledge of structure-activity relationships of defense genes and greatly increase our general knowledge of immune system function and regulation. In addition, accurate detection of disease outbreaks will be greatly facilitated by sensitive and specific polymerase chain reaction based identification methods.

The histopathological analysis of invertebrate tissues is another area that has seen an increase in interest in ecotoxicological surveys as it can be used to reveal the extent, severity and novelty of disease symptoms and pathological damage, particularly in more long lived, sessile species such as corals. A combination of scanning electron microscopy and histology was used to characterize the different stages of an apparent fungal disease syndrome in coral species off the coast of Kenya, thought to be linked to detrimental environmental conditions.[78] Extended histopathology has proven to be a useful adjunct to functional assays in vertebrate immunotoxicity testing[79] and the same is proving true for invertebrate histopathology, exemplified by projects such as the Registry of Tumours in Lower Animals (http://www.pathology-registry.org). A histopathological survey of crustaceans around the coastline of England, collecting baseline data on multiple organ pathologies, parasite loads and general condition in the shore crab *Carcinus maenas* and the brown shrimp *Crangon crangon.*[80] There was a wide variation in the prevalence of viral and yeast infections at estuary sites impacted to varying degrees by pollution, prompting the authors to recommend *C. maenas* as a useful disease model for studying the links between stressful environmental conditions and disease prevalence.

CONCLUSIONS

It seems clear from the foregoing account that invertebrate immune systems are far from simple and different organisms vary greatly in the levels of complexity in their forms of defense**.** Immunity is an essential physiological system at the interface of the organism and the environment, and its adequate functioning is fundamental to the survival and fitness of populations. Many of the molecules and pathways used in invertebrate immune defense have their counterparts in vertebrates, but others are quite distinct, having evolved convergently or are unique adaptations to particular ecological niches. Thus the pattern of vulnerability to immunotoxicants in invertebrates is similar to that in vertebrates in some respects, but very different in others. Invertebrates are vitally important both as commercially important and farmed food species, as disease vectors and as components of healthy ecosystems. A more detailed knowledge of normal invertebrate immune function is a vital research objective if we are to fully appreciate the ways in which habitat degradation and pollution contribute towards deteriorating health and disease.

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22 Amphibian, Fish, and Bird Immunotoxicology

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INTRODUCTION

Among wildlife groups, amphibians, fish, and birds are of importance for immunotoxicological studies because all three groups are sensitive bioindicators of ecosystem health. Their immune systems are nearly as complex as those of mammalian species, and harmful effects of environmental chemicals on their immune defenses can suggest potential danger for humans who are exposed to similar chemicals. What follows is an introduction to immunotoxicology in each of these vertebrate groups with references to review articles that provide greater detail.

AMPHIBIAN IMMUNOTOXICOLOGY

Amphibians are ancient creatures that inhabit aquatic and semi-aquatic environments on all continents except Antarctica. A recent global amphibian assessment concludes that amphibians are declining more rapidly than either birds or mammals [1]. A number of possible factors may contribute to amphibian declines. They include overexploitation, habitat destruction, climate change, and disease [1]. In addition to these factors, environmental chemicals could suppress immune defenses and magnify the effects of disease. Amphibians are uniquely dependent on clean sources of water. The immune system is vulnerable to toxic assault at embryonic, larval, or adult stages. Most species (except direct developing species) deposit their eggs in streams or still bodies of water where the tadpoles develop until metamorphosis [2]. After metamorphosis, the adults of many species spend time away from the water, but most species remain dependent on permanent bodies of water [2]. Amphibian skin is highly permeable, and it plays a major role in transport of essential ions, water, and oxygen [3]. Therefore, amphibians may be very sensitive to "poisoning" by uptake of environmental chemicals across the skin. Here we briefly review the literature describing immunosuppression by environmental chemicals in amphibians.

Amphibian Immunity

Although a limited number of amphibian species have been studied in detail, information gained about the immune system of the South African clawed frog, *Xenopus laevis,* and the Mexican axolotl, *Ambystoma mexicanum,* reveals that the amphibian immune system is nearly as complex as that of mammals. The organs that constitute the adaptive immune system are the thymus, spleen, and gut-associated lymphoid tissue. There are also areas of lymphopoiesis in the kidney, liver, mesentery, and gills. Bone marrow is present in some anuran species [4]. Anurans (frogs and toads) possess T and B lymphocytes that express rearranging T cell receptors (TCR) and immunoglobulin (Ig) receptors [4]. Adaptive immune responses are directed by a well-developed major histocompatibility complex (MHC) [5,6]. Three Ig isotypes have been described (IgM, IgY, and IgX) [7]. Leukocyte-derived cytokines including IL-1, and IL-2-like molecules, transforming growth factor beta (TGFβ), and macrophage migration inhibitory factor (MIF) have also been described [8–11]. MHC-restricted cytotoxic and helper T-cell responses have been characterized [4, 12].

In addition to these well-characterized adaptive immune defenses, amphibians have an array of innate immune defenses. These include complement-mediated defenses [13], Natural Killer cells (NK) [14], phagocytic cells [15], and antimicrobial peptides secreted into the gut and skin mucosa [16, 17].

Immunotoxic Effects of Environmental Chemicals on Amphibian Embryos

Unlike mammalian embryos that are protected within the uterus, amphibian embryos are protected only by a fertilization membrane and jelly coat. Although there is some

disagreement in the literature on this point, some toxins clearly can penetrate the fertilization membrane and jelly coat [18]. At early developmental stages, the mesoderm that contributes to the hematopoietic stem cell (HSC) compartment lies beneath a single layer of ectodermal cells, and therefore the entire HSC compartment is vulnerable to effects of xenobiotic chemicals [19]. To determine whether embryonic exposure to an immunotoxic agent can affect hematopoiesis, the Rollins-Smith laboratory used an amphibian diploid/triploid transfer model. Diploid embryos of *X. laevis* were exposed briefly to a test chemical. After 2 hours of exposure to chemical, the HSC compartment was transplanted from a test embryo or control embryo to an untreated triploid host embryo. Later, the contribution of the donor HSC compartment to cell populations in the blood, thymus, and spleen was assessed. Diazinon, but not lead acetate, interfered with the ability of transferred stem cells to contribute to hematopoiesis. At the concentration at which an effect on hematopoiesis was observed, diazinon was not directly toxic to the embryos [20].

Immunotoxic Effects of Environmental Chemicals on Amphibian Tadpoles

The amphibian immune system is developing and expanding rapidly during the tadpole period [4]. Following the first wave of development of lymphocytes in the larval period, some are eliminated by corticosteroid-hormone-driven apoptosis [21, 22], and then the lymphocytes expand again in the postmetamorphic period. The postmetamorphic renewal of lymphocytes is dependent on thyroid hormones (TH), and agents that interfere with uptake of iodine from the blood, such as perchlorate, impair lymphocyte expansion [23, 24]. Perchlorate is one ingredient in solid rocket propellant and is present in some fertilizers; and thus, it is a serious contaminant of some aquatic systems, especially in the western U.S. [25]. Bullfrog tadpoles from a contaminated site in Texas tested positive for perchlorate in the tissues. In such animals, thyroid function and development of the immune system of exposed tadpoles may be impaired [25]. In a similar way, agents that interfere with the action of the corticosteroid hormones (CH) can inhibit the naturally occurring loss of lymphocytes at metamorphosis [21]. It is thought that deletion of tadpole lymphocytes is necessary to avoid development of autoimmunity due to adult-specific self-antigens that emerge at metamorphosis [21]. Because metamorphosis is driven by the combined actions of TH and CH, it is likely that agents that disturb the normal neuroendocrine control of metamorphosis could alter immune system development [26]. Treatment of tadpoles with malathion [27, 28] or atrazine [29] resulted in delayed metamorphosis. Although TH levels were not measured in these studies, interference with TH function by malathion was suggested as the possible cause for this developmental delay [28]. Whether a malathion-induced inhibition of metamorphosis would alter immune defenses is not yet known. Malathion and atrazine did affect the degree of infection of wood frog (*R. sylvatica*) tadpoles by trematodes capable of inducing limb deformities (*Ribeiroia sp.* and *Telorchis sp.*). Increased malathion or atrazine resulted in a greater degree of parasitism and fewer circulating eosinophils [30]. Instead of delayed metamorphosis induced by malathion and atrazine, the herbicide acetochlor was shown to accelerate metamorphic changes in *R. pipiens* [31], *R. catesbeiana* [32], and *X. laevis* [33]. Accelerating metamor-

phosis may impact immune responses. Studies of *X. laevis* tadpoles showed that accelerating metamorphosis with excess TH resulted in impaired allograft rejection capacity [34]. Thus, environmental chemicals that alter the rate of metamorphosis could impair immune defenses of amphibian tadpoles in a variety of ways that are not well understood.

Immunotoxic Effects of Environmental Chemicals on Adult Amphibians

Because amphibians are often present in aquatic habitats that are disturbed by agricultural activities, it is likely that they are exposed to a variety of pesticides used to control weeds or insects. Juvenile frogs (*R. pipiens or X. laevis*) exposed to environmentally relevant concentrations of six pesticides (atrazine, metribuzin, aldicarb, endosulfane, lindane, and dieldrin) showed immunosuppression. Spleen cell numbers were reduced and phagocytic activity was impaired. Lymphocytes from pesticide-treated leopard frogs exhibited reduced T-cell proliferation *in vitro* in response to mitogens, and the frogs developed increased parasitism when challenged with a parasitic nematode (*Rhabdias ranae*). The parasitic worms migrated more rapidly to the lungs and reproduced earlier in pesticide-exposed frogs [35–37]. Adult Woodhouse's toads (*Bufo woodhousii*) were more susceptible to development of hepatomegaly and death due to experimental infection with the bacterium *A. hydrophila* if they were exposed to sub-lethal doses of malathion than toads that were not treated with the pesticide [38]. In *R. pipiens* treated with malathion, DDT, or dieldrin, specific IgM antibodies were suppressed in comparison with untreated controls. Oxidative burst products measured in whole blood were also decreased. A delayed-type hypersensitivity (DTH) response was enhanced in pesticide-treated frogs in comparison with control frogs. This pattern of altered immune responses observed in the laboratory was also noted in wild frogs collected in pesticide-exposed locations but not in those collected from pesticide-free locations. This suggests that the laboratory results may predict immunosuppression in natural populations [39].

There have been a very limited number of immunotoxicity studies of urodele amphibians. In general, the immune responses of urodeles have been more difficult to measure than those of anurans. Skin graft rejection is chronic rather than rapid [40], and *in vitro* mitogen responses are modest [41, 42]. For immunotoxicity studies in tiger salamanders, phagocytosis and oxidative burst assays have been studied [42, 43]. Peritoneal neutrophils can be collected after injection with thioglycollate. These neutrophils ingest foreign material and produce hydrogen peroxide indicative of oxidative burst activity. Phagocytosis was measured using flow cytometry and was correlated with manual counts of the numbers of fluorescent latex beads that had been engulfed by the neutrophils and the total number of neutrophils taking up the beads [43]. Immune endpoints including phagocytosis, respiratory burst, and histopathology have been examined in tiger salamanders after experimental exposure to the military explosive trinitrotoluene, but only histopathology of the liver showed evidence of change due to contaminants [44].

FISH IMMUNOTOXICOLOGY

Fossil records show that fishes are descendants of the first vertebrates to evolve, and they have filled virtually every aquatic niche beginning before the Devonian period, roughly 400 million years ago. While most lineages of early fishes became extinct, modern fishes are comprised of a few species of jawless fish (lampreys and hagfish), many chondrichthyians (sharks, rays, and chimeroids), and several groups of bony fishes that include the highest order of modern fishes, the teleosts, or modern bony fishes [45].

In terms of fish immunotoxicology, most of the scientific attention is focused on modern bony fish because of their economic importance as food and for recreational fisheries, and as sentinels of environmental health. For nearly three decades since the inception of aquatic immunotoxicology as a discipline, numerous publications show clearly that fish immune systems are sensitive to chemically-induced perturbation just as are higher vertebrates like mammals [46–48]. However, unlike mouse and rat models in immunotoxicology, the differences in physiology, immunology, and evolutionary history among the 20,000 different species of fishes are astounding. Therefore, most of what is known about the impact of xenobiotics on fish immunity is based on a limited number of species chosen on the basis of ease of culture, ecological and economic importance, and, in some instances, the history of the particular model's use in general fish biology. For example, the rainbow trout, *Oncorhynchus mykiss*, (and other salmonids), the channel catfish, *Ictalurus punctatus*, the medaka, *Oryzias latipes,* the carp, *Cyprinus carpio*, the mummichog, *Fundulus heteroclitus*, and the zebrafish, *Danio rerio*, have contributed greatly to experimental fish immunotoxicology [46, 47].

Basic Fish Immunology

The basic anatomy and physiology of the immune system of most modern bony fishes is similar and established the pattern for the development and evolution of immunity in all vertebrates (Figure 22.1) [49]. For a detailed overview of innate and adaptive immunity in fishes, as well as the developmental immunobiology of fishes, several excellent recent reviews are recommended [50–52] and are summarized as follows. Like all higher vertebrates, fishes have a spleen with red and white pulp, and they have a true thymus. They lack bone marrow as a primary organ, and they lack lymph nodes as secondary organs. The bone marrow equivalent of fishes is the anterior kidney, often referred to as the head kidney, which is either anatomically separate from the posterior renal kidney as seen in the channel catfish and mummichog, or is a continuation of the renal kidney as seen in the rainbow trout. The innate immune system of fishes is comparable to that of higher vertebrates, including mammals, and is replete with a full array of Toll-like receptors and other pattern recognition molecules and their signaling pathways, as well as complement, protease inhibitors, lysozyme, and pentraxins for pathogen deactivation and opsonization. Moreover, many proinflammatory cytokines and chemokines have been identified in fishes. Unlike higher vertebrates, the primary and only functional Ig of teleost fishes is tetrameric IgM. Thus, they do not have class-switching associated

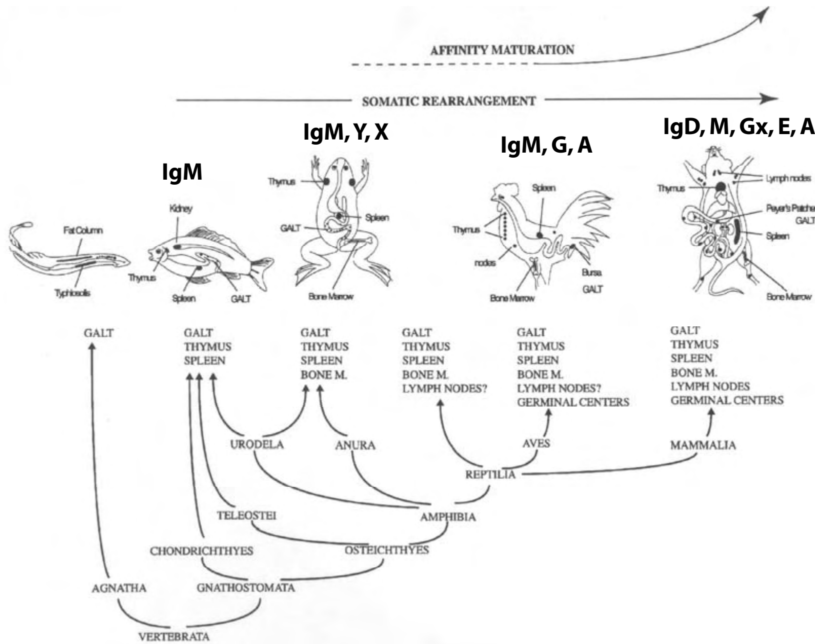

FIGURE 22.1 Evolution of vertebrate lymphoid systems. Bone M: bone marrow; affinity maturation, ability to increase antibody affinity after immunization; somatic rearrangement, antigen receptor variable genes generated by somatic rearrangement of gene segments mediated by RAG enzymes (from Du Pasquier[60]).

with a rapid and more intense secondary antibody response to antigens. The secondary antibody response in fish is very slow and prolonged, lasting for several months in some experimental situations.

Assessing Immune Function in Fish

Basic immune functions in fish are routinely quantified using techniques borrowed from mammalian immunologists and modified according to the particular species under study. For example, the primary IgM antibody response can be quantified in fish, either as serum antibodies against a variety of antigens using ELISA, or as numbers of specific antibody secreting cells in EliSpot assays, or as a primary plaque forming cell assay with sheep red blood cells (SRBCs). Innate immunity is most often evaluated using phagocyte function assays (phagocytosis and oxidative burst activity), by natural killer-like activity, or by proinflammatory protein expression (e.g., nitric oxide synthase, cyclooxygenase-2, P-4501A activity). Lymphoid organ and blood cellularity and mitogen-induced lymphocyte proliferation are also routinely measured in fish health assessments.

Of particular note, fish immunologists generally lack some reagents to quantify leukocyte subsets, yet this area of research is improving as genomics and proteomics (mostly from zebrafish) are finding homologues of many cell surface markers. Almost

all of the TLRs have been identified, and their signaling pathways appear to be highly conserved. The general question of which species of fish to use in standard immunotoxicology tests is still debated, however the general consensus is that the question at hand should drive that decision. Larger species of fish (e.g., channel catfish and rainbow trout) will provide more tissues for measuring a complete set of functional endpoints associated with immune status, however smaller species of fish like zebrafish, medaka, or the mummichog are easier to culture in limited laboratory spaces, and thus may be well suited for genomic- and proteomic-based studies. Moreover, the data derived from an estuarine species like the mummichog may not be relevant to a freshwater species like the channel catfish.

Relationships Between Contaminants and Immune Dysfunction in Fishes

As observed in mammalian models, the immune system of fishes is a sensitive target organ system to evaluate toxicity. For a more thorough review of environmental immunotoxicology in fishes, with reference to specific classes of xenobiotics, readers are referred to several reviews that deal with the subject over a span of nearly three decades [45–47, 54-57]. While fish in the environment may be exposed to a variety of xenobiotics, the most frequently investigated xenobiotics are the polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) due to the presence and activation of the aryl hydrocarbon receptor (AhR) in fish, and heavy metals due to their ubiquitous environmental distribution.

Polycyclic aromatic hydrocarbons (PAHs) are combustion byproducts of fossil fuels and a variety of other organic compounds. Many PAH congeners are potent immunotoxic agents in fish and mammals, at least in experimental systems [58–62]. Arkoosh and colleagues [63, 64] demonstrated that salmon from Puget Sound, Washington, a system heavily contaminated with PAHs, have reduced immunological memory and reduced peripheral blood mitogenic responses. Genotoxicity, as a specific mechanism for PAH-induced immunotoxicity, is supported by the findings of Faisal and colleagues [65]and Rose and colleagues,[66] that lymphoid tissues and isolated leukocytes of mummichogs metabolize benzo[a]pyrene to structures associated with DNA adducts in those tissues. However, changes in intracellular calcium flux and oxidative stress as mechanisms of PAH-induced immunotoxicology have also been reported [67].

Halogenated aromatic hydrocarbons (HAHs) include the polyhalogenated biphenyls, polychlorinated dioxins, furans, and naphthalenes. 2,3,7,8-tetrachlorodibenzo-*p*-dioxon (TCDD) is the prototype congener of this class and one of the most immunotoxic xenobiotics known to date, at least in mice. Despite the wide range of studies in mammals, only a few published studies examined the effects of TCDD on fish immune function. Spitzbergen and colleagues. [68, 69] noted only moderate suppression of the antibody response, but demonstrated enhanced susceptibility to IHN virus in rainbow trout. Rice and Schlenk [70] demonstrated that PCB-126, a TCDD-like congener often used as a potent AhR ligand, suppressed nonspecific cytotoxic cell (NCC) and phagocyte function in catfish, but did not affect the humoral response to pathogenic *Edwardsiella ictaluri*.

Most studies that have examined the effects of PCBs on fish immunity used com-

mercial mixtures such as Aroclor 1254, which suppresses the antibody response in rainbow trout and increases disease susceptibility [71, 72]. In channel catfish, however, it increased anterior kidney B-cell populations and enhanced phagocyte activity, while having no effect on antibody responses [73].

The immunotoxicology of metals in fishes has been reviewed elsewhere [74–76, 45]. Overall, the immune systems of fishes are highly sensitive to metals, although the effects are not always suppression of immune functions. Burnett [76] demonstrated that low levels of metals increased intracellular calcium, increased protein phosphorylation, and stimulated lymphocyte proliferation in fish. Since most metals are toxic to both the nervous system and the immune system, a neuroendocrine-immune link to immunotoxicity from metal exposure is likely.

Suggested Approach to Immunotoxicity Testing in Fishes

It is now clear that most fish immunologists and toxicologists agree that a rather simple approach to fish immunotoxicity testing is very informative of overall immune function. First and foremost, general indices of health should be monitored on a routine basis, with particular attention focused on swimming behavior and feeding behavior. Organ weights and cellularity, as well as hematological parameters, may provide some information regarding general stress, but they may not be xenobiotic-specific. Simple phagocyte functions like phagocytosis and oxidative burst activities seem to be as informative as other more complete endpoint of innate immunity, and these two endpoints are fairly easy to measure and require little training to perform. As modeled after mammalian Tier I toxicity testing, the primary IgM response to a T-dependent antigen is easily measured in fish, either by simple ELISAs to measure serum antibody levels or as numbers of specific antibody secreting cells. It may take as long as 21 to 28 days before primary IgM responses peak, with antibody secreting cell numbers peaking about a week before. It is interesting to note that fish IgM readily precipitates with fibrinogen upon freezing, thus it is important not to freeze fish serum or plasma samples. To avoid bacterial contamination and degradation of IgM, a small amount of concentrated sodium azide or pentachlorophenol can be added to a sample prior to storage at 4ºC. Plasma lysozyme is easily quantified using enzyme activity and is routinely measured in both aquaculture and immunotoxicological studies.

AVIAN IMMUNOTOXICOLOGY

In one of the founding studies in immunotoxicology, Friend and Trainer [77] demonstrated that exposure to polychlorinated biphenyls (PCBs) increased mortality of mallard (*Anas platyrhynchos*) ducklings challenged with duck hepatitis virus. Since then the field of avian immunotoxicology has expanded to show that both the structure and function of the avian immune system often is affected by a diverse array of environmental contaminants, including heavy metals, pesticides, petroleum hydrocarbons, and organic industrial chemicals. Wild birds have proven to be excellent sentinel species for assess-

ing ecosystem health because of their sensitivity to contaminants and high probability of exposure based on habitat use (e.g., presence in agricultural fields for pesticides) and (or) food habits (e.g., higher trophic levels for contaminants that biomagnify). However, immunological studies of some wild bird species may be limited by their high mobility (complicating recapture needed for some tests), conservation status (i.e., sampling restrictions), and small body size (i.e., small blood or tissue samples). Here we briefly describe the avian immune system, highlight some of the investigative approaches and significant findings in avian immunotoxicology, and discuss the emerging support for the ecological relevance of immunosuppression in wild birds. Comprehensive reviews of avian immunotoxicology are available elsewhere [78, 79].

Avian Immunology

In general, the avian immune system is similar to that of mammals, with several significant exceptions. Avian white blood cells involved in specific immunity include T and B lymphocytes, and nonspecific cells include monocytes/macrophages, NK cells (which are often most abundant in gut-associated lymphoid tissue), and various granulocytes (basophils, eosinophils, and heterophils). Heterophils have different staining characteristics but similar functional properties as mammalian neutrophils. Avian T cells mature in the thymus, which is a bi-lobed organ extending along the neck from the base of the skull to the thyroid glands in the upper chest cavity. Avian B cells mature in the bursa of Fabricius, which is a blind sac connected to the large intestine near the cloaca. The bursa provides a discreet organ for studying B cell maturation in birds, an advantage for using avian models in developmental immunotoxicology. Three Ig isotypes have been described for birds: IgM, IgG, and IgA. Avian IgG is approximately 12 kDa heavier than mammalian IgG. The generation of diversity of antigen binding regions within avian Igs uses primarily a gene conversion process rather than the more diverse mechanisms of generation of diversity employed by mammals [80].

Toxic Effects of Environmental Contaminants on Immune Organs, Cells, and Plasma Proteins

The impacts of contaminants on the structure of the immune system can be assessed by examining white blood cell (WBC) numbers and the mass and cellularity of immune organs, although these indicators are usually not as sensitive as measures of immune function. Avian immunotoxicity studies frequently assess total and (or) differential WBC counts [79], and immunosuppression can be indicated by reduced numbers of WBCs or elevated WBC numbers caused by recurrent infections. An elevated heterophil to lymphocyte ratio can indicate altered immune status in response to corticosteroid stress hormones or other factors [78,7 9]. Exposure to lead shot or lead acetate has been shown to alter total and (or) differential WBC numbers in Japanese quail (*Coturnix coturnix*) and mallards [81–83]. In western grebes (*Aechmophorus occidentalis*) from California, concentrations of mercury in the kidney were positively correlated with heterophil

number and negative correlated with eosinophil number [84]. Various measures of organochlorine exposure are correlated with altered WBC numbers in herring gulls (*Larus argentatus*) and Caspian terns (*Sterna caspia*) from the Great Lakes [85–87].

The primary immune organs, the thymus and bursa, are key immunological structures for the development of lymphocytes, and their mass and lymphoid cellularity are often sensitive to contaminants. For instance in chicken embryos, developmental exposure to PCB 126, a planar, dioxin-like congener, reduced the mass of the thymus and bursa indicative of a reduced number of developing lymphocytes in these organs [88]. This thymic atrophy was associated with other cellular processes such as reduced expression of TCR and increased thymocyte apoptosis [89, 90]. Similarly, thymic and bursal atrophy and altered thymocyte apoptosis were associated with PCB exposure in herring gull embryos from the Great Lakes [91]. Mercury exposure was associated with atrophy of both the thymus and bursa in great egrets (*Ardea alba*; 92).

Analysis of blood plasma proteins holds much promise because these proteins are indicators of immunological status. Of particular interest are the Igs and the α- and β-globulins (contain acute phase proteins whose concentrations often change with infection and inflammation). α- and β-globulin concentrations were associated with organochlorine contaminants in prefledgling herring gulls and Caspian terns from the Great Lakes [93]. Air pollution was associated with increased pre-albumin and decreased β-globulins in rock buntings (*Emberiza cia*) in Spain [94]. Plasma proteins are usually quantified following separation by gel electrophoresis, which allows identification of globulin groups but not individual bands. New proteomic techniques for protein isolation and identification should greatly facilitate the future use of plasma proteins as immunological indicators in wild birds.

Toxic Effects of Environmental Contaminants on Immune Function

Assays of immunological function are often more sensitive to contaminants than the indicators of immune structure described above. While a variety of functional tests have been employed in avian immunotoxicology, this discussion will focus on those used most frequently: *in vivo* skin tests for T cell function, *in vivo* antibody responses, *in vitro* lymphocyte proliferation, and phagocytosis assays. DTH assays test T cell-dependent inflammation. Intradermal or subdermal injection of a specific antigen to which the animal has been previously immunized stimulates T cells to release cytokines, causing a localized inflammation characterized by the influx of WBCs and fluid. Exposure to selenomethionine reduced the DTH response to *M. bovis* in mallards [95]. Because DTH tests often require multiple immunizations several weeks apart, their application is limited in wild birds, which are difficult to recapture over long time periods. Instead, a more commonly used assay for T cell function in birds is the phytohemagglutinin (PHA) skin response [78]. PHA injected intradermally or subdermally stimulates T cells to cause a localized inflammation response similar to a DTH response, except no prior sensitization to PHA is required. In prefledgling herring gulls and Caspian terns from the Great Lakes, the PHA response showed strong negative associations with PCBs and other organochlorines [85, 87]. High doses of lead suppressed the PHA response

in Japanese quail [82] and western bluebirds (*Sialia mexicana*) [83].

B cell function can be assessed by measuring antibody titers or antibody-secreting B cells (i.e., plaque assay) following immunization with an antigen. SRBCs are the most common antigens used in avian immunotoxicology [78]. Lead exposure decreased anti-SRBC titers in Japanese quail [82] and mallards [96] but not in western bluebirds [83]. Anti-SRBC antibody titers were positively associated with PCBs and DDE in prefledgling Caspian terns [87]. In female glaucous gulls (*Larus hyperboreus*), anti-diphtheria toxoid antibody responses were negatively associated with hexachlorobenzene and oxychlordane [97].

In vitro immune function assays such as mitogen-induced lymphocyte proliferation and macrophage phagocytosis have been employed most commonly in laboratory experiments with wild bird species because access to cell culture facilities near study sites in the wild is often limited. Furthermore, the small body size of many birds (e.g., passerines) limits the volume of blood samples and hence the number of WBCs for cell culture assays. Despite the small body size of tree swallows (*Tachycineta bicolor*), Bishop and colleagues [98] successfully used small volumes of blood (1-1.2 ml) and splenocytes to study the immunotoxic effects of orchard insecticides and fungicides. Splenocyte proliferation induced by pokeweed mitogen was significantly increased in swallows from orchards. Responses of splenocytes to other mitogens were not affected. Flow cytometry assays of phagocytosis and respiratory burst by monocytes also were not influenced by pesticides. In larger birds where larger blood samples are possible, lymphocytes and monocytes can be isolated by density gradient or slow spin methods and cryopreserved on liquid nitrogen in the field for later analysis in the laboratory [99, 100]. These cells have good viability after thawing and can be cultured in lymphocyte proliferation and phagocytosis assays. These methods hold much promise for future field studies in avian immunotoxicology.

Ecological Relevance of Immunosuppression in Wild Birds

There is emerging evidence that contaminant-induced immunosuppression is, in some instances, associated with increased rates of infection and decreased survival in wild birds. In laboratory rodents, suppressed immune function is usually associated with increased morbidity and (or) mortality upon challenge with infectious organisms [101]. An important caveat is that such associations are usually only apparent when there is a good match between the specific immunological mechanisms that are suppressed and those that are required to fight a particular disease. For instance, a contaminant that suppresses primarily cytotoxic T cells will increase susceptibility to viral infections but not necessarily extracellular bacteria. Despite the fact that one of the first studies in avian immunotoxicology employed challenge infections [77], most studies in this field have not examined responses to actual pathogens. Exposure to Bunker C fuel oil and South Louisiana crude oil increased mortality following challenge with *Pasteurella multocida* in adult male mallards [102]. Anecdotal evidence suggests that exposure to lead shot in wild birds may be associated with increased prevalence of *Trichomonas gallinae* (a potentially fatal protozoan), aspergillosis, avian cholera, and coccidiosis

[82]. In double-crested cormorants (*Phalacrocorax auritus*) from the Great Lakes, the prevalence of severe eye infections (*Pasteurella sp.*) was strongly correlated to egg PCB concentrations [103, 104]. A comprehensive study of contaminants and parasites in glaucous gulls (*Larus hyperboreus*) near the Svalbard archipelago in the Barents Sea showed that total organochlorine exposure was significantly positively correlated with the intensity of the overall gastrointestinal parasite load [105]. In the same population of birds, reduced immune functions were associated with contaminants [97]. A similar study of gastrointestinal parasites in herring gull chicks from the Great Lakes showed significant differences in parasite load between sites but no associations with organochlorines [106]. However, this latter study was complicated by significant ecological differences amongst the geographically diverse study sites (e.g., different fish in the diet, which act as secondary hosts for these parasites). This study illustrates the difficulty of sorting out the effects of contaminants versus ecological factors in disease studies of wild birds.

The expanding field of avian immunoecology has provided strong evidence that suppressed immunity leads to ecologically relevant consequences in wild birds. In a review of 12 immune function studies, immune responses were the most significant predictors of subsequent survival of young wild birds [107]. In an assessment of 280 introduction attempts in 38 avian species, immunocompetence as assessed by the PHA skin response was an important positive predictor of the ability of birds to colonize new areas (i.e., found new local populations) [108]. Similar assessments of survival, fitness, and dispersal ability should be conducted within the context of immunotoxicological studies of wild birds.

SOME UNANSWERED QUESTIONS: OPPORTUNITIES FOR FUTURE STUDIES

Wildlife immunotoxicology including studies of amphibian, fish, and birds lags behind that of mammalian immunotoxicology, and this is due mainly to a lack of critical reagents. More investigators need to produce reagents such as monoclonal antibodies and freely distribute these to the community of scientists investigating immunotoxicology in these groups. The same is true for molecular probes, cDNA libraries, and microarrays currently being developed by several labs around the globe. With these critical reagents in hand, some additional questions can be answered. For example, we do not yet know if the immune system of fish is responsible for hepatic lesions often seen in feral fish sampled from heavily polluted environments. While chemical-induced hepatotoxicity is well characterized in susceptible mammals through autoimmune reactions, this has not been examined in fish [46]. Likewise, suppression of immunity in fish and amphibians due to neuroendocrine-immune interactions as a mechanism of immunotoxicity has not yet been addressed. With the enormous efforts directed at endocrine disruption, and the large body of evidence demonstrating immune modulation by sex hormones, immune dysfunction in amphibians, fish, and birds from endocrine disruptors is likely. Finally, one of the most important questions in immunotoxicology of these groups is whether or not immunomodulation by xenobiotics contributes to disease outbreaks in

the wild, resulting in changes in populations and ultimately natural communities. As immunotoxicologists move from *in vitro* experiments towards whole animal laboratory experiments, and ultimately to field studies, the general lack of control over environmental factors becomes problematic. Future studies should include studies of the impacts of additional potentially immunotoxic compounds on each group, and studies of the impacts at concentrations present in the environment. A better understanding of the mechanism of action of each immunotoxic agent may lead to the development of safer chemicals that remain effective but protect wildlife species.

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23 Marine Mammal Immunotoxicology

Peter S. Ross and Sylvain De Guise

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INTRODUCTION

Interest in the area of marine mammal immunotoxicology is not incidental. Their charismatic nature, prominence in public zoos and aquaria, exposure to often very high contaminant levels, and a series of mass strandings, have all contributed to high public and scientific interest in marine mammals. This has driven the need for improved husbandry practices and veterinary diagnostic tools for captive individuals, and calls for explanations of strandings, disease outbreaks, and mass mortalities in wild populations.

Marine mammals do not all fall within closely related taxa, and comprise members with ancestors common to the terrestrial carnivores (in the case of pinnipeds, otters, and the polar bear: *Ursus maritimus*), the artiodactyls, such as the hippopotamus, *Hippopotamus amphibius* (in the case of cetaceans), or to the proboscidea, such as the elephant, *Elephas maximus* (in the case of sirenians). Within these lineages, marine mammals have diversified, as evidenced by the variety of forms within the pinnipeds (Phocidae or true seals; Otariidae or sea lions and fur seals; and Odobenidae with its solitary member, the walrus: *Odobenus rosmarus*), the cetaceans (Whales, dolphins and porpoises), the sirenians (manatees, *Trichechus spp.*; and the dugong, *Dugong dugon*; Order Sirenia), as well as marine-dwelling otters (the sea otter: *Enhydra lutris* and the

marine otter: *Lutra felina*), and the polar bear. The term "marine mammal," therefore, is a functional one, rather than a phylogenetic one, something that has direct relevance for researchers looking to conduct immunological or immunotoxicological studies in this category of vertebrate.

Marine mammals have adapted to an aquatic or semi-aquatic lifestyle with such features as reduced or eliminated limb structures, a blubber layer for thermoregulatory, nutritional, buoyancy and locomotory roles, and reproductive strategies conducive to fitness in a given biogeographic zone (habitat). The environmental challenges faced by newborn marine mammals at birth may explain there largely precocious nature and the apparent maturity of the immune system of newborn marine mammals [1].

In crossing species barriers, the marine mammal immunotoxicologist must consider some combination of (1) universally workable immunological reagents and assays; (2) newly developed, species-specific reagents; (3) an understanding of comparative immunology; (4) consideration of different developmental immunology models; and (5) knowledge of the complex mixture of environmental contaminants to which they are exposed.

Natural biological and ecological factors play a role in the way in which the immune systems of different marine mammal species deal with pathogens. These include placentation type, gestational length, degree of maternal care, duration of lactation, social interactions, nutrition and condition, age, sex, stress, interactions with other species, genetic susceptibility to the pathogen itself, and the co-evolution of the hosts and pathogens. Marine mammals exhibit a tremendous variety of forms and strategies, all of which have some bearing on the immunotoxicologist concerned about the real-world impacts of environmental contaminants. The science of marine mammal immunotoxicology quickly encompasses more than a "simple" chemical—immune system interaction, delving into multiple natural and anthropogenic factors, as the contaminant-immune system-pathogen interactions play out in different ways on an ecological scale.

Anthropogenic factors that play a role in the way in which different marine mammal species are affected by pathogens include those factors typically associated with emergent infectious disease (EIDs). These include human disturbance, climate change, competition through fisheries, habitat destruction, encroachment by human-associated animals such as pets and livestock, and contamination of aquatic food webs by immunotoxic chemicals [2, 3]. Thus, by encroaching on the natural habitat of marine mammals, humans inadvertently introduce new pathogens (i.e. "biological pollution") to marine mammal populations, disturb individuals leading to stress, reduce the availability of prey, and contaminate their food supply with chemicals that may modulate a normal immune response. Humans may therefore contribute directly to an outbreak of an EID, or contribute indirectly to those factors that predispose marine mammal populations to reduced fitness or increased risk of serious disease.

The vulnerability of marine mammals to environmental pollution provides an ecologically-relevant context for marine mammal immunotoxicology. Often situated at the top of aquatic food chains, marine mammals are exposed to high concentrations of a complex mixture of persistent contaminants, many of which are known to be immunotoxic in laboratory animals. Marine mammal immunotoxicology came into its own in

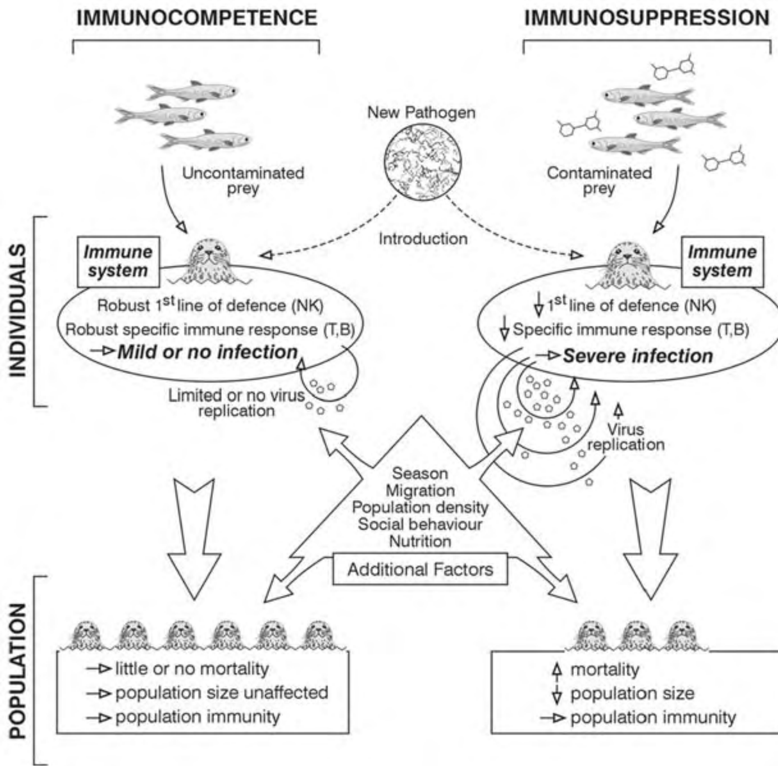

FIGURE 23.1 Immunotoxic contaminants often reach very high concentrations in marine mammals, and may have population-level consequences. The real-world of immunotoxicity in marine mammals, therefore, must capture other factors that shape the outcome of, for example, a pathogen-associated outbreak or mass mortality. These can include natural factors including age, sex, stress, nutrition, season and population parameters, as well as anthropogenic factors including overfishing, disturbance, habitat destruction and climate change. Environmental contaminant associated immunotoxicity may facilitate the spread and/or the severity of disease outbreaks with this backdrop. (From Ross, P.S., Human Ecol. Risk Assess. 8, 277, 2002, with permission).

the late 1980s and the 1990s, following high-profile outbreaks of infectious disease and related mass mortalities of seals, dolphins and porpoises inhabiting industrial coastal regions. The outcome of such disease incidents is often governed by a variety of interacting natural and anthropogenic factors, underscoring the need for multidisciplinary research and informed conservation strategies that address the complexity of the issues at hand (see Figure 23.1).

Additional information is available to the reader in the way of reviews on disease outbreaks in marine mammals, the anthropogenic factors affecting disease outbreaks in marine mammals, and considerations for study design in marine mammal immunotoxicology [3–5].

COMPARATIVE IMMUNOLOGY OF MARINE MAMMALS

Marine mammals share fundamental immunological features with their terrestrial counterparts. For example, as in veterinary medicine, hematological variables have long been used as diagnostic tools in marine mammal medicine, providing elementary clinical information on quantitative aspects on white blood cell subpopulations [6]. Such work provided early evidence of inflammatory responses by a variety of marine mammal species to viral, bacterial and macroparasitic infection akin to those responses that were better understood in domestic animals.

An increasing understanding of the immune systems of marine mammals arose with interest in comparative immunology in the 1970s. One of the first steps to assess whether marine mammals were different from terrestrial mammals involved the histological examination of lymphoid organs. Most studies have confirmed that there were few differences, besides the fact that mesenteric lymph nodes were grouped into a large aggregate sometimes referred to as the "pseudopancreas" in beluga whales, *Delphinapterus leucas* [7], and that marine mammal lymphoid organs in general appeared little stimulated [8].

Early characterization of immunoglobulin structures and classes established that several pinnipeds and cetaceans share basic features with their terrestrial counterparts [e.g., 9, 10, 11]. Early functional studies of bottlenose dolphin (*Tursiops truncatus*) and pilot whale (*Globicephala melaena*) lymphocytes established the utility of mitogens in eliciting proliferative responses, although these approaches did not discern the identities of the responding cells [12–14].

DEVELOPMENTAL IMMUNOLOGY OF MARINE MAMMALS

Critical to the long-term viability of a given species is the rigor with which newborn animals cope with pathogens they encounter in their surrounding environment. Marine mammals exhibit a number of species-specific adaptations which reflect their habitat needs. Maternal care, placentation type (reflecting their taxonomic lineage), and duration of gestation and nursing periods, figure prominently in the developmental immunology of marine mammals.

Pinnipeds, sea otters, and the polar bear share the endotheliochorial placentation that is also found in their terrestrial counterparts (e.g., cats and dogs). This limits the amount of immunoglobulins transplacentally transferred, and underscores the importance of acquiring maternally-derived antibodies via milk for passive protection against an array of pathogens. Studies of free-ranging harbor seals established that immunoglobulin G (IgG) levels in newborns rise from ~ 5% of maternal IgG levels at birth to ~ 65% at 15 days of age [1, 15, 16]. The transfer of IgG from female grey seals to their pups was demonstrated following the development of polyclonal antiserum [17]. Relatively slow increases in circulatory IgG, IgM and IgA were noted through the nursing period in northern fur seals (*Callorhinus ursinus*), perhaps a reflection of the more protracted 3-month nursing period [10]. Low levels of IgG were found in 1-week-old grey seal pups, followed by an increase during their 3-week nursing period [17]. Rapidly increas-

ing levels of IgG, an IgG subclass, and an IgM-like protein were observed during the nursing period of the northern elephant seal (*Mirounga angustirostris*) [18].

Placentation in whales is considered epitheliochorial, consistent with the structure found in their terrestrial counterparts, which include pigs (family Suidae) [19]. While little is known about the relative importance of transplacentally-derived immunoglobulins for newborn cetaceans, epitheliochorial placentation almost completely restricts any transplacental transfer [20]. This was confirmed by the incidental finding of the absence of gamma-globulins in an orphaned beluga whale neonate [21]. Colostral intake immediately following birth is therefore likely to be critical to the survival of newborn cetaceans.

Sirenians are characterized by hemochorial placentation, a model which favors a more important role for transplacental transfer of immunoglobulins, and less via colostrum.

Although little is known of the immunocompetence of newborn marine mammals, there is support for the idea that they are born with a functional immune system that is ready to respond to pathogens encountered in their environment. Strong lymphoproliferative responses to the mitogen Concanavalin A (ConA) relative to their mothers, and strong rabies vaccine-directed antibody responses relative to young cats and dogs, suggest that young pinnipeds may be well able to mount a protective response against pathogens in their environment [1, 15].

ADVANCES IN MARINE MAMMAL IMMUNOLOGY AND IMMUNOTOXICOLOGY TOOLS

Rodent and human immunology has served to guide our understanding of the mammalian immune system, where the highly conserved innate immune system, and the more complex acquired or adaptive immune system, interact to protect the host from infection.

The acquired immune system has probably received most of the attention in marine mammals, as has been the case in rodent and human immunology. Recent advances include the production of monoclonal [22–24] and polyclonal [25] antibodies to marine mammal immunoglobulins, enabling more precise, and often species-specific, reagents for the detection and quantification of antigen-specific antibodies.

Lymphocytes, the effector cells of the acquired immune system, include morphologically indistinguishable T and B cells, the former divided into CD4+ T helper cells and CD8+ cytotoxic T cells. Since the functions of those cell subsets differ so drastically, it became important to develop tools to distinguish them from each other. Efforts to identify cell subsets according to their expression of different surface antigens have been successful, including various Cluster of Determination (CD) markers (Table 23.1). In addition, cross-reactive monoclonal antibodies, and subsequently developed species-specific polyclonal and monoclonal antibodies towards the major histocompatibility complex (MHC) have been used to label cells in circulation and in tissue sections (Table 23.1).

Other cell-surface molecules important to the acquired immune system have been characterized at the molecular level, including beluga whale CD4 [26], bottlenose

TABLE 23.1
Cell Surface Markers Characterized for Marine Mammals.

Specificity	Species	Target cells	Type	Reference
CD2	Beluga	T cells	Cross-reactive	[91]
CD2	Bottlenose dolphin	T cells	Species-specific	[92]
CD3	Striped and common dolphins	T cells	Cross-reactive	[93, 94]
CD3	Sea otter	T cells	Cross-reactive	[37]
CD4	Beluga	T-helper cells	Cross-reactive	[91]
CD19	Bottlenose dolphin	B-cells	Species-specific	[92]
CD21	Bottlenose dolphin	B cells	Species-specific	[92]
CD45R	Bottlenose dolphin	Activated T cells	Species-specific	[95]
CD79 alpha	Sea otter	B cells	Cross-reactive	[37]
MHC I	Beluga	All nucleated cells	Cross-reactive	[91]
MHC I	Bottlenose dolphin	Lymphocytes	Species-specific	[96]
MHC II	Bottlenose dolphin	T and B cells	Cross-reactive	[97]
MHC II	Beluga	Leukocytes	Cross-reactive	[91]
MHC II	Striped and common dolphins	Macrophages, dendritic-like cells and some lymphocytes	Cross-reactive	[93, 94]
MHC II	Harbor porpoise	T and B cells	Cross-reactive	[98]
MHC II	Bottlenose dolphin	Dendritic cells	Cross-reactive	[99]
MHC II	Bottlenose dolphin	Lymphocytes	Species-specific	[100]
MHC II	Sea otter	B cells, monocytes, activated T cells	Cross-reactive	[37]
TCR gamma delta	Beluga	Gamma delta T cells	Cross-reactive	[91]
Pan leukocyte	Harbor porpoise	Leukocytes	Cross-reactive	[98]
Macrophage markers	Short-finned pilot whale and Risso's dolphin	Tissue macrophages	Cross-reactive	[101]
Macrophage scavenger receptor antigen	Baird's beaked whale, short-finned pilot whale, Risso's dolphins, bottlenose dolphins, and pantropical spotted dolphins	Liver macrophages	Cross-reactive	[101]
Beta-2 integrin	Bottlenose dolphin, killer whales	Activated neutrophils	Species-specific	[102]
Ig	Bottlenose dolphin	B cells	Species-specific (polyclonal)	[97]
IgG	Striped and common dolphins	B cells	Cross-reactive	[93, 94]
IgM	Beluga	B cells	Cross-reactive	[91]
Lysozyme	Striped dolphin	Monocytes	Cross-reactive	[93]

dolphin IgM [27], as well as portions of the MHC in the beluga whale and the narwhal, *Monodon monoceros* [28], bottlenose dolphin [29], California sea lion [30], and the southern elephant seal, *Mirounga leonina* [31]. It is important to note that the diversity in the MHC dictates the genetic extent to which a host is susceptible to infection by a given pathogen, an important consideration in the many MHC "bottlenecked" marine mammal populations.

In addition to being able to recognize the different cells involved in the acquired immune system of marine mammals, it is important to assure that the cells perform their functions appropriately. The ability of lymphocytes to proliferate upon stimulation (usually with mitogens) has been studied for several decades [1, 12, 14, 15, 32-35]. Recent advances include the demonstration of a conserved specificity for standard mitogens used in beluga whales [32] and harbor seals [33]. An assay to assess the expression of the receptor for interleukin-2 (IL-2), an early event in lymphocyte activation, was adapted in harbor seals [35], bottlenose dolphins [36], and sea otters [37]. Molecular and biochemical mechanisms of activation of beluga T lymphocytes do not vary substantially from those in other mammals [38].

Several cytokines have been characterized at the molecular level in different species of marine mammals (Table 23.2). In addition, limited evidence exists for conserved functionality of cytokines in marine mammals, such as the ability of human recombinant IL-2 to stimulate T cell proliferation [32, 33] and natural killer cell activity [39, 40] in beluga whales and harbor seals. Assays were developed to quantify circulating levels of cytokines [41, 42], as well as C-reactive protein, a marker of acute inflammation [43].

TABLE23.2
Cytokines Characterized for Marine Mammals.

Cytokine	Species	Reference
IL-1	Bottlenose dolphin	[103]
IL-2	Beluga and grey seal	[104]
IL-2	Manatee	[105]
IL-2	Manatee, Killer whale	[106, 107]
IL-2	Northern elephant seal, harbor seal, California sea lion	[108]
IL-4	Bottlenose dolphin	[109]
IL-6	Beluga	[110]
IL-6	Killer whale	[111]
IL-6	Harbor seal, sea otter, killer whale	[112]
IL-8	Bottlenose dolphin	[113]
IL-10	Killer whale	
IL-1-Beta	Beluga	[114]
TNF	Beluga	[114]
TNF	Bottlenose dolphin	[115]
IFN-gamma	Bottlenose dolphin	[116]

TABLE 23.3
Relevance of the Assays and Reagents Available to Assess Immune Function in Marine Mammals.

Assay	Significance
Immunophenotyping	Identification of lymphocytes subtypes
Phagocytosis	Ability of neutrophils to engulf foreign particles
Respiratory burst	Ability of neutrophils to destroy or kill phagocytized particles
NK cell activity	Response to tumors and early phase of viral infections
Lymphocyte proliferation	Ability of lymphocytes to proliferate upon stimulation
CD45R expression	Measure of lymphocyte activation/memory
IL-2R expression	Measure of lymphocyte activation
ß2-integrin	Measure neutrophil activation
Immunoglobulins	Measure of the levels of specific or non-specific antibodies
Cytokines	Messages between cells
Immunogenetics	Diversity between individuals and ability to respond to specific pathogens

Functional assays have also been adapted and validated in the study of the innate immune system of marine mammals. Phagocytosis represents the ability of cells to engulf pathogens or particles, which are then destroyed by the generation of oxygen free radicals, a phenomenon described as the respiratory burst. Phagocytosis and respiratory burst have been evaluated using different methods in beluga whales [44] and bottlenose dolphins [45]. Respiratory burst was also demonstrated in bottlenose dolphins with the expression of conserved genes for this function [46]. The activity of natural killer (NK) cells, an important first line of defense against tumors and viral infection, has been demonstrated in harbor seals [39] and beluga whales [40].

Overall, a battery of reagents and assays has been developed that enable the assessment of several aspects of the immune system of marine mammals. These are summarized in Table 23.3.

IMMUNOTOXICOLOGICAL STUDIES IN MARINE MAMMALS

There is growing evidence that environmental contaminants represent a conservation-level concern in some marine mammal populations, by contributing to an increased vulnerability to infection by natural or emergent pathogens. In this light, those marine mammals that are most contaminated with persistent organic pollutants (POPs) are most vulnerable to adverse health effects. Those species that occupy high trophic levels in aquatic food webs are typically the most contaminated [47–49].

Most marine mammals are exposed to relatively high concentrations of those contaminants considered to be persistent (do not breakdown readily in the environment), bioaccumulative (are not readily metabolized and excreted by biota in aquatic food webs), and (immuno)toxic. Candidates in this category include various congeners of

polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs or dioxins) and polychlorinated dibenzofurans (PCDFs or furans), and related compounds. And while the environmental concentrations of many of the 'legacy' POPs have declined over recent decades, there is increasing concern that some current use chemicals may also prove to be persistent, bioaccumulative, and (immuno)toxic, such as the polybrominated diphenylethers (PBDEs) [50]. Organotins, which are associated with proteinaceous tissues in marine mammals, rather than with fatty tissues, also appear to be immunotoxic, as mitogen-induced lymphocyte proliferation is reduced upon *in vitro* exposure to monobutyltin, dibutyltin, and tributyltin [51].

Conversely, marine mammals are not generally exposed to high levels of those contaminants that are non-persistent (readily breakdown in the environment or at lower levels in aquatic food webs) or non-bioaccumulative (are readily eliminated by either marine mammals or at lower levels in aquatic food webs). Nonetheless, there is *in vitro* evidence that the latter category of contaminants can be immunotoxic to marine mammals, such that those occupying lower trophic levels or inhabiting industrial regions may be at risk. For example, Zn and Cd exposure *in vitro* increased metallothionein expression and reduced phagocytic activity in grey seal peripheral blood mononuclear cells (PBMC) [52, 53].

The virus-associated mass mortalities that took place among marine mammals in the 1980s and 1990s elicited an interest in co-factors that may have contributed to the severity of these events. The deaths of 20,000 harbor and several hundred grey seals in northern Europe in 1988 prompted questions about climate change, algal blooms, environmental pollutants, exotic diseases and other perceived stressors [54]. After the identification of a newly described virus, phocine distemper virus (PDV) [55], concern still lingered that immunotoxic environmental contaminants could have facilitated the outbreak.

Captive Feeding Studies

Following the 1988 PDV epizootic, a study was carried out to assess the effects of environmental contaminants on immune function by feeding two groups of captive harbor seals either herring (*Clupea harengus*) from the Atlantic Ocean or from the relatively contaminated Baltic Sea. After 30-months on the diets, the authors reported reductions in NK cell activity, *in vitro* mitogen-induced T-cell proliferation, *in vivo* delayed-type hypersensitivity (DTH) responses following immunization with ovalbumin, *in vitro* antigen-specific proliferative responses of PBMC following *in vivo* immunization with tetanus and rabies antigen, and mixed lymphocyte responses in the harbor seals fed the more contaminated Baltic Sea herring [39, 56–58].

Analysis of the contaminants in herring and in the seals at the end of the study revealed that PCBs accounted for the majority of Toxic Equivalents to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TEQ). Combined with the pattern of immunotoxic effects at the level of the thymus and the T-cell, and a literature indicating an AhR-mediated immunotoxic vulnerability of the thymus [59, 60], the authors concluded that AhR-mediated effects, caused in large part by PCBs, led to the observed effects [57, 61]. They speculated that

many populations of free-ranging harbor seals studied in Europe and North America may be vulnerable to immunotoxic effects as they exceeded the PCB levels measured in the Baltic group of seals (17 mg/kg or 209 ng/kg TEQ)[62]. The captive feeding study provided direct evidence of contaminant-related immunotoxicity, having explained or eliminated some of the many confounding factors of concern, including age, sex and condition. The authors concluded that environmental contaminants likely contributed to the severity of the PDV-associated mass mortality of harbor seals as a consequence of immunotoxicity and associated reduced resistance to disease.

Laboratory Animals as Surrogates for Marine Mammals

Further evidence to support the notion of a role for immunotoxic environmental contaminants in the 1988 outbreak came from two studies of laboratory rats carried out in tandem with the seal studies. PVG rats were fed the same two batches of herring used in the seal study, with a similar pattern of effects observed in the seals [63, 64]. However, there were additional indications of immunotoxicity that could not be evaluated in seals for ethical or technical reasons, including increased virus titers in a rat cytomegalovirus (RCMV) host resistance model, and reduced thymus cellularity in the rats fed Baltic Sea herring. A positive control group of rats in one of the studies was exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin, thereafter exhibiting an amplified pattern of the effects that had been observed in the Baltic group. The collective results from the captive seal studies and the laboratory animal studies were seen to implicate an AhR-mediated immunotoxicity, in which dioxin-like PCBs played a dominant role [64, 65].

Conflicting results have been obtained in other laboratory animal studies, likely reflecting differing study design and exposure regimes. Fischer rats fed a diet containing 5% lipid content from blubber of contaminated St Lawrence belugas had no differences in immune function when compared to a control group fed Arctic beluga blubber [66]. In contrast, the more sensitive C57Bl/6 mice had reduced peritoneal macrophage phagocytic activity and specific humoral response to sheep erythrocytes in a similar exposure design [67]. Mink were used as a sea otter surrogate species to estimate the immunotoxic effects of chronic exposure to bunker C fuel oil [68]. While effects were observed on cell populations, expression of surface antigens, and function of lymphocytes, the exact relevance to the health of sea otters is unclear.

Epidemiological or Associative Studies

Several studies have used field approaches to evaluating the possible role of environmental contaminants on immune function in free-ranging marine mammals. A negative correlation between mitogen-induced PBMC proliferation and PCB concentrations was observed in bottlenose dolphins [69], although the small sample size and possible confounding effects of age precluded firm interpretation. A negative correlation was observed between serum total IgG levels and PCB concentrations in live-sampled, free-ranging polar bears, after correcting for age and sex [70], although condition (e.g., body

weight: length index) was not reported. Northern fur seal pups born to young mothers had both higher POP concentrations and reduced immune responses, when compared to pups born to older mothers (less contaminated because of multiple births) [71]. However, pups born to young female pinnipeds are generally less healthy than those born to larger, more experienced females, independent of a contaminant influence [72, 73]. Such studies provide useful information on some of the factors influencing immune function, but whether they provide evidence of immunotoxicity must be scrutinized in the context of confounding factors [74].

More recently, mitogen-induced proliferative responses of PBMC were positively correlated with total PCB and total TEQ concentrations in free-ranging harbor seal pups of the same age, after the effects of body weight were accounted for [75]. The authors suggest that the relatively low contaminant exposure levels in the seals studied may have led to an early phase stimulatory response akin to the hormetic responses observed at low toxicant doses in other physiological studies. A subsequent study included harbor seal samples obtained from a wider geographic area, including the PCB-contaminated Puget Sound, and found a negative correlation between T cell proliferation and blubber PCB concentrations [76]. These studies suggest that environmental contaminants are disrupting normal functional immune responses in free-ranging harbor seals, although more research will be needed to better characterize the pharmacokinetics of this effect (dose response), and which contaminant class(es) is (are) responsible.

Contaminant Levels in Disease Victims

In addition to the inherently difficult immunotoxicological studies of free-ranging marine mammals, a strategy involving a comparison of contaminant levels in marine mammals that have died from either infectious disease or from trauma has been used to glean insight into the link between contaminants, the immune system and vulnerability to disease. Harbor seals that died during the 1988 PDV epizootic in Europe had higher concentrations of PCBs than those that survived [77]. Striped dolphins (*Stenela coeruleoalba*) that died during a morbillivirus epizootic in the Mediterranean Sea in 1990–92 had higher concentrations of PCBs than did free-ranging individuals that were biopsy-sampled before the outbreak [49]. California sea otters that died from infectious disease had higher levels of butyltins and organochlorine pesticides than those killed by trauma [78, 79]. Interpreting such results must be done cautiously, as sick or dying individuals have invariably fasted, something which leads to increasing concentrations of POPs in blubber as lipids are burnt off [77]. Simple observations of higher levels of POPs in victims of disease outbreaks compared to victims of trauma that are likely in good or normal condition is insufficient to support a mechanistic linkage between the contaminants and disease-induced mortality [74, 77].

After adjusting for the confounding factors of age, sex, nutritional status, and season, harbor porpoises (*Phocoena phocoena*) that died from disease had higher levels of PCBs than victims of trauma [80], providing a more rigorous evaluation of the link between contaminants, the immune system and disease outcome in marine mammals. Such studies can yield insight into the "real world" of immunotoxicity among marine

mammals, but caution is needed to ensure that confounding factors are eliminated in the study design or taken into account in statistical evaluation.

IN VITRO MARINE MAMMAL IMMUNOTOXICOLOGY

The inherent logistical and ethical challenges associated with studies of marine mammals have triggered the evaluation of *in vitro* alternatives. This has also enabled a direct characterization of the toxic effects of chemicals at the cellular level. *In vitro* exposure experiments were useful to demonstrate the immunotoxic potential of heavy metals in beluga whale lymphocyte proliferation [81] as well as in grey seal lymphocyte proliferation and neutrophil phagocytosis [82]. A similar approach demonstrated the differences in susceptibility of beluga whale lymphocyte proliferation to different PCB congeners and DDT metabolites, and for the first time suggested synergistic interactions between PCB congeners when in mixtures [83]. Butyltins appeared more toxic than PCB congeners in Dall's porpoises, bottlenose dolphins, California sea lions, and largha seals (*Phoca largha*), with evidence of synergistic interactions between butyltins and PCBs in mixtures [51]. Harbor seal T lymphocyte proliferation was significantly reduced upon exposure to benzo-a-pyrene but not PCB 156 and PCB 80 [84]. Ongoing studies of the interactions of organochlorines in mixtures in marine mammals suggest marked differences among species, which do not appear to be predicted using a mouse model [85, 86]. For example, it appears that phagocytosis in bottlenose dolphins and beluga whales was modulated by non-planar PCBs, which are usually considered to be relatively non immunotoxic [87].

The results of *in vitro* exposures to different classes of chemicals can be useful when assessing the direct effects of chemicals on certain immunological endpoints, differences in susceptibility among species (at the cellular level), and differences in susceptibility within a species but between different immune functions. However, such an approach on its own cannot take into account such factors at the whole-organism level including exposure, absorption, metabolism and excretion, the built-in redundancy of the immune system, as well as host resistance.

CONCLUSIONS

Although marine mammals differ in fundamental ways from their terrestrial counterparts, they continue to share many features of the immune system that are typical of all mammals, and indeed, vertebrates. Carrying out immunological or immunotoxicological research in marine mammals is fraught with the obvious challenges associated with the logistics of studying often very large animals in an aquatic environment that is foreign to our own (some means of overcoming or addressing confounding factors in immunological studies of free-ranging marine mammals can be gleaned from [4]). In addition, ethical considerations play a significant role, as experimentation with marine mammals is heavily scrutinized, with an increasing emphasis on minimally-invasive or *in vitro* techniques. These challenges have not impeded a number of insightful immunological and immunotoxicological studies from being carried out. However, such

considerations lend support to inter-species extrapolation and a "weight of evidence" approach to marine mammal immunotoxicology [88]. Overall, the weight of evidence approach suggests that the immunotoxic potential of pollutants in marine mammals should be considered seriously in the context of marine mammal health.

The conserved nature of physiological systems among vertebrates has enabled extrapolation from experimental animal models (e.g., mice and rats) to humans. Indeed, while interspecies differences in sensitivities are evident in toxicological studies, common patterns of effects among species provide a basis for extrapolation. Ultimately, though, perhaps the most effective argument in favor of extrapolation is related to the complex mixtures of contaminants to which marine mammals are exposed through their diet. Mechanistic studies aimed at establishing cause-and-effect for multiple chemicals and endpoints are perhaps better left to the carefully controlled laboratory animal studies, while minimally invasive studies of free-ranging marine mammals can provide an ecologically-relevant real world context.

At the end of the day, the marine mammal immunotoxicologist must not only consider the contaminant-immune system-pathogen link, but also the numerous other factors that may shape the outcome of a disease event in a wild population. Anthropogenic impacts on the immune system are not limited to immunotoxic chemicals alone. Noise, disturbance, and temperature stress have been implicated in a reduction in immune function in marine mammals [89, 90]. The immunotoxicologist has two challenges. First, research must carefully document the effects, or likely effects, of environmental contaminants on immune function and host resistance in free-ranging marine mammals while controlling for confounding factors. Second, this research must be placed in a conservation context that takes into account multiple stressors in a wild population and informs wise decision making.

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Part VII

Autoimmunity and Autoimmune Diseases

24 Immunopathogenesis of Autoimmune Diseases

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INTRODUCTION

The immune system specifically recognizes and eliminates foreign antigens thereby protecting the host against infection. During maturation of the immune system lymphocytes specific for the universe of possible antigens arise, but tolerance mechanisms minimize harmful responses against self-tissues. However, the inappropriate activation of surviving self-reactive T or B lymphocytes can result in systemic or organ-localized autoimmune damage.

Autoimmune diseases affect approximately 5 to 8% of the population in the United States, or up to 22½ million people,[1] and are the third most common category of disease in industrialized countries following heart disease and cancer.[2,3] Because several of the diseases start at a relatively young age and continue throughout life, autoimmune diseases have a disproportionate affect on public health with an estimated annual cost of over 100 billion dollars in the United States. Often patients require a lifetime of care with, at this time, no prospect of a cure. Previously, autoimmune diseases were studied mainly on the basis of the organ affected, but more recently the focus has switched to investigating autoimmune diseases as a group in order to better understand the common mechanisms underlying the immunopathogenesis of these related disorders.

OVERCOMING TOLERANCE: AUTOIMMUNITY VERSUS AUTOIMMUNE DISEASE

Autoimmunity is a common phenomenon, since naturally occurring autoantibodies and self-reactive T and B cells are present in all normal individuals.[4,5] The development of autoimmune disease, however, is a relatively uncommon consequence of autoimmunity.[3] The immune response to autoantigens follows the same rules as the response to foreign antigens and is normally tightly regulated. Only when the autoimmune response is poorly controlled does an autoimmune disease result.[6–8] Thus, autoantigens need to be perceived in a manner that makes them strongly immunogenic, such as in the context of infection, before autoimmune disease develops.[7,9] Autoimmune disease results, then, from dysfunction of the immunoregulatory mechanisms that usually limit immune responses.

Mechanisms of self-tolerance, defined as a state of non-responsiveness to self, can be divided into central and peripheral. In central tolerance, immature lymphocytes in the bone marrow (B cells) and thymus (T cells) that recognize self-antigens with high affinity die by apoptosis.[5,10] In peripheral tolerance, mature self-reactive lymphocytes are inactivated, killed or turned off by regulatory mechanisms. The principle mechanisms of peripheral tolerance include functional anergy, ignorance, and suppression by regulatory T cells.[5,11] Defects in a single tolerance mechanism can be associated with a particular autoimmune disease, or defects in multiple pathways may be involved in various diseases or even a single condition.[12] The size of the peripheral T cell pool is remarkably stable throughout life, indicating precise regulation of cell survival, proliferation, and apoptosis.[13]

APOPTOSIS

Apoptosis, or programmed cell death is a normal physiologic process, occurring in senescent and damaged cells, that maintains homeostasis. Cells undergoing apoptosis demonstrate characteristic biochemical and morphological changes, such as alterations in the distribution of membrane lipids, membrane blebbing, and fragmentation of the

nuclear and cellular constituents into membrane-enclosed apoptotic bodies.[14] Phagocytosis is the final fate of many cells undergoing apoptosis. This contrasts with cell death by necrosis, which is a random process without orderly nuclear changes. Changes in the apoptotic cell death process, leading to inappropriate cell death or survival, or disturbances in clearance of apoptotic cells, are thought to be involved in the pathogenesis of a number of autoimmune diseases.[14–17] For example, defects in the apoptosis of fibroblasts within the inflamed synovium have been implicated in the hyperplasia associated with rheumatoid arthritis.[18] Defects in the uptake of apoptotic cells or cell material by macrophages is believed to contribute to immune complex deposition in lupus,[16] while increased thyrocyte apoptosis is implicated in Hashimoto's thyroiditis.[17]

T Regulatory Cells

Activation of self-reactive $CD4^+$ helper T cells is essential to the development of most autoimmune diseases, and therefore T cell tolerance is critical in maintaining unresponsiveness to self-antigens. $CD4^+$ $CD25^+$ T regulatory cells are a minor population of T cells that develop in the thymus and migrate to the periphery where they maintain tolerance by suppressing self-reactive T helper cells.[12,19,20] Multiple events can impair tolerance. For example, deletion or mutation of the transcription factor Foxp3 or the growth factor interleukin (IL)-2 interferes with the generation and/or function of T regulatory cells.[12,21] Loss of Foxp3, IL-2 or the inhibitory receptor CTLA-4 leads to lymphoproliferation and multiorgan autoimmune disease, indicating the critical role for T regulatory cells in preventing autoimmune disease.[21] T regulatory cells also inhibit the rejection of transplants, prevent the induction of antitumor responses, and regulate the immune response to infections.[20]

EFFECTOR MECHANISMS: ANTIBODY-MEDIATED VERSUS CELL-MEDIATED DAMAGE

Antibody-mediated Damage

A common feature of all autoimmune diseases is the presence of autoantibodies. Even in instances where the autoantibodies are not believed to be primarily involved in the pathogenesis of disease, they make an important contribution in the diagnosis or classification of the autoimmune disease.[3,22] In order for autoimmune disease to occur, the responsible antigen must be presented in an accessible form to a competent immune system.[3] For antigens located on the cell surface, the pathogenic agents are generally circulating antibodies, especially those classes of antibodies that are capable of activating the complement cascade. In certain forms of hemolytic anemia, therefore, autoantibodies to red cell surface antigens may induce either lysis or phagocytosis of red blood cells. In other cytopenias, platelets and leukocytes can also be depleted by antibodies. Another important group of autoantibodies react with cell surface receptors.

They include antibodies to the acetylcholine receptor that block transmission at the neuromuscular junction in myasthenia gravis, and autoantibodies to the thyrotropin receptor that block thyroid cell stimulation in Graves' disease. Antibodies to intracellular enzymes, such as dehydrogenases and transferases, have been increasingly implicated in autoimmune diseases, such as primary biliary cirrhosis and celiac disease.[3] In many systemic autoimmune diseases, a variety of autoantibodies occur that can bind widely distributed antigens thereby increasing damage to the host. For example, intracellular constituents, such as nuclear antigens, are released as cells die or undergo apoptosis and then interact with corresponding autoantibodies to form injurious immune complexes, such as those observed in lupus.[23,24]

Since most autoimmune diseases are chronic in nature, autoantibodies are likely to appear long before clinical symptoms, providing a good predictive marker for the potential to develop disease.[25] Estimates based on first degree relatives showed that the likelihood of developing type 1 diabetes within five years was 10% in the presence of one autoantibody, but 60 to 80% if three autoantibodies were present.[25] In a recent study, antinuclear autoantibodies were found in 80% of the subjects that went on to develop lupus and were present up to 9.4 years before diagnosis.[23,25] Thus, multiple autoantibodies are a good predictor of the risk of developing certain autoimmune diseases.

Cell-Mediated Damage

In many autoimmune diseases antibodies do not seem to play the major pathogenic role.[3] Rather, autoreactive T cells or their products are responsible for tissue injury. Type 1 diabetes and multiple sclerosis are classic examples in which pathology is primarily due to T cell-mediated immunity, particularly associated with T helper-type 1 (Th1) responses. The actual mechanism of damage may be attributed to direct attack by cytotoxic T cells or to indirect effects of cytotoxic cytokines, prostaglandins, reactive nitrogen or oxygen intermediates produced and released by stimulated T cells or macrophages. A common pathogenic mechanism involved in cell-mediated immunity is the release of the proinflammatory cytokines, IL-1β and tumor necrosis factor (TNF-α).[9] Agents that block these cytokines have clinical benefit in treating patients with certain autoimmune diseases.[18,26,27] Even in cell-mediated autoimmune diseases, however, autoantibodies may contribute secondarily to the damage induced by inflammatory cells.

INDUCTION OF AUTOIMMUNE DISEASE: GENES VERSUS ENVIRONMENT

The development of autoimmune disease depends on a combination of genetic and environmental factors (Figure 24.1).[3,5,28] Most autoimmune diseases are thought to be polygenic, involving more than one gene, and depend on the accretion of a number of genetic traits.[5,10]

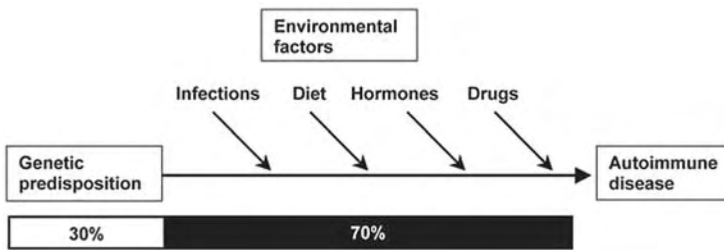

FIGURE 24.1 The development of autoimmune disease depends on a combination of genetic and environmental factors.

Genes

Familial Frequency

The suggestion of a genetic predisposition to autoimmune disease arose first from clinical reports stating that patients often describe a family history of the same or related autoimmune diseases.[3] For example, clinicians noticed that patients with the autoimmune thyroid diseases Graves' disease and Hashimoto's thyroiditis have a family history of one or the other of these diseases. Moreover, even those family members who do not develop clinically apparent disease have a high probability of developing the characteristic thyroid autoantibodies. There is also a greater than expected occurrence of other autoantibodies to gastric mucosa, pancreatic beta cells, and adrenal cortex.[3] The fact that autoimmune diseases tend to cluster in families and in individuals indicates that common factors are involved in disease susceptibility.[3,9,28–30]

HLA Association

Human lymphocyte antigen, or HLA haplotype, is the best available predictor of developing an autoimmune disease.[3,31] The likelihood of developing similar autoantibodies relates directly to sharing HLA haplotypes with family members, and the probability is even greater if two haplotypes rather than one are shared. This clinical evidence is strengthened by animal studies, including our initial demonstration in mice and chickens that susceptibility to experimentally induced or spontaneously developing thyroiditis is associated with the major histocompatibility complex (MHC).[3,32-34] The role of MHC in affecting susceptibility to autoimmune disease (MHC class II is expressed on antigen presenting cells) implies that antigen presentation during the innate immune response plays a critical role in the initiation of autoimmune disease.[9,35] MHC is proposed to increase autoimmune disease by enhancing antigen presentation in the periphery, which results in increased helper T cell activation.[36] It is also widely recognized that genes outside of the MHC contribute to the risk of autoimmune disease.[3,37] Extensive studies of type 1 diabetes mellitus and lupus, or their animal models, have revealed a number of non-MHC genes that contribute to susceptibility.[3,31,38] Common susceptibility loci

have been found for a number of different autoimmune diseases, including diabetes and myocarditis, suggesting that shared genes are involved in the pathogenesis of disease;[37] recent evidence suggests that these genes control immunoregulatory factors.

Microchimerism

The presence within one individual of a small population of cells from another genetically distinct individual is referred to as microchimerism.[39] Recent studies indicate that cell transfer between fetus and mother during pregnancy can persist in both, decades later.[39,40] Microchimerism can have beneficial or detrimental effects either by contributing to tissue repair or as a target for autoimmunity. Maternal microchimerism occurs more frequently in patients with systemic sclerosis compared to controls (72% vs. 22%, respectively), but no clear association has been shown for other autoimmune diseases like thyroiditis, Sjogren syndrome or lupus.[39,41] The role for microchimerism in autoimmunity is not clear, but it is possible that genetically foreign cells stimulate the immune response similar to chronic graft-versus-host disease.

Environment

Based on a preponderance of evidence, heredity accounts for only about one third of the risk of developing an autoimmune disease.[3,42,43] This estimate is based on studies of genetically identical, monozygotic twins compared to nonidentical, dizygotic pairs where the concurrence rate of around 2% to 7% approximates that of other siblings. Noninherited factors, therefore, account for the remaining approximately 70% risk of developing an autoimmune disorder (Figure 24.1). Thus, it is highly likely that external environmental factors influence the induction of autoimmunity.[3,9,10,42]

Infections

Historically, infection was the environmental factor first associated with autoimmune disease. A body of circumstantial evidence links type 1 diabetes, multiple sclerosis, myocarditis and other autoimmune diseases with preceding infections (Table 24.1).[28] In general, however, it seems that the infection occurs well before the onset of autoimmune disease, making it difficult to isolate a particular causative agent responsible for the disease.[3,9] The fact that many different microorganisms are associated with a single autoimmune disease (Table 24.1) suggests that infectious agents induce disease through similar mechanisms.[9,42]

Animal models have established that infections can induce autoimmune disease. For example, coxsackievirus B3 infection of susceptible strains of mice results in inflammation in the heart that resembles the myocarditis and dilated cardiomyopathy that occurs in humans.[28,44] The same disease can be induced by injecting mice with cardiac myosin mixed with adjuvant, thereby reproducing the disease in the absence of virus infection, indicating that an active viral infection is not necessary for the development of autoimmune disease.[9,29,44] Likewise, a number of autoimmune diseases can be

TABLE 24.1
Infections in Humans Associated with Autoimmune Diseases

Disease	Infection[a]
Multiple sclerosis	EBV, measles virus
Lyme arthritis	*Borrelia bergdorferi*
Type I diabetes	CVB4, Rubella, CMV, mumps virus
Rheumatoid arthritis	*E. coli*, Mycobacteria, EBV, HCV
Lupus	EBV
Myocarditis, Rheumatic Fever, Chagas' disease	CVB3, CMV, Chlamydia, *Streptococci*, *Trypanosoma cruzi*
Myasthenia gravis	HSV, HCV
Guillian-Barre syndrome	CMV, EBV, *Campylobacter*

[a] Abbreviations: CVB, coxsackievirus B; CMV, cytomegalovirus; EBV, Epstein Barr virus; HCV, hepatitis C virus; HSV, herpes simplex virus.

induced experimentally by administering self-antigen in the presence of adjuvant, such as rheumatoid arthritis with collagen, multiple sclerosis with myelin basic protein, and thyroiditis with thyroglobulin. Several mechanisms have been postulated to be primarily responsible for the development of autoimmune disease following infection including direct viral damage,[45] release of cryptic self-peptides,[46] molecular mimicry,[47,48] bystander activation,[47,49] and the adjuvant effect.[9]

Molecular Mimicry

The mechanism most commonly invoked to explain the association of infection with autoimmune disease is molecular mimicry; that is, the concept that antigens (or more properly, epitopes) of the microorganism closely resemble self-antigens.[50] The induction of an immune response to the microbial antigen thus results in cross-reactivity with self-antigens and the induction of autoimmunity. Although epitope specific cross-reactivity has been shown in some animal models,[48,51-53] molecular mimicry is clearly demonstrated to be the causative mechanism in few, if any, human diseases.[3,54,55]

The Bystander Effect

Another possibility is that microorganisms expose self-antigens to the immune system by directly damaging tissues during an active infection. This mechanism has been referred to as "the bystander effect."[49,56] It must be remembered that the invading microorganism not only elicits a specific immune response against the microorganism, but also generally provides increased activation of the immune system. An alternative possibility is that the infecting microorganism presents a superantigen that activates an entire family of T cells, some of which can lead to an autoimmune response.[57] By activating the innate immune response, infection itself can overcome tolerance by providing co-

stimulatory signals that overcome clonal anergy and ignorance against self. This action of microorganisms has been referred to as "the adjuvant effect." Recently, a renewed understanding of the critical role of innate immunity in influencing the development of the adaptive immune response has led researchers to a better understanding of the adjuvant effect.[9,35,58]

The Adjuvant Effect

Adjuvants improve antigen uptake and processing by antigen presenting cells (APC), and are considered to "non-specifically" stimulate the immune response in experimentally induced models of autoimmune disease.[9,59,60] Interaction of the microorganism component of adjuvants with Toll-like receptors (TLR) or other pattern recognition receptors (PRR) on APC results in activation of APC and upregulation of molecules essential for antigen presentation, such as MHC class II and CD80/86, as well as production of proinflammatory cytokines.[9] The self-antigen administered with the adjuvant is then presented to the adaptive immune system resulting in activation and expansion of autoreactive T and B cells. Although adjuvant or self-antigen usually does not induce overt disease if administered separately, adjuvants alone can sometimes induce autoimmune disease in some animal models,[61,62] indicating the important role that activating the innate immune response plays in the development of autoimmune disease.[9,35,58] Even though the innate immune response does not respond "specifically" to antigen epitopes like the adaptive immune response, it does produce a "restricted response" to particular classes of pathogens via PRR. Thus, the adjuvant effect is not as non-specific as previously believed and is important in determining whether autoimmune disease develops following infection.[9] It is possible that infections could break tolerance by stimulating TLR-mediated proinflammatory responses during antigen presentation resulting in altered regulation of the immune response and the development of chronic autoimmune disease.[9,63]

Hormones

Another common feature of autoimmune diseases is a sex-related bias. Most autoimmune diseases are more prevalent in women than in men.[3,28,64] Conservative estimates indicate that 78.8% of the persons with autoimmune diseases are women.[2] This observation underscores the intimate interrelationship between the hormonal and immune systems. The hypothalamic-pituitary-adrenal axis has a profound effect on the induction of the immune response, and steroid hormones, including estrogens, are known to affect antibody production and T cell proliferation.[28,65] Although some diseases, such as Hashimoto's thyroiditis, occur predominantly in women, related endocrinopathies, such as type 1 diabetes, are slightly more common in men.[3,64] For some time, qualitative and quantitative differences in the immune response have been known to exist between men and women, with women producing increased antibodies and men producing increased cell-mediated responses with more severe inflammation.[28,64,66] Many animal models of autoimmune disease show a similar sex bias, with a higher incidence of disease in females but increased severity in males.[28] Recently, estrogens and androgens have been found to

directly influence whether a Th1 or Th2-type immune response develops by interacting with hormone receptors on immune cells.[66] Not only are a variety of sex hormone receptors found on immune cells, but also cytokine receptors, such as the IL-1 receptor, have been discovered on hormone-producing tissues, suggesting a bi-directional regulation of the immune response. *In vitro* studies of immune cells cultured in the presence of hormones have shown that estrogens can significantly increase proinflammatory cytokine production (e.g., TNF, IL-1).[64] Thus, an increased proinflammatory immune response in women may lead to an increased incidence of autoimmune disease.[28]

Diet

Many environmental factors have been implicated in the induction of autoimmunity. One dietary component, iodine, has been well studied as a factor that increases autoimmune thyroid disease.[67,68] Based on studies in the OS chicken, the increasing prevalence of autoimmune thyroid disease observed in U.S. and Western European populations has been ascribed to increased use of iodized salt.[69] Experimentally, it can be shown that iodinated thyroglobulin is a more potent autoantigen than the equivalent noniodinated molecule. Food additives or pesticides may also be important in contributing to the development of autoimmune disease in susceptible individuals.[70]

Drugs

In recent years, the introduction of many new drugs into clinical practice has been accompanied by an increasing number of reports of drugs causing disease syndromes resembling autoimmune disorders.[3,24,36,71] Many drugs induce expression of autoantibodies in a significant number of patients without inducing drug-induced autoimmune disease.[71] Procainamide and hydrolazine, for example, induce a lupus-like disorder in certain patients.[72,73] Penicillamine has been associated with myasthenia gravis and other autoimmune diseases, and α-methyldopa is known to cause a form of hemolytic anemia.[3,72] In all cases of drug-induced autoimmune disease described thus far in the literature, the disease disappears when the drug is removed.[24,71] Procainamide-induced autoimmunity in mice can also be prevented by T regulatory cells.[74]

Toxins

Various heavy metals, such as mercury, silver, or gold, can induce an antibody response to cell nuclear antigens in susceptible strains of mice, suggesting that heavy metals may sometimes be the instigating agents of autoimmune disease in humans.[3,70,75,76] Mercury can induce a T cell-dependent systemic autoimmune disease in mice with many of the characteristics of human lupus, which requires costimulation via CD28 and CD80/86 and can be prevented by treatment with antibody that blocks CTLA-4 signaling or by T regulatory cells.[74,77] Halogenated hydrocarbons also have profound effects on the regulation of the immune response and, therefore, represent candidates for enhancing autoimmune responses in humans.[3,78] Aromatic amines, such as those derived from petroleum distillation, have also been blamed as possible environmental inducers of

lupus or lupus-like disorders, and silica has been reported to increase the prevalence of scleroderma.[70,79] These studies have focused a great deal of attention on the possibility that pollutants in general are agents that increase the probability of developing autoimmune disease. As we begin to unravel the role of environmental pollutants and toxic metabolites in the pathogenesis of autoimmune diseases, we may find novel strategies to reduce the occurrence of autoimmune disease in susceptible individuals.

THERAPIES

Patients usually come to medical attention only after epitope spread and autoimmune escalation have greatly expanded the immune response, making it difficult to intervene at the point of initiation of disease.[3,80] Experience has shown, however, that it is difficult to turn off an ongoing autoimmune response, and that intervention at the earliest stage of antigen recognition is likely to be necessary if antigen-specific treatment is to succeed. In this way, the physician can eliminate a harmful autoimmune response without dampening or compromising the general immune response. To accomplish this goal, it will be necessary to identify subjects at risk of developing autoimmune disease and to intervene at the earliest possible moment.[81] An alternative approach is to identify and eliminate the environmental trigger in individuals who are genetically predisposed. A precedent for this approach is the marked reduction and virtual elimination of rheumatic heart disease in many of the industrialized countries where antibiotic treatment for streptococcal infection is employed.[3,81] In fact, administration of penicillin in patients with a history of rheumatic fever can prevent rheumatic heart disease.[81] In the past, therapies for autoimmune diseases have included "blanket" immunosuppressive or antiviral/antibacterial treatments. Recent therapies, however, are selectively targeting pathways common to a number of autoimmune diseases.[82] Therapies include treatments that target proinflammatory cytokines like TNF and IL-1β, or block costimulatory molecules using CTLA-4-Ig, or use therapeutic vaccination with T cells.[83-85] Blocking TNF has remarkable effects on several autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, ankylosing spondylitis, psoriasis and multiple sclerosis. Recently, familiar oral medications, such as statins and angiotensin blockers, widely used to treat other disease conditions such as allergy and hypertension, have been shown to inhibit autoimmune inflammation.[85] Along with the therapeutic successes, however, has come the realization that these drugs can also aggravate autoimmunity in a significant number of treated patients.[26,71] Various effector mechanisms accompany autoimmune responses and may contribute to the immunopathogenesis of autoimmune disease. Thus, it is unlikely that blocking a single effector mechanism will be effective in treating autoimmune diseases.

Many mysteries remain to be solved in the immunopathogenesis of autoimmune diseases. Clearly, this group of disorders constitutes an important threat to human health and well-being. They, moreover, represent a fascinating problem for the investigator at the interface of immunology and toxicology.

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25 Environmental Influences on Autoimmunity and Autoimmune Diseases

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INTRODUCTION

Autoimmunity, Autoimmune Diseases and the Role of Environmental Exposures

Autoreactive T cells and autoantibodies directed against self-antigens (autoimmunity) may be produced as part of the normal process of immune surveillance and response to infections or other stimuli. What becomes "abnormal," however, is the progression from autoimmunity to pathologic conditions characterized by tissue damage and destruction (autoimmune diseases). The specific target of the damage determines the clinical expression of the disease, and autoimmune diseases can involve virtually any organ or system (Table 25.1).

TABLE 25.1
Prevalence, Sex Distribution, Autoantibody Targets and Major Areas of Environmental Research Concerning Selected Autoimmune Diseases.

Disease	Prevalence per 100,000*	Percent Female	Autoantibody Targets	Major Areas of Environmental Research
Organ-Specific				
Addison's disease	14	93%	21-Hydroxylase	
Diabetes (type 1)	192	48%	Islet IA-2, glutamic acid decarboxylase	Infectious agents, ultraviolet radiation/vitamin D
Grave's disease/hyperthyroidism	1300	88%	thyroglobulin	
Multiple sclerosis	58	64%	myelin oligodendrocyte glycoprotein	Infectious agents (Epstein-Barr virus), solvents, ultraviolet radiation/vitamin D, tobacco
Myasthenia gravis	5	73 %	Acetylcholine receptor	
Primary biliary cirrhosis	3	89 %	Antimitochondrial antibodies	
Thyroiditis/hypothyroidism	4600	83 %	17 alpha-hydroxylase, thyroid peroxidase	Iodine
Uveitis	2	50 %	retinal S-antigen	
Vitiligo	400	52 %	Melanin-concentrating hormone receptor 1	
Systemic				
Polymyositis/dermatomyositis	5	67%	Translational factors, others	Ultraviolet radiation/vitamin D
Rheumatoid arthritis (adult)	860	75%	Citrullinated peptide	Silica, farming, tobacco
Scleroderma	4	92%	Topoisomerase I RNA polymerase	Silica, solvents
Sjögren's disease	14	94%	Ro/SS-A	
Systemic lupus erythematosus	24	88%	dsDNA, Sm	Silica, infectious agents (Epstein-Barr virus)
Wegener granulomatosis or small vessel vasculitis	3	51%	Proteinase-3 (cANCA)	silica, solvents

*Adapted from Jacobson3, Cooper and Stroehla4; thyroid disease prevalence data from Hollowell5 and includes clinical and subclinical disease; limited to clinical disease estimates are 500 per 100,000 for hyperthyroidism and 300 per 100,000 for hypothyroidism.

A recent report by the National Institutes of Health estimated that at 14 to 22 million people in the United States are affected by an autoimmune disease.[1] As a group, these diseases represent a leading cause of death among women under age 65, with systemic lupus erythematosus, multiple sclerosis, and type 1 diabetes being the major sources of this impact on mortality.[2] The autoimmune thyroid diseases, type 1 diabetes and rheumatoid arthritis are the most common of the autoimmune diseases (Table 25.1).[3–5] Most autoimmune diseases disproportionately affect women. In the thyroid diseases, primary biliary cirrhosis, scleroderma, systemic lupus erythematosus, and Sjögren's syndrome, more than 85% of patients are female, but it is not known why the female predominance is so high in these specific diseases.

The rate of disease concordance among monozygotic twins is less than 40% even for the autoimmune diseases with the highest concordance (systemic lupus erythematosus, type 1 diabetes, hyperthyroidism, and multiple sclerosis). For other diseases (rheumatoid arthritis and scleroderma), the concordance rates are quite low (< 15%).[6] These figures suggest that environmental factors, and the interactions of genes and environmental factors, play a substantial role in the development of disease. The definition of "environment" is quite broad and can include infectious agents, occupational exposures, pollutants, medication use, stress and the response to stress, and personal behaviors such as diet and smoking. Studies in mice and in humans suggest that there may be several shared or common "autoimmunity genes" that increase risk for the development of different autoimmune diseases.[7] As discussed in this chapter, there are also examples of environmental exposures that affect risk of multiple autoimmune diseases.

For some autoimmune diseases, little is known about environmental factors involved in the initiation or progression of the disease. For other diseases, however, considerable research has been conducted on one or more types of exposures. Most epidemiologic studies of environmental influences have focused on multiple sclerosis, rheumatoid arthritis, scleroderma, systemic lupus erythematosus, and small vessel vasculitis, but experimental studies using murine models of these diseases is limited (Table 25.1).

Mechanisms of Disease

The underlying pathogenesis of autoimmune diseases involves the loss of tolerance to self-antigens. There are multiple steps and avenues through which environmental exposures could affect this process. These steps include, but are not limited to, the alteration and translocation of normally sequestered autoantigens onto the surface blebs of apoptotic cells,[8] inability to effectively clear apoptotic debris,[9,10] abnormal activation of or selection of autoreactive B and T cells,[11-13] and altered production of pro- and anti-inflammatory cytokines.[14] The mechanisms involved in initiating loss to tolerance may differ from the mechanisms involved in accrual of damage as the disease progresses, so it is important to differentiate between studies of disease etiology and studies of disease progression. Recent studies in type 1 diabetes,[15] systemic lupus erytheatmosus,[16] and rheumatoid arthritis[17] have demonstrated the presence of autoantibodies in some patients several years before the onset of symptoms or diagnosis. Thus, to understand the role of environmental factors in autoimmune diseases will require studies of the

influence of environmental exposures on the development of autoreactive cells and on the persistence of these cells and progression to clinically-overt disease.

Examples of Specific Occupational, Environmental, and Personal Exposures

Infectious Agents

There is considerable interest in the role of infectious agents in the development of autoimmune diseases. Some of this interest is based on the concept of molecular mimicry as a causal mechanism. Molecular mimicry refers to the possible pathologic role of cross-reactive antibodies or T cells to a self-antigen that is structurally similar to, and thus shares epitopes with, a viral or other infectious agent. For most autoimmune diseases, however, evidence of molecular mimicry leading to disease is not conclusive.[18,19] Viruses and other infections also have a less-specific immune effect, stimulating toll-like receptors and proinflammatory cytokine secretion, which is another mechanism that has been postulated to influence autoimmune disease risk.[20]

Many different viruses, including enteroviruses (Coxsackie B), cytomegalovirus, and Epstein-Barr virus, have been studied in mouse and rat models of type 1 diabetes, and there is evidence that these infections can lead to production of autoantibodies or destruction of the pancreatic beta cells by T cells.[21] There is less support for this process from epidemiologic studies examining enteroviruses infection (as determined serologically or using polymerase chain restriction-based techniques) in relation to risk of developing diabetes-related autoantibodies or clinically-diagnosed disease.[22,23] Epstein-Barr virus has also been a major area of research within the context of systemic lupus erythematosus[24] and multiple sclerosis.[25] Epstein-Barr virus is a very common infection (more than 90% of adults in the United States have been exposed), and persists in a latent state. Additional aspects of exposure (e.g., age at infection, number, and type of strains), factors affecting reactivation, or aspects of genetic susceptibility need to be examined in studies of the causal relation between this virus and these diseases.

Silica Dust

Crystalline silica, or quartz, is an abundant mineral found in sand, rock, and soil. Respirable silica dust (particles <5µm) is a known occupational hazard of the "dusty trades" (e.g., pottery or china manufacturing, work involving sandblasting or abrasive grinding, some construction trades). High level exposure to respirable silica can result in the chronic, progressive lung disease silicosis, characterized by inflammation and fibrosis.

Occupational exposure to silica dust has been identified as a risk factor for several systemic autoimmune diseases. This literature dates back almost 100 years, to the description by Bramwell of diffuse scleroderma in stone masons.[26] Rheumatoid arthritis and scleroderma in miners were described in the 1950s, and more formal cohort studies of miners and of granite workers were conducted in the 1980s. Other studies focusing on silicosis patients, and several case-control studies of these diseases and of Wegner

TABLE 25.2
Recent (1990–2005) Epidemiologic Studies of Occupational Silica Exposure and Risk of Autoimmune Diseases

Disease and Design	Sex (n)*	Exposure Classification and Results†	References
Systemic sclerosis			
Cohort	M (94; 243,900)	High exposure RR 7.8 (6.5, 9.5)	Mehlhorn[28]
Uranium miners	M (60; 12,400)	Silicosis RR 97 (75, 125)	
	M (34; 211,500)	High exposure, no silicosis RR 3.1 (2.2, 4.3)	
	M (3; 50,000)	Low exposure RR 1.2 (not significant)	
Silicosis patients	M (5; 1,130)	RR 37.0 (11.9, 86.3)	Brown[29]
Case-control	M (56, 56)	Possible or probable- job/task history OR 1.0 (0.13, 7.2)	Silman[30]
	M + F (21, 42)‡	Silica — job/exposure history OR 2.1 (0.34, 13.6)‡	Bovenzi[31]
	M + F (55, 171)‡	Silica — job/exposure history OR 1.7 (0.4, 7.6)‡	Bovenzi[32]
	F (274, 1184)	Silica — job/exposure history OR 1.5 (0.7, 2.9)	Burns[33]
	F (274, 1184)	Sculpting/pottery — job history OR 2.1 (1.1, 4.1)	Burns[33]
	M (160, 83)	Definite or probable — job/task history OR 3.9 (1.8, 8.5)	Englert[34]
	M (11, 22)	Ever — job/exposure history OR 3.6 (0.64, 20.4)	Diot[35]
	F (69,138)	Ever — job/exposure history OR 13.0 (1.5, 110)	Diot[35]
	M + F (80, 160)	Ever — job/exposure history OR 5.6 (1.7, 18.4) high (> 1) cumulative exposure score' 3.7 (1.1, 13.2)	Diot[35]
Rheumatoid arthritis			
Nested case-control (pottery workers)	M (43, 6353) F (15, 1972) M + F (58, 8325)	No association with duration, cumulative exposure', or mean silica concentration	Turner[36]
Silicosis patients	M (54; 1,130)	RR 8.1 (5.9, 10.9)	Brown[29]
	M (24, 463)	OR 2.7 (1.7, 4.1)	Rosenman[37]
Case-control	M (176, 630)	Mineral dust —job/exposure history OR 1.9 (0.8, 2.5) $p < 0.05$ for trend with duration; no association seen in females (339 cases, 627 controls)	Olsson[38]
	M (276, 276)	Rock drilling, stone crushing, stone dust — job/task history OR 2.2 (1.2, 3.9)	Stolt[39]

(*Continued*)

TABLE 25.2 *(Continued)*

Disease and Design	Sex (n)*	Exposure Classification and Results†	References
Systemic lupus erythematosus			
Cohort	M (28, 300,000)	Prevalence 10 times expected	Conrad[40]
Uranium miners			
Silicosis patients	M (8, 1130)	RR 23.8 (10.3, 47.0)	Brown[29]
Case-control	M (25, 34)	Job/task history — medium OR 3.0 (0.6-16.7); high OR 6.0 (0.7-48.0)	Parks[41]
	F (240, 321)	Job/task history — medium OR 2.7 (0.6-11.8); high OR 1.6 (0.8-3.0)	Parks[41]
	M + F (265, 355)	Job/task history — medium OR 1.7 (1.0-3.2); high OR 3.8 (1.2-11.6) $p < 0.05$ for trend across dose levels	Parks[41]
Wegener glomerulonephritis			
Case-control	M (16, 32)	Job/task history OR 14.0 (1.7, 113.8)	Gregorini[42]
	M + F (16, 32)	Job/task history OR 5.0 (1.4, 11.6)	Nuyts[43]
	M + F (65, 65)	Task history — OR 4.4 (1.4, 14.4)	Hogan[44]
	M + F (75, 273)	High —job/task history — ever OR 1.4 (0.7, 2.7); year before onset OR 3.0 (1.0, 8.4)	Lane[45]
	M + F (60, 120)	job/task history OR 3.4 (1.1, 9.9); dose-response across exposure score‘	Beaudreuil[46]

* for cohort studies, n = number of cases, total cohort size in cohort studies; for case-control studies, n = number of cases, number of controls. † Risk ratio (RR) or odds ratio (OR) and 95% confidence interval. ‡ Combining the two studies, the odds ratio was 2.4 for men, 1.5 for women, and 2.4 for the combined sample. ‘ based on probability, intensity, frequency and duration measures.

glomerulonephritis and small vessel vasculitis were published in the past 15 years. The clinical and epidemiologic studies published through 1999 were reviewed by Parks and colleagues,[27] and the recent studies (since 1990) are summarized in Table 25.2.[28–47] This collection of studies is striking in terms of the relative consistency and strength of the observed associations. Almost all point estimates of the risk ratio or odds ratio are at least 1.5, with most in the "medium" (2–4) or "strong" (5 or higher) range. Although there are fewer studies that include women, similar patterns of association were seen in analyses stratified by sex in a recent case-control study of systemic lupus eruthematosus.[41]

Silica is a strong adjuvant, leading to an increased production of the pro-inflammatory cytokines including tumor necrosis factor and interleukin-1.[27] Adjuvant properties do not necessarily account completely for the development of autoimmune disease, however, since other mechanisms are likely involved that result in the loss of self-tolerance. Silica exposure also leads to increased cell apoptosis and necrosis, which may result in increased exposure to immunogenic self-antigens. Recently a series of studies of silica exposure in lupus-prone mice were conducted. In the New Zealand mixed lupus mouse strain, silica exposure was shown to accelerate disease development (i.e., increased autoantibody production, immune complexes, proteinuria, and glomerulonephritis).[47,48] These studies provide insights into potential mechanisms through which silica exposure may affect the development of autoantibodies and autoimmune disease, including the recognition of specific epitopes on apoptotic macrophages by the autoantibodies in the exposed mice, and changes in CD4 T-cell counts and other T cells.

Issues regarding the influence of duration or intensity of exposure in relation to effect on autoimmune disease processes are questions that have not been established, with some inconsistencies seen in the epidemiologic studies (Table 25.2). Dose or intensity of silica exposure affects the clearance from the lung and silica-containing macrophages can be translocated to pulmonary lymph nodes. Increased production of immunoglobulins and of lymphocyte-derived interferon-gamma is seen at these sites.[49]

Another interesting issue with respect to silica exposure is the potential effect modification based on smoking history. The association between silica and systemic lupus erythematosus[41] and rheumatoid arthritis[39] was significantly higher among smokers than among non-smokers in two case-control studies. This effect could represent a reduced ability to clear small particles from the lung, or differences in localized or systemic immune responses.[41]

Solvents

Solvents are liquid compounds used extensively as degreasers and cleansers in a variety tasks and workplaces (e.g., cleaning metal, dry-cleaning establishments). Solvents include many different agents with different chemical properties, such as alcohols, glycols, aromatic hydrocarbons (e.g., benzene, toluene, xylene), and chlorinated products (e.g., carbon tetrachloride, trichloroethylene). Exposure to solvents occurs in occupational settings through inhalation and dermal routes. In the general population, exposure may occur from contaminated water and air pollution (in the case of volatile organic compounds) from industrial sources and from traffic. Solvents are metabolized

relatively quickly so biologic measurements in blood or urine reflect short-term rather than long-term or cumulative exposures.

One of the first links between solvents and autoimmune diseases was the recognition of the induction of a scleroderma-like disease by vinyl chloride. This disease is characterized by skin thickening, Raynaud's phenomenon, acroosteolysis (shortening of the terminal digital phalanges due to bone resorption), and pulmonary involvement.[50] Several epidemiologic studies pertaining to other solvents and scleroderma have been conducted. A meta-analysis of the studies published from 1990 to 1998 estimated a combined odds ratio of 2.9 (95% confidence interval, CI, 1.6, 5.3) for the general category of occupational exposure to solvents.[51] Five other case-control studies of solvents and scleroderma have been reported since 1999. These studies also reported associations with the broad classification of solvents, chlorinated solvents, paint thinners and removers, and mineral spirits, with most estimates 2.0 or higher.[32,35,52–54] Similar associations were also reported in a large population-based study of women with undifferentiated connective tissue disease,[55] and in studies of multiple sclerosis.[56-58] In contrast, associations were not seen between solvents and systemic lupus erythematosus[59] or rheumatoid arthritis,[38] although the data for these two diseases are limited. An increased prevalence of antinuclear antibodies was one of the immunologic parameters described in residents exposed to trichloroethylene-contaminated water in Woburn, Massachusetts.[60]

Exposure to trichloroethylene has been studied extensively in MRL +/+ mice. Accelerated disease expression is seen in mice exposed to trichloroethylene in drinking water or by intraperitoneal injection, as indicated by increased autoantibody and immunoglobulin production.[61,62] These changes involve activation and decreased apoptosis of CD4+ T cells, increased production of interferon–gamma and reduced levels of interleukin-4.[62-64] Similar effects were seen with exposure to highly reactive metabolites (e.g., trichloroacetaldehyde hydrate and trichloroacetic acid)[61,64,65] but the effects were not seen with the addition of diallyl sulfide, which blocks the cytochrome P450 CYP2E1 pathway through which trichloroethylene is metabolized.[66] These reactive compounds may induce lipid peroxidation, and oxidative stress has been hypothesized to affect severity and risk of renal damage in lupus.[67] It is not yet known whether similar effects are seen with other types of solvents, in other lupus-prone mice or in the rat model of experimental autoimmune encephalomyelitis (multiple sclerosis).

Pesticides

Pesticides can be classified by function (e.g., herbicide, insecticide, fungicide) or class (e.g., organophosphates, organochlorines). Pesticides do not generally target the immune system in targeting unwanted organisms. Most of the immunotoxicology research on pesticides focuses on immunosuppression and hypersensitivity effects rather than on autoimmunity,[68] and compared with neurologic or reproductive end-points, there have been relatively few epidemiologic studies of pesticide exposure in relation to autoimmune diseases. Epidemiologic studies of systemic lupus erythematosus and rheumatoid arthritis have not generally reported strong associations with pesticide use in general, or with serologic or questionnaire-based data pertaining to specific pesticide

exposures.[49,69,70] However, some studies of rheumatoid arthritis have reported stronger associations with farming,[38,70] and one study of systemic lupus erythematosus reported a strong association with mixing, but not applying, pesticides.[59]

Hexachlorobenzene is an organochlorine that was used previously as a fungicide, particularly on seeds of wheat and other grains. Although it has not been used as a pesticide in the United States since 1965, it is a persistent environmental pollutant and is also produced as an industrial byproduct in the manufacture of other pesticides and solvents. In Turkey, an accidental poisoning in the late 1950s through contaminated seeds resulted in severe liver and dermatologic damage (hepatic porphyria or porphyria turcica), particularly in children. Photosensitive cutaneous lesions, hyperpigmentation, hepatomegaly, splenomegaly, and painless arthritis were some of the long-term complications seen.[71] Some clinical and experimental data suggest immune-mediated, and possibly autoimmune-related aspects of the disease.[72,73]

Malathion is a widely used organophosphate insecticide. In 2000 to 2001, 20 to 25 million pounds were used, making it the sxith most commonly used pesticide in the United States.[74] Experimental studies of malathion exposure demonstrated accelerated expression of rheumatoid factor and anti-DNA antibodies and of glomerular disease (as evidence by proteinuria and inflamed glomeruli) in MRL-lpr mice.[75] Another feeding study in SJL/J mice reported increased IgM antibody production to sheep red blood cells with increasing doses of malathion, with effects seen even at the lowest dose (0.018 mg/kg) administered.[76] In a recent *in vitro* study of rat macrophages, TNF-α production was decreased in cells cultured with malathion.[77] Malathion may act through alterations in macrophage phagocytosis or other aspects of macrophage activity, but there are some inconsistencies in the available studies.[78,79]

Some pesticides are endocrine-disruptors, with potential effects on steroidal hormones (estrogens, androgens, and progesterone), gonadotropin hormones and thyroid hormones.[80] Sobel and colleagues examined the effect of three organochlorine pesticides (*o,p'* – dichlorodiphenyl-trichloroethane (*o,p'* – DDT), methoxychlor, and chlordecone) in ovariectomized female (NZB × NZM)F_1 mice. This lupus mouse model develops renal disease and anti-double stranded DNA antibodies.[81] Acceleration of the disease process (time to renal damage) was seen with each chemical, but was strongest for chlordecone. Autoantibody production was increased with chlordecone exposure. These effects were not related to the estrogenic effect of the pesticides as measured by uterine weight, suggesting that research on mechanisms through which a pesticide may influence autoimmune disease should not be limited to estrogen disruption.

Ultraviolet Radiation (and Vitamin D)

One of the primary reasons ultraviolet radiation is of interest in the context of autoimmune diseases is because of induction of photosensitive cutaneous lesions (cutaneous lupus erythematosus)[82] and dermatomyositis.[83] A recent global evaluation of the frequency of dermatomyositis and associated autoantibodies in referral centers around the world showed a positive correlation with the intensity of ultraviolet irradiation at those locations.[84]

Ultraviolet radiation also has potential immunosuppressive effects, which may influence the risk for other (non-cutaneous) diseases. This effect is mediated in part through the production of 1,25-hydroxycholecalciferol [1,25-$(OH)_2D_3$], the active form of vitamin D. The vitamin D receptor has been detected in lymphocytes and the thymus, and vitamin D plays a role in T-cell mediated immune response.[85] Higher risks of type 1 diabetes and multiple sclerosis are generally seen at higher latitudes (e.g., further north in the northern hemisphere and further south in the southern hemisphere), which would be areas of relatively low exposure to ultraviolet radiation.[86] Case-control and prospective studies of vitamin D intake have provided some evidence of a protective effect of vitamin D on risk of developing type 1 diabetes.[87] Prospective studies have also reported a reduced risk of multiple sclerosis[88] and rheumatoid arthritis[89] with higher intakes of vitamin D. In murine models of experimental autoimmune encephalomyelitis (multiple sclerosis), type 1 diabetes, and inflammatory bowel disease, treatment with 1,25-$(OH)_2D_3$ in conjunction with adequate calcium intake inhibits the development of disease.[90–92] In lupus mouse strains, however, there is some evidence of worsening of disease (particularly with respect to measures of renal damage) with 1,25-(OH)2D3 treatment.[93]

Tobacco Use

Smoking has been strongly associated with several types of cancer (e.g., lung, bladder, cervical) and cardiovascular disease for more than 40 years. More recent studies have focused on tobacco use in relation to organ-specific and systemic autoimmune diseases, and to the prevalence of various autoantibodies in the non-diseased population. Smoking is anti-inflammatory in some respects, resulting in impaired secretion of pro-inflammatory cytokines and decreased activity of natural killer cells.[94] Other immune-modulating effects may also occur since the effects of smoking differ considerably among the different autoimmune diseases. However, there has been relatively little research pertaining to specific mechanisms through which tobacco use may affect specific autoimmune diseases.

A meta-analysis of 25 studies of autoimmune thyroid diseases[95] indicated that current smoking was associated with Grave's disease (hyperthyroidism) (odds ratio, OR, 3.3, 95% CI, 2.1, 5.2) for current smokers, with a weaker association (OR 1.9, 95% CI 1.4, 2.6) seen among former smokers and between ever smoking and Hashimoto's thyroiditis (hypothyroidism) (OR 1.9, 95% CI 1.4, 2.6). The strongest associations were seen with Grave's disease with ophthalmopathy. However, a recent analysis using the National Health and Nutrition Examination Survey III data, a representative sample of the United States population, reported that smokers had a lower prevalence of anti-thyroperoxidase antibodies and anti-thyroglobulin antibodies. The distribution of thyroid stimulating hormone levels was shifted (reduced), resulting in a lower prevalence of elevated thyroid stimulating hormone levels (hypothyroidism) and an increased prevalence of mild hyperthyroidism (thyroid stimulating hormone levels < 0.4 mU/liter) in smokers compared with non-smokers.[96]

There are fairly consistent data showing associations between smoking history

and rheumatoid arthritis, although this may be limited to rheumatoid factor-positive patients.[97,98] Smoking has also been associated with the production of rheumatoid factor in the general (non-diseased) population.[99] Padyoko and colleagues observed an interaction between the HLA-DRB1 genotype and smoking among rheumatoid-factor-positive rheumatoid arthritis cases in Sweden (odds ratio 7.5 for this combination of genes and exposure, compared with an odds ratio of 2.4 for smoking in the absence of this genotype.[100] In contrast, the association seen between smoking history and systemic lupus erythematosus is weaker than that seen with rheumatoid arthritis.[101] Three prospective studies examined risk of multiple sclerosis in relation to smoking history, and weak associations (odds ratios between 1.5 and 2.0) were seen in each of these studies.[102-104]

Ulcerative colitis and Crohn's disease are interesting in that the effects of smoking seem to be quite different in these diseases. In Crohn's disease, an increased risk among current and former smokers has been reported.[105,106] In contrast, a reduced risk of ulcerative colitis, and reduced severity of disease, is seen among current smokers compared with never smokers, and there is some evidence of a dose-response with amount smoked (greater reduction in risk with increased cigarettes per day). This pattern is not seen among former smokers, however. Type 1 (autoimmune) diabetes is another disease in which an inverse association has been reported between smoking and risk in adults[107]and between nicotine exposure and risk in mice.[108]

SUMMARY AND FUTURE DIRECTIONS

This chapter reviewed current research pertaining to selected environmental agents and autoimmune diseases (Table 25.3). Other infectious agents (e.g., parvovirus, varicella), occupational exposures (e.g., mercury), dietary factors (dietary supplements, nutrients such as antioxidants, and specific proteins in wheat and other grains implicated in celiac disease), and stress have been the focus of additional research that was not included in this review.

There are clear gaps in our understanding of the role of specific agents in the development of autoreactive cells, and in the progression to clinical disease that could be addressed by research in immunotoxicology (Table 25.3). Although there are examples of exposures that appear to have similar effects across several diseases (e.g., silica), for other exposures the effects differ markedly between diseases (e.g., smoking and ultraviolet radiation). Mechanistic research pertaining to specific exposures that have been the focus of epidemiologic studies is quite limited, and few epidemiologic studies have focused on the specific agents within some broad categories of exposures (e.g., solvents or pesticides) which immunotoxicology research suggest may affect autoimmunity and other aspects of immune-modulation. Understanding the etiology of autoimmune diseases will also require research on genetic susceptibility in conjunction with environmental exposures, an area of study that is just beginning. Despite the resources required, the many experimental and clinical approaches outlined above are likely to be highly cost-effective in defining more accurately the genetic and environmental risk factors for autoimmune disorders and in ultimately leading to preventative strategies for these increasingly recognized and costly diseases.

TABLE 25.3
Summary of Research on Specific Environmental Exposures and Autoimmune Diseases

Exposure	Summary of Current Evidence
Infectious agents	Strong mechanistic evidence from rodent models of autoimmune disease of viral or other infectious agents affecting autoimmunity or progression to overt disease, but harder to demonstrate in humans. Enterovirus (Coxsackie virus) focus of epidemiologic studies in type 1 diabetes, Epstein-Barr virus focus of epidemiologic studies in multiple sclerosis and systemic lupus erythematosus.
Silica	Many epidemiologic studies of scleroderma, lupus, rheumatoid arthritis with fairly consistent and strong associations seen; adjuvant- pro-inflammatory properties. Limited mechanistic research in MRL +/+ lupus mice
Solvents	Several epidemiologic studies of scleroderma, undifferentiated connective tissue disease, and multiple sclerosis suggest modest associations with "any" solvents or with organic or chlorinated solvents; Trichloroethylene, paint removers, and mineral spirits are some of the specific solvents implicated in these studies. Mechanistic research in MRL +/+ lupus mice.
Pesticides	Few epidemiologic studies of pesticide use in general, or specific pesticides, in relation to any autoimmune disease. Mechanistic research primarily for hexachlorobenzene and malathion. Mechanisms other than endocrine-disruption should be considered, even for pesticides with endocrine-disrupting properties.
Ultraviolet radiation	Positive association with frequency of dermatomyositis in one study; inverse association with risk of diabetes and MS. Inhibition of disease in rodent models of multiple sclerosis, type 1 diabetes and inflammatory bowel disease but acceleration of disease in lupus-prone mice.
Tobacco	Different effects seen among diseases; increased risk seen in hyperthyroidism, rheumatoid arthritis, and Crohn's disease but reduced risks seen in ulcerative colitis and possibly adult-onset type 1 diabetes. Limited mechanistic research.

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26 Drug-Induced Autoimmune Disease

Jack P. Uetrecht

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INTRODUCTION

Drug-induced hypersensitivity is a major clinical problem, and it also significantly increases the uncertainty of drug development. Between the years 1975 and 2000, over 10% of newly approved drugs either had to be withdrawn from the market or achieved "Black Box" warnings because of adverse reactions that were not predicted by preclinical testing and clinical trials [1].

Drug-induced autoimmunity is one type of drug hypersensitivity reaction. It is usually characterized by autoantibodies; however, autoreactive T cells are likely to be equally important, but it is more difficult to demonstrate their pathogenic role. The classic type of drug-induced autoimmunity is a lupus-like syndrome, which is characterized by antinuclear antibodies (ANA), and various clinical manifestations of autoimmunity, such as arthralgias, glomerular deposits of immune complexes, pleurisy, etc. [2, 3]. Pathogenic autoantibodies can also be responsible for many types of drug hypersensitivity reactions that target a specific cell or organ (e.g., hemolytic anemia). In addition, idiosyncratic drug reactions, such as halothane-induced hepatitis, are often associated with both drug-specific and autoantibodies, but it is less clear what role

the autoantibodies play in such reactions. It remains to be determined to what degree autoimmunity, especially autoreactive T cells, contributes to the pathogenesis of other types of idiosyncratic drug reactions.

CLINICAL SYNDROMES

Drug-Induced Lupus-Like Reactions

Systemic lupus erythematosus (SLE), or simply lupus, is a systemic autoimmune disease of unknown etiology. The diagnosis is made on the basis of the presence of a combination of symptoms and autoantibodies [4]. Common symptoms include rash, especially a malar rash, arthralgias/arthritis, dry eyes, pleuritis, and various central nervous system-related symptoms. Laboratory findings commonly include lymphopenia, proteinuria, and immune complexes. Almost all patients with SLE have ANA of various specificities. Although most lupus is idiopathic, it has been estimated that about 10% is caused by drugs [2, 3]. Although in a specific case, drug-induced lupus is clinically indistinguishable from idiopathic lupus, it is usually less severe. The diagnosis of drug-induced lupus is based on the same criteria as SLE, and in addition, it must have started after beginning a drug and it usually resolves when the drug is discontinued. The lag time between starting drug treatment and the onset of symptoms is longer than for most other drug hypersensitivity reactions: usually at least a month and often more than a year. Although the symptoms of lupus usually resolve rapidly upon discontinuation of the offending drug, ANA can persist with diminishing titer for years. Drugs that can cause lupus often induce ANA, without any apparent clinical effects, at a much higher incidence than they induce a lupus-like syndrome [5], and autoantibodies in the absence of clinical manifestations of autoimmunity should not be considered lupus.

There are many drugs that have been associated with drug-induced lupus [6]. The drug associated with the highest incidence is procainamide; with chronic therapy at high dose the incidence approaches 20% and the incidence of ANA is almost 90% [5]. Procainamide is also associated with a relatively high incidence of drug-induced agranulocytosis [7], but it is not known whether this is autoimmune in nature. Although there are few other drugs that contain a primary aromatic amine functional group, most, such as sulfonamide antibiotics and nomifensine, are also commonly associated with drug-induced lupus [2]. The exception is dapsone. The recognition that minocycline is associated with a lupus-like syndrome is more recent, which is surprising because it is a relatively old drug [8, 9]. Minocycline is the only tetracycline with a tertiary amine *para* to a hydroxy group, which makes it possible to oxidize this tetracycline to a reactive quinone iminium ion [10]. Minocycline is also associated with a relatively high incidence of liver toxicity [11] and a variety of drug-induced autoimmune syndromes [12, 13].

Another chemical class of drugs associated with inducing a lupus is the hydrazines. As with aromatic amines, there are few examples because most hydrazines are toxic. Hydralazine is second only to procainamide with respect to the incidence of lupus associated with its use [14]. Although its present use is infrequent, the finding that it is

especially effective for treatment of hypertension in blacks may lead to an increase in its use [15]. The other major drug that contains a hydrazine group is isoniazid, which is also associated with a significant incidence of lupus [16]. The major serious adverse reaction associated with isoniazid is liver toxicity [17]; it is unknown to what degree autoimmunity may play a role in this adverse reaction.

Thiono and sulfhydryl drugs are also associated with a significant incidence of a lupus-like syndrome. Propylthiouracil is associated with a significant incidence of lupus [18] as well as liver toxicity [19, 20] and agranulocytosis [21]. Penicillamine is associated with lupus, agranulocytosis [22] and a variety of autoimmune syndromes as discussed later.

A more recent addition to the list of drugs that have been associated with a lupus-like syndrome is terbinafine [23, 24]. This drug is also metabolized to a reactive metabolite [25], and it has a very long half-life in the skin, which likely explains why the dominant manifestations of lupus associated with this drug are in the skin.

A new class of drugs that is associated with autoimmunity, including a lupus-like syndrome, is cytokines (e.g., IFN-α [26, 27]) and antibodies against cytokines (e.g., anti-TNFα, [28, 29]) that are used to treat immune-mediated diseases such as rheumatoid arthritis and Crohn's disease. Presumably, the mechanism by which these agents induce a lupus-like syndrome involves direct dysregulation of the immune system and is likely different than the mechanism by which small molecules induce autoimmunity. Many chronic illnesses are likely directly or indirectly mediated by the immune system, and immune-modifying drugs used to treat such illnesses are likely to make a larger contribution to the incidence of drug-induced autoimmunity in the future.

Autoimmune Hemolytic Anemia

Although it is seldom used anymore, the classic drug associated with autoimmune hemolytic anemia is α-methyldopa [30]. Between 10 to 20% of patients treated with α-methyldopa developed autoantibodies that, in most cases, were autoantibodies directed against an epitope in the Rh complex that do not bind to Rh negative cells [31]. Only a fraction of patients actually developed clinically important hemolytic anemia; however, in some cases the anemia was life-threatening. Although I do not know of any studies that have identified reactive metabolites of this drug, it is easy to speculate that it would be oxidized to an ortho-quinone and/or quinone-methide. It is known that α-methyldopa binds tightly to red cells and the binding is promoted by oxidants and inhibited by reducing agents [32]. Other catecholamines could be oxidized to similar reactive species, but the dose of α-methyldopa is quite high. Levodopa can also cause autoimmune hemolytic anemia, but it is less common and may resolve with a decrease in dose [33].

In contrast to α-methyldopa-induced hemolytic anemia, most drug-induced hemolytic anemia, especially for penicillins [34] and cephalosporins [35], involves drug-dependent antibodies, presumably because the drug acts as a hapten to directly modify erythrocytes or form immune complexes [36]. However, there are many examples where a drug, such as nomifensine, induces both drug- (or metabolite)-dependent and drug-

independent antibodies, some with a specificity similar to that induced by α-methyldopa [37]. The high incidence of nomifensine-induced hemolytic anemia ultimately led to its withdrawal. This drug is a primary aromatic amine, which would likely be oxidized to reactive metabolites; however, as with α-methyldopa, I do not believe that this has been demonstrated, nor do I believe that there have been attempts to use reactive metabolites as opposed to stable metabolites to test antibody specificity.

Another drug that is associated with a relatively high incidence of both drug-dependent and autoimmune antibodies is diclofenac [38]. In some cases the specificity of the diclofenac-induced autoantibodies is very similar to that induced by α-methyldopa [39]. Diclofenac is a secondary aromatic amine and is oxidized to reactive metabolites by both cytochromes P450 and myeloperoxidase [40]. When patient sera were tested, it was addition of the 4-hydroxy metabolite that most commonly led to agglutination of red cells [41]; this metabolite has the potential to be air oxidized to a reactive iminoquinone.

Autoimmune Thrombocytopenia

Many drugs, especially quinidine and heparin, induce antibodies leading to thrombocytopenia. In most cases the antibodies are drug-dependent; however, there are many examples in which the antibodies are autoimmune in nature [42], even for drugs, such as quinidine that are classically associated with drug-dependent antibodies [43]. Gold therapy, in particular, is associated with autoimmune-thrombocytopenia [44].

Another type of autoantibody leading to thrombocytopenia that is induced by ticlopidine is directed against von Willebrand factor metalloproteinase [45]. This prevents the normal clearance of large multimers of von Willebrand; this can lead to platelet aggregation, and in some cases, to thrombotic thrombocytopenic purpura, commonly referred to as TTP. Many other drugs have been implicated as cases of TTP, but the mechanism is unclear and appears to differ with different drugs [46].

Drug-Induced Pemphigus

Pemphigus is a serious autoimmune disorder of the skin leading to blisters. It is characterized by IgG on the surface of keratinocytes, and the autoantibodies are usually directed against desmoglein 1 and/or desmoglein 3 [47]. It can either be idiopathic or drug-induced, and it has not been possible to differentiate the two histologically [48]. Therefore, as with lupus, the diagnosis of drug-induced pemphigus is based on drug history with resolution when the drug is discontinued. By far the major drug associated with pemphigus is penicillamine, but other sulfhydryl-containing drugs, penicillin, pyrazolones, and cytokines, such as interferon-α have also been implicated [49].

Autoimmune Liver Toxicity

In the previous examples of drug-induced autoimmunity there is little question that the reactions were immune-mediated. For example, it is easy to demonstrate that the

autoantibodies induced by α-methyldopa cause aggregation of red cells, and although most patients who have such antibodies do not have clinically evident anemia, few would question that these antibodies are responsible for anemia. The hepatotoxicity induced by several drugs, including halothane, tienilic acid, and dihydralazine, is also associated with autoantibodies, but it is not clear that these antibodies actually mediate the toxicity, and some have questioned whether this toxicity is really immune-mediated. Although few immune responses are limited to a pure cellular response or antibody response, the type of reactive metabolite associated with drugs such as tienilic acid binds almost exclusively to intracellular proteins (vide infra), and would therefore be expected to be presented in the context of MHC-I and induce a cell-mediated immune response rather than an antibody response. However, the cytochrome P450's involved have been found to be transported to the plasma membrane [50, 51]; therefore, it is quite possible that the autoantibodies do participate in the cell injury caused by these drugs. Irrespective as to whether the antibodies are pathogenic, the clinical features of these adverse reactions strongly suggest that this type of hepatotoxicity is immune-mediated. For example, halothane hepatitis rarely occurs on first exposure, presumably because halothane exposure is always limited to a relatively short period and so prior sensitization is required to produce a strong immune response [52].

Tienilic acid- and dihydralazine-induced hepatitis are associated with antibodies against Cyp 2C9 [53] and Cyp 1A2 [54, 55], respectively. These are also the same cytochrome P450s that are responsible for the formation of reactive metabolites of these two drugs. Anticonvulsant hepatotoxicity is associated with antibodies against rodent Cyp 3A and related human enzymes such as thromboxane synthase [56, 57]. It is interesting to note that cytochromes P450 are often the target of autoantibodies in idiopathic autoimmune hepatitis [58].

A classic drug associated with hepatotoxicity is halothane. It is associated with antibodies against trifluoroacetylated protein due to the binding of the reactive metabolite of halothane [59, 60]. However, in addition, it is also associated with autoantibodies, such as antibodies against Cyp 2E1, the major P450 responsible for oxidation of halothane [61].

Minocycline is associated with a relatively high incidence of hepatotoxicity. In many cases it is quite distinct from minocycline-induced lupus, occurs earlier in the course of treatment (about 1 month), and the mechanism is unknown [62]. However, in some cases the liver toxicity merges with the lupus-like syndrome, occurring after about a year of therapy, and is associated with ANA. This form is indistinguishable from idiopathic autoimmune hepatitis [63], and antibodies against Cyp 3A6 and Cyp 2C4 have been reported [64]. Diclofenac has also been reported to cause hepatitis with autoimmune features such as ANA [65].

Miscellaneous

Drugs can cause a wide variety of other autoimmune reactions. One example is myasthenia gravis, which is characterized by muscle weakness and is mediated by antibodies against the acetylcholine receptor at the neuromuscular junction. It has been reported in association with penicillamine [66], gold salts [67], and procainamide [68]. Another form of drug-induced autoimmunity is polymyositis, which is an autoimmune disease

involving muscle and can also be caused by penicillamine [69]. Propylthiouracil has been reported to cause autoimmune vasculitis leading to Wegener's granulomatosis, respiratory distress syndrome, and pericarditis [70–72]. Other drugs, such as minocycline, penicillamine, hydralazine, and allopurinol can also cause other forms of vasculitis, such as polyarteritis nodosa, which are associated with antineutrophil antibodies [73–75]. Anti-TNFα antibody therapy has led to a demyelinating syndrome resembling multiple sclerosis, which is an autoimmune disease, that resolved when the drug was stopped [76].

The anticonvulsant phenytoin, and to a lesser degree carbamazepine, can inhibit the synthesis of antibodies, and in some cases these drugs can result in lymphoproliferation [77–79]. These effects on the immune system could be viewed as a type of autoimmunity. The relationship between such effects and autoimmunity are still not clear, although the more recent observations that cytokines and anti-cytokines can cause autoimmunity support the existence of such a relationship. The previous edition of this book contained an extensive discussion of the possible relationship between immunosuppression and autoimmunity [80]. Phenytoin and carbamazepine can also cause a lupus-like syndrome although the incidence is lower than with many other drugs.

POSSIBLE MECHANISMS

There are many hypotheses for the mechanism of drug-induced autoimmunity, but none have been clearly demonstrated. It is unlikely that any one mechanism will explain all drug-induced autoimmunity. Table 26.1 lists some of the drugs most commonly associated with autoimmunity and their spectra of toxicity. There are some similarities but each drug also has its own pattern of toxicity.

Most of the drugs in Table 26.1 form reactive metabolites [2]. I have included agranulocytosis in the table even though there is no good evidence that it is an autoimmune reaction because there is some correlation between oxidation of a drug to a reactive metabolite by neutrophils/monocytes and their ability to cause agranulocytosis. However, there are certainly drugs, such as hydralazine, that are oxidized to reactive metabolites by neutrophils and yet are not associated with agranulocytosis [81]. Even the gold in gold salts is oxidized by the hypochlorous acid generated by neutrophils/monocytes to a higher oxidation state, which is chemically reactive [82]. There is a large amount of circumstantial evidence that many hypersensitivity drug reactions, including drug-induced autoimmune reactions, are caused by reactive metabolites of drugs rather than by the drug itself [83, 84]; however it is not clear what role the reactive metabolites play. On the other hand, autoimmune reactions due to immune modulators, such as infleximab, presumably involve a more "pharmacological mechanism" and some of the autoimmune reactions due to small molecules may also be mediated by pharmacological mechanisms rather than reactive metabolites.

COVALENT BINDING AND THE HAPTEN HYPOTHESIS

A classic mechanism by which some reactive metabolites are believed to mediate hypersensitivity reactions is by acting as a hapten (i.e., the reactive metabolite binds to

TABLE 26.1
Patterns of Adverse Reactions of Drugs Commonly Associated with Autoimmunity

	Lupus	Hemolysis/ Thrombocytopenia	Agranulocytosis	Hepatitis	Misc.
Procainamide	++	+	++	±	+
Sulfamethoxazole	+	+	+	+	+
Hydralazine	++	±	–	±	–
Isoniazid	+	–	–	++	–
α–Methyldopa	+	++	+	++	+
Minocycline	++	–	–	++	+
Diclofenac	–	+	+	+	+
Phenytoin	+	±	+	+	+
Carbamazepine	+	±	+	+	+
Penicillamine	+	+	+	+	++
Propylthiouracil	++	±	++	++	+
Ticlopidine	+	++	++	+	–
Gold Salts	+	++	++	+	+
ß-lactams	±	++	+	+	+
Interferon-α & ß	+	+	+	±	++
Anti-TNFα	+	–	–	–	+

Note: This table is not meant to be quantitative and is based on a general knowledge of these drugs. It is very difficult to determine incidence, and it can also be difficult to determine causality, especially with most case reports.

protein or other macromolecules making the protein "foreign" and leading to an immune response [85]). In general, this leads to an immune response that is focused on the hapten; however, there are mechanisms by which this reaction may lead to an autoimmune response. One mechanism may be through cross-reactivity; specifically, the immune response is polyclonal and invariably involves portions of the protein to which the hapten is bound [86, 87]. A related concept is molecular mimicry in which a drug-modified protein "looks" like some other self protein, which may lead to breaking tolerance to the self molecule. Alternatively, if a drug-modified protein "looks" like a molecule to which the immune system is already sensitive to, such as a pathogen, it could also lead to an immune response. However, despite the attractiveness of the molecular mimicry hypothesis, there is no clear evidence to support it even in the classic case of rheumatic fever [88]. An alternative mechanism by which binding of a reactive metabolite may lead to an autoimmune response is through a change in processing of antigen leading to the presentation of cryptic antigens [89]. Binding of a reactive metabolite may lead to a change in conformation of the protein, an increased rate of ubiquitination and a change in the major sites of cleavage into peptides when the protein is processed. This change in pattern produces peptides to which tolerance has not been induced and can lead to an autoimmune response.

There are several good examples were binding of a reactive metabolite to a protein leads to antibodies against that protein as mentioned above. Halothane can cause hepatitis and it is metabolized by P450 to a reactive trifluoroacetyl chloride. This reactive metabolite binds to many proteins and induces an immune response against many proteins [90]. Some of the antibodies that are generated recognize hapten-modified proteins, but others recognize native proteins. Also as mentioned before, the reactive metabolites of tienilic acid and dihydralazine also lead to autoantibodies against the enzyme that produced the reactive metabolite. These observations support the concept that reactive metabolites acting as haptens can lead to autoantibodies through cross-reactivity or generation of cryptic antigens. However, I should stress again that there is no evidence that these antibodies are pathogenic.

As noted above, most of the drugs that have been associated with a relatively high incidence of a drug-induced lupus-like syndrome are metabolized to reactive metabolites by neutrophils and monocytes, which is mediated by the enzyme myeloperoxidase [91]. Drug-induced lupus and other forms of autoimmunity are also often associated with an autoantibody referred to as pANCA (perinuclear antineutrophil cytoplasmic antibody) [13, 70–72, 92]. A principle target of the pANCA antibody is myeloperoxidase. This may be another example of a reactive metabolite binding to a protein and leading to autoantibodies against that protein.

Danger Hypothesis

Polly Matzinger has questioned the concept that the immune system responds to "foreignness" and proposes that instead an organism must respond to danger [93]. Reactive metabolites can cause cell damage and cell stress in several different ways, and in this way may stimulate an immune response [94, 95]. One intriguing aspect of drug-induced autoimmunity is that when the drug is discontinued, the adverse reaction generally resolves rapidly even though, by definition, the reaction is autoimmune and so the antigen is still present. One possible explanation for this observation is that the drug is acting as a source of reactive metabolite, which in turn, is acting as a danger signal. When the danger signal is gone, the autoimmunity resolves.

Interference with Tolerance

Autoimmunity can be viewed as a failure of tolerance [96]. The observation that most of the drugs that induce autoimmunity are oxidized to reactive metabolites by monocytes suggests that these reactive metabolites may activate these cells, which could lead to a loss of tolerance either directly or by modification of MHC-II, thus leading to a graft-versus-host reaction [91]. Penicillamine and hydrazines (i.e., hydralazine and isoniazid) may also interfere with tolerance by binding to aldehydes on antigen presenting cells that are involved in signaling with T cells through Schiff base formation and this, in turn, could lead to activation of the antigen presenting cells [97].

Rubin found that injection of the hydroxylamine of procainamide, a reactive

metabolite, into the thymus interfered with central tolerance and led to autoimmunity with autoantibodies similar to those observed in procainamide-induced lupus [98, 99]. Although this is an attractive hypothesis, the local concentration of this reactive metabolite achieved by injection is very high relative to anything that might occur in vivo and would likely cause direct cytotoxicity. Such direct damage to thymocytes could interfere with tolerance in ways that would not occur under usual conditions of procainamide treatment.

Richardson found that procainamide and hydralazine inhibit DNA methylation, although the mechanism is somewhat different for these two drugs [100]. DNA methylation is an important mechanism for the control of gene expression. It was also found that if T cells were treated with procainamide or hydralazine it led to increased expression of LFA-1. When these cells were injected into a syngeneic animal, it led to the induction of autoantibodies and other manifestations of autoimmunity [101]. DNA methylation is also impaired in idiopathic lupus [102]. These observations provide a compelling argument for inhibition of DNA methylation playing a role in the lupus-like syndrome induced by these two drugs. However, it seems unlikely that this is the mechanism for all drug-induced lupus or even that it is the whole story for these two drugs, and treatment of animals with these two drugs does not, in general, induce a lupus-like syndrome.

An important animal model of lupus is the lpr mouse. It was discovered that the abnormality leading to autoimmunity in this model is a defect in the gene coding for Fas protein and this leads to impaired apoptosis [103]. This, in turn, leads to lymphadenopathy and prevents elimination of autoimmune T cells, thus interfering with tolerance. Minocycline inhibits apoptosis, and it has been postulated that this contributes to the mechanism of minocycline-induced lupus [104].

Other Pharmacologic Mechanisms Leading to Autoimmunity

Interferons alpha and beta have major effects on the immune system, such as increasing the expression of MHC-I and MHC-II. Interferon-α is elevated in patients with idiopathic lupus and it is believed to be involved in its pathogenesis [105]; therefore, it is not surprising that therapy with these interferons can lead to autoimmunity.

It is less clear how agents such as infiximab lead to autoimmunity. One possibility is that inhibition of TNF-α may shift the Th1/Th2 balance toward a Th2 response and increase the production of autoantibodies. It may also lead to the production of immune complexes or exposure to previously hidden antigens [106].

CONCLUSIONS

The immune system is a very complex system with many redundant control systems. One hint of this complexity is that idiopathic lupus has a high genetic component, and there are 48 chromosomal regions that have been linked to the risk of idiopathic lupus; yet, the child of a person with lupus only has a 5% chance of developing lupus [107].

Thus, environmental factors must also play an important role. With this complexity it is unlikely that there is a single mechanism of drug-induced autoimmunity and, even with one drug, several factors probably contribute to the mechanism of drug-induced autoimmunity. Although there may be common patterns, as hinted at by Table 26.1, certainly the contributing factors are likely different for different drugs. Although the classic drugs associated with the highest incidence of drug-induced autoimmunity are infrequently used at the present time, new drugs have taken their place and this is a problem that is not likely to go away.

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27 Experimental Models of Autoimmunity

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INTRODUCTION

Immunostimulatory chemicals may pose a threat to human health, because such chemicals, which include a variety of drugs and environmental pollutants, may induce allergic or autoimmunogenic responses in susceptible individuals. One important goal of current immunotoxicology is to develop models or design strategies to assess the hazard of chemicals to induce allergic or autoimmune phenomena.

It is important to note that, particularly with respect to xenobiotics, allergy and autoimmunity are flip sides of the same coin, in that stimulated activity may be directed against self-specific as well as chemical-specific components [1]. In addition, pathologic

self-specific responses following exposure to xenobiotics may be a reflection of the fact that normal healthy individuals possess a substantial number of auto-reactive lymphocytes and antibodies [2,3].

Development of models to assess chemical-induced allergic or autoimmune reactions is difficult in that both types of reactions are subject to complex processes, and are idiosyncratic in nature. Factors that must be considered include a large number of genetic as well as phenotypic, neuroendocrine, or environmental factors that are only in part related to the immune system.

In general, the number of animal models of autoimmunity is quite extensive. These models represent a variety of systemic and organ-specific diseases, and are mostly used to study the etiology of certain autoimmune diseases and to explore therapeutic possibilities. Models can be based either on spontaneous (e.g., genetically predisposed) development of disease or on induction by self-specific or microbial antigens (typically in combination with an adjuvant) or chemicals. But, irrespective of how the disease is induced, all models rely on inbred animals, indicating the importance of genetic background and in accordance with genetic predisposition as one of the factors that relate to the idiosyncratic nature of autoimmune diseases.

In the case of spontaneous autoimmune diseases mice are the most frequently used animal model. With the advent of transgenic and genetically modified (knockout, KO) mice, the number of genetically predisposed autoimmune models has substantially increased. Other species that have been useful include rats, monkeys, cats, dogs, rabbits, and chickens for some specific forms of autoimmune diseases [4, 5].

Chemical-induced autoimmune and allergy models are actually rare (Table 27.1), which is not so surprising considering the idiosyncratic nature of the phenomena. In this chapter, a number of models currently available to assess immunostimulating (allergenic or autoimmunogenic) potential of xenobiotics will be presented.

RAT MODELS

The Brown Norway Rat Model

Of all rat strains, the Brown Norway (BN) rat is used most frequently in relation to chemical-induced autoimmunity. This strain displays clinically manifested autoimmune disease following exposure to a number of chemicals. $HgCl_2$ is the most scrutinized compound in the BN rat but D-penicillamine [6–8], gold-salts [9,10], hexachlorobenzene (HCB) [11–14] and recently, nevirapine [15], have all been shown to induce clinical effects. Captopril [7] and felbamate [16] appeared not to induce autoimmune effects in BN rats.

Pathological changes reported to occur in the BN rat are not unique to any one compound and include both organ-specific (glomerulonephritis, splenomegaly, skin rashes) and systemic effects (hyperimmunoglobulinemia, in particular IgE, and increased levels of autoantibodies) and is accompanied by polyclonal T and B cell lymphoproliferation [17, 18].

TABLE 27.1
Noncomprehensive Overview of Chemical-Induced Autoimmune Phenomena in Test Animals

	Compound	Effects
Rat		
Brown Norway rat	$HgCl_2$ AU-salts D-Penicillamine Nevirapine	IC-glomerulonephritis Skin pathology, dermatitis Polyclonal IgE AutoAb (Type IV-collagen, ANA, anti-ACh, thyroglobulin)
	HCB	Systemic inflamatory response with autoimmune symptoms
Lewis	CyA	Alopecia Graft vs. Host
BioBreeding (BB)	Iodine	Autoimmune thryoiditis
Mouse		
A/J	Procainamide	ANA
BALB/cJ	Procainamide	Splenomegaly
B10s, BALB/c, SJL	$HgCl_2$ Gold salts	Anti-fibrillarin, ANA
A.SW, C3H/HeJ, C57BL/Ks	D-Pencillamine	ANA, anti-insulin
C57BL/Ks	STZ	Type 1 Diabetes
BALB/c,	Hydralazine	Splenomegaly
A/J	Hydralazine	Smooth muscle antibodies
BALB/c	Pristane	AA
Guinea pigs	D-Penicillamine	Myositis, myasthenia gravis
Cat	6-propylthiouracil	SLE: ANA, Sm antigen Lymphadenopathy, Hemolytic anemia
Dog		
Beagle	Procainamide	ANA
Doberman	Sulfonamides	Blood dyscrasias, skin pathology Hepatopathy, fever
Chickens		
OS	Iodine	Autoimmune thyroditis
Monkey	L-canavanine (alfa-alfa seeds)	SLE, ANA

BN rats are known as so-called Th2-prone animals; that is, they demonstrate many characteristics of a type 2 immune response, including relatively high levels of IgE. Th2-dominated responses and underlying genetic traits are thought to be responsible for the high susceptibility of this strain to chemical-induced autoimmune effects. This susceptibility is often cited as a drawback of using this strain. However, as is the case with disease-prone mouse strains, it can also be argued that the inherent susceptibility of the BN rat resembles the inherent susceptibility in human cases of chemical-induced autoimmune disorders.

Metals

The most obvious clinical effect of $HgCl_2$ exposure is autoimmune glomerulonephritis, including proteinuria and serum antibodies to glomerular basement membrane, which can be induced by multiple subcutaneous injections in susceptible strains of animals such as the BN rat. Gold salts (as gold chloride, s.c. for 10 days, about 10 mg/kg body weight) [10] also induce an autoimmune syndrome in the BN rat that is similar, although less intense, to that observed with $HgCl_2$, with increased IgE levels and vasculitis in the gut [6, 10].

Susceptibility to $HgCl_2$-induced autoimmune effects depends mainly on the MHC-II haplotype, but non-MHC genes also influence the autoimmune process [19]. Interestingly, Lewis rats are resistant to $HgCl_2$-induced autoimmunity; rather, exposure is associated with immunosuppression [20]. Notably, this extreme example of opposing effects of the same chemical in two different animal strains illustrates to what extent genetics determine the outcome of an immune reaction and is regarded a classical example to support the use of inbred strains of animals of a certain genotype for the study of chemical-induced autoimmunity.

Resistance to $HgCl_2$-induced autoimmunity and suppression of immune function in Lewis rats is mediated by $CD8^+$ regulatory T cells (previously designed "suppressor" T cells) [20]. Autoimmune effects in BN rats are also subject to regulatory processes, as autoimmune phenomena peak around day 15 to 20 and decrease again thereafter [21]. In addition, low dose pretreatment of BN rats with $HgCl_2$ prevents development of adverse immune effects [22] and neonatal injection of $HgCl_2$ in BN rats renders them tolerant to mercury-induced autoimmune phenomena [23]. This tolerance is compound-specific, as $HgCl_2$-tolerant rats are still susceptible to gold-induced autoimmunity. The transience of autoimmune effects as well as low-dose protection induced by $HgCl_2$ may involve IFN-γ-producing $CD8^+CD45RC^{high}$ regulatory T cells [22, 24–26] or $RT6.2^+$ T cells [27].

D-Penicillamine

The anti-rheumatic drug D-penicillamine induces autoimmune phenomena in BN rats that are similar to those induced by $HgCl_2$ and resemble those observed in patients undergoing an adverse reaction to the drug [8]. Phenomena include transient increases in T and B cell numbers, elevated serum IgE levels, IgG deposition in glomeruli and skin reactions. Tournade and colleagues [8] reported effects in rats exposed to 450 mg D-penicillamine/kg body weight by gavage for 2 months, whereas Masson and colleagues [28] reported that 60 to 80% of treated BN rats develop autoimmune disease at 20 mg/day via drinking water. Interestingly, low dose pretreatment (5 mg/day, for 14 days) with D-penicillamine protected animals subsequently exposed to autoimmunogenic doses [7, 28].

The transience of autoimmune effects, as well low-dose tolerance, suggests the induction of immunoregulatory processes. A recent series of studies have further explored these processes in D-penicillamine-induced autoimmunity [28]. After a high dose of the drug, $CD4^+$ cells expressing increased levels of IL-4 have been found, indicating involvement of Th2 cells, whereas after low dose pretreatment and high dose challenge,

increased production of IFN-γ has been demonstrated in $CD4^+$ cells, which is suggestive of Th1 phenotype stimulation. In the same study, a low dose of D-penicillamine also induced IFN-γ-producing $CD8^+$ cells. And, in addition to this apparent skewing of the immune response to a predominant Th1 phenotype, low doses of D-penicillamine appeared to stimulate the formation of TGF-β- and IL-10-expressing CD4 and CD8 cells.

Cells other than T lymphocytes also appear to be involved in tolerance induction. Depletion of macrophages inhibited tolerance induction and transfer studies with non-T cell fractions from tolerant animals was shown to confer tolerance to naive animals [29] Thus, tolerance induction by low doses of D-penicillamine appears to have a complex mechanism that includes various T cell subsets as well as non-T cells, that may be antigen presenting cells.

Interestingly, poly I:C, which resembles viral RNA and is an agonist of toll-like receptor (TLR) 3, has been found to reverse tolerance induction. LPS, acting through TLR4, had a similar but less profound inhibitory effect on tolerance induction [30] The effects of both these microbial factors may be linked to the fact that infectious agents are risk factors of adverse immune responses to chemicals [31, 32]. In aggregate, these results illustrate that D-penicillamine-induced immune alterations are subject to complex regulatory mechanisms and indicate that bypassing mucosal tolerance might be pivotal in inducing idiosyncratic reactions.

Hexachlorobenzene

The toxic effects of the fungicide and environmental pollutant hexachlorobenzene (HCB) were first noted in the 1950s when people were exposed to high doses of HCB through intake of contaminated grain. Intoxicated individuals developed a variety of symptoms including hepatic porphyria, skin lesions, and arthritis. Symptoms were accompanied by infiltrates of inflammatory cells [11, 33].

The effects of HCB have been studied in BN, Lewis, Wistar and Sprague Dawley rats. All rat strains display symptoms reminiscent of an autoimmune disease (splenomegaly, increased serum levels of autoantibodies, inflammatory responses in lungs and skin), although BN rats are the most sensitive [34]. This relative strain-independence suggests that HCB-induced pathology may be partially or completely independent of host genotype and that effects may not be of autoimmune origin. In addition, although the immunosuppressive drug cyclosporine A (CsA) prevents or delays a number of T cell-dependent responses in HCB exposed rats, including elevated levels of IgE, pulmonary eosinophilia, and skin lesions [12], an initiating role of T cells has not been found and adoptive transfer of T cells from HCB exposed animals to naive animals did not induce symptoms of autoimmunity. Transcriptome profiling of various immune organs, liver and kidneys of rats subchronically treated with HCB revealed that inflammation-related genes are preferentially transcribed. Genes indicative of adaptive immunity (e.g., immunoglobulin genes) are less profoundly increased or are even decreased (CD3 and CD5) [13]. It should be noted that in this study gene expression was measured at a time point when clinical symptoms were already apparent. RNA was extracted from whole organs so all findings reflect relative changes. Nevertheless, the overall picture

that emerges indicates that HCB primarily causes an inflammatory response that subsequently stimulates adaptive immune responses. The inflammatory response may result from activation of macrophage that engulfed non-degradable HCB crystals, as occurs with exposure to silica. This idea is supported by a recent finding showing that, as with silica, HCB is able to stimulate TNF-α production by rat NR8383 macrophages. Furthermore, macrophage-depletion prevents the onset of clinical symptoms (skin and lung pathology) [35]. T cells, in part possibly autoreactive, would then have a secondary, enhancing role in pathology.

Nevirapine

Nevirapine is a non-nucleoside reverse transcriptase inhibitor used to treat HIV-infected patients that causes mild to severe skin rash and even Stevens-Johnson Syndrome/Toxic Epidermal Necrolysis (SJS/TEN) in a substantial proportion (16%) of patients. Nevirapine also induces hepatotoxicity. These adverse clinical symptoms may also occur in non-HIV subjects taking the drug as postoperative prophylaxis [15].

Recently, nevirapine has been found to cause skin rash in 100% of high dose (150 mg/kg by oral gavage) female BN rats [15]. Female Sprague Dawley rats were less sensitive (21% or rats showed a rash), while male BN or Sprague Dawley rats, and female Lewis rats, were resistant. Low dose pretreatment with nevirapine induced tolerance to a subsequent challenge with high doses, although it is unknown as yet whether this tolerance is immunologic (similar to D-penicillamine and $HgCl_2$) or metabolic in nature.

The development of nevirapine-induced disease is clearly immune-mediated as upon re-challenge with nevirapine the rash developed faster in previously exposed and fully recuperated rats. In addition, memory for skin reactions in response to nevirapine were transferable by splenocytes from treated to naive animals [15]. In summary, nevirapine-induced skin reactions in rat are immune-mediated and dependent on genetic background, including gender.

Other Rat Models

Cyclosporin A-induced Autoimmunity (CsA-AI)

Cyclosporin is able to induce an autoimmune syndrome in Lewis rats, but only when these rats are first subjected to lethal (8.5 Gy) X irradiation and reconstituted with syngeneic or autologous bone marrow [36]. In this model, CsA exposure is begun on the day of bone marrow transplantation, and signs of autoimmune disease are apparent after approximately two weeks. Acute symptoms of CsA-AI are similar to Graft versus Host Disease (GvHD), with erythroderma, dermatitis and alopecia while chronic symptoms include progressive alopecia combined with scleroderma-like skin pathology.

CyA-AI is clearly immune-dependent and involves autoreactive T cells specific for an MHC class II-peptide named CLIP and requires both an intact thymus and the absence of regulatory T cells ($CD45RC^{low}CD4^{+}$ phenotype) [37]. CsA inhibits differentiation of $CD4^{+}CD8^{+}$ thymocytes, possibly by interference with activation-induced (IL-2-dependent) cell death [38], and is the probable mechanism underlying increased

release of auto-reactive T cells from the thymus [39]. In line with this interference with early T cell differentiation, neonatal administration of CsA in mice also induces a multiorgan-type autoimmune disease [40].

MOUSE MODELS

Over the past 20 to 30 years, the mouse has been used extensively to investigate chemical-induced systemic immunosensitization and autoimmunity. Mice have traditionally been used instead of rats in immunotoxicology and immunology studies because of the greater availability of mouse-specific immunologic reagents and a wider selection of strains, including genetically modified animals.

METALS

Subcutaneous administration of $HgCl_2$ [41–43] or intramuscular treatment with Au(I)-salts [44] has been shown to induce anti-nuclear (ANA) and anti-nucleolar autoantibodies (AnolA) approximately 4 weeks after exposure, particularly in high responding A.SW mice (MHC-H2s haplotype). The response to $HgCl_2$ in mice displaying autoimmune symptoms is Th2-mediated, involving IL-4 production and increases in IgE and IgG1 levels [45]. Other H2s mice, such as B10s mice are also susceptible, but congenic H2d mice (e.g., B10D2) or H2k mice (B10.BR) are resistant to $HgCl_2$-induced autoimmune effects. As is the case for rats, this indicates the importance of genetic background, in particular that of MHC haplotype [41]. Interestingly, both H2s and H2d mice respond to $HgCl_2$ exposure with an increase in activated $CD4^+CD45R^{low}$ effector T cells [46], implying that the difference between these strains also involves immunoregulatory processes. Indeed, it was recently found that $HgCl_2$ stimulates $CD4^+CD25^+$ T regulatory cells in C57BL/6J (H2b) mice and moreover, that these T regulatory cells confer long-lasting unresponsiveness to $HgCl_2$. With respect to route of exposure, $HgCl_2$ given orally via the drinking water also induced AnolA (of IgG-class) in SJL/N (H2s) mice after 10 weeks [47].

DRUGS

Only a few drugs have been identified as being capable of inducing autoimmune phenomena in mice. Among these are D-penicillamine, quinidine, streptozotocin (an anti-neoplastic drug that is also used as a model compound to induce diabetes) and procainamide.

D-penicillamine has been shown to induce anti-ssDNA and anti-insulin antibodies in C57BL/Ks (H2d) and C3H/He (H2k) but not in BALB/c (H2d) or C57BL/6 (H2b) mice after subcutaneous exposure for 4 weeks [48]. Oral exposure to D-penicillamine or quinidine in the drinking water for 7 to 8 months, also caused an increase in levels of auto-antibodies in A.SW/Sn (H2s) mice [49].

Streptozotocin (STZ) is capable of inducing immune-dependent diabetes mellitus (IDDM) when administered intraperitoneally at low dose on 5 to 6 consecutive days. Induction is dependent on strain and/or gender, with male C57BL/Ks (H2d) mice being the most sensitive [50, 51]. STZ is a good example of chemicals that elicit a typical Th1-dependent immune response, including a strong activation of macrophages, IFN-γ-producing $CD4^+$ and $CD8^+$ T cells and production of IgG2a antibodies [52, 53].

Procainamide has been found to induce an increase in ANA in A/J mice after 8 months of exposure via the drinking water [54]. This increase appeared to be mediated by $CD25^-CD4^+$ T cells and regulated by $CD25^+CD4^+$ regulatory T cells.

Diphenylhydantoin, which has been demonstrated to cause autoimmune phenomena in man (SLE, vasculitis and scleroderma and skin) has also been tested (via drinking water for 6 months) in genetically predisposed mice (C57BL/6-lpr/lpr strain) but the compound depressed rather than increased the levels of ANA (55 and section 4.1, below). In another study [56], a slight shift towards a Th2 response was demonstrated as an increase in the KLH-induced production of IL-4 and IgE (IgE was detected by direct ELISA, which makes these data suspect) in a 4 weeks exposure study. In this same study, proliferative responses of splenocytes to KLH (using spleen cells of KLH-sensitized mice), mitogens (ConA, LPS) or anti-CD3 were also reduced, possibly through interference with accessory cell function.

Robinson and colleagues [57] compared 16 different MHC-defined mouse strains in a single study for induction of ANA by $HgCl_2$ (subcutaneously, detected after 0.5 to 2 months), gold salts (intramuscularly, detected after 1 to 5 months) and D-penicillamine (orally, detected after 4.5 to 5 months) and have reported that A.SW mice were high responders to all three chemicals.

Pristane

Pristane (2,6,10,14-tetramethylpentadecane) is a mineral oil known to induce arthritis, a disease also referred to as pristane-induced arthritis (PIA) [58]. Susceptibility to PIA is MHC-haplotype dependent, in that DBA/1 (H2q) mice are susceptible whereas DBA/2 (H2d) are not, and is accompanied by a broad spectrum of autoantibodies, including anti-Rheuma Factor (RF), anti-collagen and antibodies to heat shock proteins (HSP). PIA is clearly immune dependent since nu/nu mice and irradiated mice do not develop PIA. PIA involves polyclonal T cell activation [59], particularly $CD4^+$ cells [58]. Intriguingly, mice can be protected from developing PIA by HSP65-specific $CD4^+$ Th2 cells [60].

GENETICALLY PREDISPOSED ANIMAL MODELS

Genetically predisposed animals or induced animal models may also be used to study and predict chemical-induced autoimmunity. In induced models, a susceptible animal strain is immunized with a mixture of an adjuvant and an autoantigen isolated from the target organ. Examples are adjuvant arthritis (AA), experimental allergic encephalomyelitis (EAE) and experimental uveitis in the Lewis strain rat. Examples of spontaneous models

of autoimmune disease are the BB-rat and the NOD-mouse that develop autoimmune pancreatitis and subsequently diabetes, or the (NZBxNZW)F_1 or MRL/lpr mouse [61, 62] that spontaneously develop systemic lupus erythematosus (SLE)-like disease.

The rationale behind using autoimmune-prone animal strains to study and predict autoimmunogenic potential of chemicals is that exacerbation of disease is one of the possibilities by which chemicals may elicit autoimmune phenomena [5, 61].

Induced models are often used to the study the pathogenesis of, and therapeutic venues for relevant autoimmune diseases. Some of these models, in particular EAE and AA models, have also been proposed as means to evaluate the immunomodulatory effects of chemicals on established autoimmune diseases in the Tiered Approach of immunotoxicity testing.

SLE-Prone Strains of Mice

As many drugs are linked to lupus-like symptoms (e.g., drug-related lupus) and although drug-induced lupus differs from SLE in certain aspects [61, 62] it has been proposed to use SLE-prone strains of mice as model animals to test for the exacerbating and even initiating potential of drugs. Among the mice strains proposed for this purpose are the spontaneous SLE models (BWF1, NZB × SWRF1, MRL/lpr/lpr/Mp, BXSB/Mp, (NZB × NZW)F1, NZM, NZBWF1, AKR). Experience with many of these strains is scarce, and is restricted mainly to heavy metals such as $HgCl_2$ which had clear immunostimulatory effects in NZBWF1 mice [61]. In a study examining the effect of phenythoin [55], MRL-mice were exposed to the drug in drinking water for a period of 6 months, although no indications of adverse immune reactions were found. Future studies should include more autoimmunogenic pharmaceuticals and negative controls in order to decide whether SLE-prone models are indeed applicable to study or predict chemical-induced autoimmunity.

OTHER SPECIES

Dogs

The dog is a species that is frequently used in toxicity studies. However, there are few reports in the open literature on dog studies with respect to chemical- or drug-induced hypersensitivity reactions or autoimmune effects, and those that are available lack consistency. For instance, procainamide has been shown to induce lupus-like symptoms (mainly an increase of ANA) in one study [63], but not in another study with younger dogs [64]. Similar discrepancies have been observed for hydralazine-induced effects in dogs [5].

A more recent report shows clear sulfonamide-induced idiosyncratic responses in dogs (mostly Dobermans) that encompasses fever, arthropathy, blood dyscrasias (neutropenia, thrombocytopenia, or hemolytic anemia) hepatotoxicity, skin eruptions, uveitis, and keratoconjunctivitis sicca [65]. These symptoms became apparent as soon

as 12 days after the start of exposure. But, as expected with idiosyncratic reactions, the incidence in dogs (and cats) was low and estimated to be around 0.25% [66].

Radiographic contrast media have been shown to induce histamine release in a dog study, indicating that these media may induce pseudo-allergic responses [67]. The anti-neoplastic agent etoposide appeared to cause cutaneous reactions (pruritis, urticaria, and swelling in head region and extremities) in dogs (Beagles) upon intravenous exposure [68].

Miscellaneous

Other species have been used to study chemical induced autoimmune phenomena or systemic hypersensitivity reactions. Experiments with cats showed that propylthiouracil (PTU) induces SLE-like phenomena including autoantibodies against nuclear antigen, Smith (Sm) antigen, red blood cells and cytoplasmic components, lymhadenopathy, and weight loss [69]. However, important PTU-induced symptoms, including agranulocytosus and liver toxicity, that are seen in humans, were not observed in cats [16]. The fact that symptoms after rechallenge occur with the same time delay as in naive animals does not support an immunological basis for these phenomena in cats. Furthermore, a subsequent study failed to replicate earlier results for unknown reasons, casting doubt on the usefulness of the cat as a model of PTU-induced autoimmunity [16].
Chemical- and diet-induced autoimmune effects in other species have also been documented, but they are limited to isolated cases (for an extensive review, see [4]). For instance, monkeys fed alfalfa-seeds developed antibody-induced anemia; chickens (Cornell C strain) receiving an excess of iodine developed antibodies against thyroid hormones and lymphocytic thyroiditis; halothane-treated rabbits displayed antibodies against a set of 5 endogenous antigens; and drug-specific antibodies (including isoniazid and procainamide) were detected in Guinea pigs following injection of drugs in combination with complete Freund's adjuvant [70, 71]. However, based on FDA records, the Guinea pig does not provide a reliable predictive animal model for adverse immune responses to therapeutics [72].

LOCAL LYMPH NODE ASSAYS

Local lymph node assays, such as the LLNA or popliteal lymph node assay (PLNA) are straightforward and robust animal test models that link lymph node reactions directly to the site of application (epidermal in LLNA, subcutaneously in PLNA) of potentially immuno-active chemicals. In particular the PLNA has been extensively used to evaluate the potency of drugs to stimulate the immune system. It is important to note that the PLNA (like all local lymph node assays) is regarded as screening assay that allows assessment of the hazard, but not of the risk, that a chemical may induce sensitization via systemic routes [73].

The PLNA is typically performed in mice [73, 74] although in some cases rats have been used [75]. The assay basically determines changes in the lymph node distal to the

paw that are induced by subcutaneous injection of a chemical into footpad. The response, which can be assessed by detecting lymphocyte proliferation, changes in leukocyte subset distribution, cytokine production, antibody responses to a co-administered antigen (see below) or by immunohistochemistry, is determined 6 to 8 days after injection.

Interestingly, by using immunologically relevant parameters instead of only proliferation indices, the PLNA seems capable of identifying immunosensitizing potential. A recent modification of this assay involves simultaneous injection of the test article and an antigen that induces antibody production with or without T cell help The known antigen is considered to be a "reporter antigen" (RA), hence the name reporter-antigen PLNA or RA-PLNA [76, 77]. The RA is regarded as a "bystander" antigen, because the nature of the antibody response to the RA is influenced by the type of response stimulated by the test article. For example, when a compound is co-injected with the T cell-independent antigen TNP-Ficoll, which is susceptible to neo-antigen-specific T cell help, and an increase of TNP-specific antibody forming cells of the IgG isotype is detected, it can be concluded that the compound induces T cell sensitization. When the T cell-dependent antigen TNP-OVA is co-injected, chemical-induced increases in the number of TNP-specific IgG-forming cells indicate that the chemical has at least adjuvant activity; when the IgG1-response to TNP-Ficoll is negative, the chemical has adjuvant activity without inducing specific T cells. This immunologically based approach improves the predictability of the PLNA and, in addition, provides additional insight into the fundamental aspects of chemical-induced immune effects [52, 53, 78].

To date, 130 to 140 compounds have been tested in the PLNA, including a substantial number of structural homologues of phenythoin (approximately 50 [79]) and zimeldine (approximately 10, [80]), and those chemicals with known immunostimulating activity in man were detected correctly [81]. However, compounds (such as procainamide) that require metabolic conversion gave false negative results unless metabolic systems (myelin peroxidase positive phagocytes or S9 mix) were injected as well [82]. Alternatively, metabolites have been tested and appeared positive whereas their precursor compounds were negative [83, 84].

Injection of certain chemicals into the footpad may cause local inflammation, raising concerns about the ethical treatment of laboratory animals. Alternatives to footpad injection were recently addressed in a study that examined the predictive value of lymph node proliferation assays when used to evaluate the immunosensitizing potential of selected drugs following systemic exposure [85]. Responses in the cervical lymph nodes following subcutaneous chemical injection on top of the head between the ears was compared to results obtained with the traditional PLNA. The cervical node protocol correctly detected 7 of the 11 drugs tested (5 as positive and 2 as negative). Of the four compounds for which false negative results were obtained, two are known to require metabolic activation and the other two were dose-limited due to toxicity [85]. In a study using the RA-protocol with TNP-Ficoll as the RA, ear-injection and subsequent detection of specific antibody formation with a range of pharmaceuticals showed comparable results with the RA-PLNA [86].

A major drawback of the PLNA and other local lymph node approaches for drug testing is that the route of exposure is irrelevant, as most drugs are taken orally. However, it was recently reported that oral exposure to D-penicillamine, diclofenac or nevirapine

stimulated the responses to systemically applied TNP-OVA [87]. D-penicillamine [87] and diclofenac [87, 88] were also capable of stimulating drug-specific anamnestic responses that could be detected by assessing TNP-specific antibody formation in the PLN after injecting TNP-Ficoll together with a non-stimulating dose of the drugs. These results suggest that the RA approach may also be useful in assessing sensitizing potential following oral drug exposures.

CONCLUDING REMARKS

Traditional regulatory toxicity studies typically do not detect auto-immunogenic and allergenic effects of compounds, in part because non-genetically homogenous out-bred animals are used but also because relevant (i.e., immune) parameters are not measured. Moreover, experimental "outliers" are usually discarded, when in fact these outliers may indicate unexpected and idiosyncratic immune effects. Although fewer outliers may result if testing was conducted in inbred strains of animals, adverse immune responses may still be missed, because chemical-induced autoimmunity (or allergy) is an idiosyncratic process. These technical issues suggest that achieving one standard model for the assessment of these effects for a wide range of chemicals might be problematic. Rather, a two- or multiple-tiered approach may be envisioned using a number of mechanism-based tools and models. A tiered toolbox approach would be particularly useful for the pharmaceutical industry that tends to develop a range of potentially active homologues of a certain drug category.

Tools and models that would fit in this approach include structure activity assessment methods and relevant in vitro methods (e.g., dendritic cell maturation), in combination with established animal models, such as the local lymph node assays and animal disease models such as the BN rat model. Hyper-reactivity responses or autoimmune diseases may often have an initial phase of immunostimulation and immunosensitization. Utilizing one of the local lymph node assays (with or without metabolic activation), with an immunological read-out parameter such as the RA approach, would allow screening for the worse case potential or hazard of a chemical's potency to stimulate the immune system. Interestingly, studies with structural homologues of phenythoin [79] and zimeldine [80] have shown that the PLNA allows assessment of structure activity relationships. Non- or minimally-stimulatory compounds could then be subjected to a second tier of tests (to be developed) in animal models that utilize strains with a known genetic predisposition to develop allergy or autoimmune disease following systemic, preferably oral, exposure routes. This should be done to confirm whether a suspected compound is also active via the most obvious route of exposure and with relevant dosing regimes. Although limited experimental data suggest that drugs with sensitizing potential may be detected when administered orally [86–88], development and validation of these models is an obvious challenge for future basic research in this field of immunotoxicology.

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Part VIII

Neuroimmunology

28 An Overview of Neural-Immune Communication in Development, Adulthood, and Aging

Denise L. Bellinger, Srinivasan ThyagaRajan, Amanda K. Damjanovic, Brooke Millar, Cheri Lubahn, and Dianne Lorton

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INTRODUCTION

The sympathetic nervous system (SNS) and the hypothalamic-pituitary axis work together as important modulators of the immune system after exposure to stressors. Norepinephrine (NE) and epinephrine (EPI) (catecholamines from the SNS) and neuroendocrine hormones modulate a range of immune cell activities, including cell proliferation, cytokine and antibody production, lytic activity, and migration. This chapter will focus on these two major pathways of brain-immune signaling, briefly summarizing the evidence for SNS and hypothalamic-pituitary-adrenal (HPA) modulation of immune function, their influence on immune-mediated diseases, immune modulation in aging, and early life influences on these pathways.

CATECHOLAMINES, SYMPATHETIC INNERVATION, AND IMMUNITY

Sources of Catecholamines

NE signals cells of the immune system after release from noradrenergic (NA) sympathetic nerves that supply lymphoid compartments of primary and secondary immune organs [1]. Sympathetic nerves closely appose T and B lymphocytes, macrophages, stromal cells, and mast cells, which express adrenergic receptors (AR). After release from sympathetic nerves, NE diffuses toward target cells, setting up a concentration gradient that fluctuates with sympathetic activity. This type of release is called paracrine release. Additionally, NA nerves can form direct synaptic-like junctions with lymphocytes, macrophages, mast cells, and other cells of the immune system. These contacts may provide a bidirectional flow of information between sympathetic nerves and immune cells. A number of neuropeptides and other signaling molecules colocalize with NE, including neuropeptide Y, enkephalins, and adenosine triphosphate (ATP), and are presumed to modulate target cell signaling by NE.

Catecholamines from non-neuronal intracellular and extracellular sources can interact with cells of the immune system. Recently, NE and EPI that can be released by activating stimuli have been detected in lymphocytes and macrophages [reviewed in 2]. These cells may synthesize catecholamines and/or take up and store catecholamines from extracellular sources (i.e., NE released from sympathetic nerves or NE and EPI present in the plasma).

Adrenergic Receptor Signaling Cells of the Immune System

NE and EPI stimulate α- and β-AR on the cell surface of target tissues. β_2-AR are expressed on almost all types of immune cells, with the notable exception of T-helper (Th)2 clones [3]. β-AR on immunocytes are coupled with Gs proteins and adenylate cyclase, with subsequent activation increasing intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) and protein kinase A (PKA). Under normal conditions, β-AR cell surface expression up- and down-regulates in response to reduced and increased catecholamine

availability, respectively. With down-regulation, β-AR internalize, reducing the signaling capacity via these receptors. β-AR expression and signaling in lymphocytes is reduced at the time of peak lymphocyte proliferation, suggesting that lowering β-AR signaling capacity may be an intrinsic property of lymphocyte activation.

The expression of α-AR on lymphocytes is not easily detected by radioligand binding assays under normal conditions. However, pharmacological studies suggest that, at least under certain conditions, lymphocytes can express this class of AR, and α_2-AR are expressed in elicited macrophages [4]. In several human autoimmune diseases, altered leukocyte AR expression has been reported. α_1-AR are present in peripheral blood mononuclear cells from children with a severe form of juvenile rheumatoid arthritis [5]. The density and signaling capacity of β-AR are altered in peripheral blood lymphocytes from adults with rheumatoid arthritis and multiple sclerosis [6, 7]. Such alterations may reflect changes in sympathetic activity and catecholamine availability, but it is also possible that the disease process itself elicits changes in lymphocyte and monocyte AR expression that compensate for, or exacerbate disease progression.

In cardiac myocytes and several cell lines, β-AR can switch coupling from Gs to Gi/Go proteins with consequent activation of different intracellular signaling pathway [8]. Activation of Gs-coupled receptors increases intracellular cAMP and cAMP-dependent PKA. PKA-mediated phosphorylation of the β_2-AR decreases and increases its affinity for Gs and Gi/Go, respectively. Subsequent activation of the PKA phosphorylated β_2-AR increases Gi signaling, with reduced and increased receptor-stimulated adenylate cyclase activation and mitogen-activated protein kinase (MAPK) activation, respectively. The extent to which β_2-AR switching from Gs to Gi/Go protein signaling occurs in immunocytes is unknown, but may explain discordant effects of β-AR stimulation on a variety of immune responses. Understanding the extent to which defects in Gs-to-Gi switching might be involved in immune-mediated diseases is necessary for development of therapies that manipulate AR signaling.

Altered Catecholamine Availability with Immune Activation

One of the first lines of evidence to suggest that NE is an important player in modulating immune responses *in vivo* was the finding of lower NE concentration [9] and higher NE turnover [10–11] in draining lymphoid organs after immunization, which suggests altered NE availability. Fuchs et al. [10] have reported a sustained rise in splenic NE metabolites and reduced NE concentration after intraperitoneal (i.p.) immunization with a particulate antigen (sheep red blood cells), indicative of increased NE turnover. Other investigators have studied NE turnover in lymphoid organs as a means of assessing NE synthesis and availability during an immune reaction. In severe combined immunodeficiency mice that were reconstituted with antigen-specific B cells and Th2-type clones [11], i.p. immunization with an antigen recognized by the donor cells, but not an unrelated antigen, increased NE turnover in the spleen, heart, and bone marrow 18 to 24 hours after challenge. Similarly, plasma NE and EPI, splenic nerve activity, and splenic NE turnover increased after administering LPS [12]. The biological impact of antigen-induced changes in NE turnover remains elusive. Presumably, changes in sympathetic

activity in secondary lymphoid organs are important for fine-tuning the immune response after activation by antigen challenge, in order to maintain homeostasis. Studies showing an inability to mount an appropriate immune response after manipulating sympathetic neurotransmission support this assumption [reviewed in 13].

Other studies suggest that the SNS provides homeostatic regulation of immune responses by modulating the balance between the production and release of (1) Th1 and Th2 cytokines during an immune response, and (2) pro-inflammatory and anti-inflammatory cytokines during an inflammatory response. For example, increased NE turnover following LPS administration reduces the pro-inflammatory response, while enhancing the anti-inflammatory response [14], indicative of a physiological mechanism to limit the magnitude of an inflammatory response. The importance of sympathetically-modulated pro- versus anti-inflammatory balance is illustrated in autoimmune diseases which have an inflammatory component. For example, in rheumatoid arthritis and experimental models of rheumatoid arthritis, the SNS is hyperactive [reviewed in 15]. Heightened sympathetic activity appears to promote pro-inflammatory cytokine production that drives joint destruction and aberrant immune reactions. In experimental arthritis, treatments with adrenergic drugs that enhance β-AR and reduce α-AR stimulation prevent joint destruction, presumably by pharmacologically restoring homeostatic sympathetic modulation of the immune system [16]. A careful examination of NE turnover over time after immunization with a variety of antigen types along with lymphocyte and macrophage α and β-AR signaling capacity, will help determine the physiological significance of altered NE availability in immune physiology.

Catecholamines Enhance and Inhibit Immune Responses

Data showing that manipulating catecholamine concentration and AR signaling *in vivo* and *in vitro* alter immune reactivity support the SNS playing an important role in immune regulation. Early reports of adrenergic stimulation being largely inhibitory of many *in vitro* immune measures led to the early view that the SNS inhibited the immune system. However, *in vivo* assessment of SNS regulation of the immune system indicates that SNS-immune interaction is much more complex. NA nerve ablation with the neurotoxin 6-hydroxydopamine (6-OHDA; chemical sympathectomy; SympX) or treatment with the β-AR antagonist, nadolol, prior to immunization reduced a Th2-driven antibody response and decreased the delayed-type hypersensitivity reaction to a contact sensitizing agent [17, 18], suggesting that the SNS can enhance immune reactivity. In transgenic mice that lack dopamine β-hydroxylase, an enzyme required for NE and EPI synthesis, immune reactivity to infectious agents and a protein antigen was impaired [19]. EPI injection before antigenic sensitization enhanced delayed-type hypersensitivity and increased draining lymph node cellularity [20]. Collectively, these results suggest that the SNS can enhance immune reactivity *in vivo*. In contrast, exposure to stressors or agents that activate the SNS reduced T cell responses, anti-viral immune reactivity, and natural killer (NK) cell activity [reviewed in 13]. Pretreatment with β-blockers or ganglionic blockade blocked these immunosuppressive effects. In other experiments, SympX enhanced antigen-induced proliferation and Th1 and Th2 cytokine production

in vitro [reviewed in 13]. NA nerve ablation prior to i.p. immunization also increased serum antibody levels in rats and in C57BL/6 mice, but not BALB/c mice [21, 22; reviewed in 13]. The discordance between these results and the reports of reduced cell-mediated immune reactivity following SympX cited above demonstrate that genetic background (i.e., animal strain), the site of immunization, and the subpopulation of Th cells involved will influence outcome.

These qualitative differences in catecholamine interactions with cells of the immune system *in vivo* suggest that a number of factors influence the outcome of SNS interactions with the immune system. Through *in vitro* studies with purified cell populations, investigators have begun to identify the mechanisms underlying catecholamine immunomodulation. Early studies using unfractionated effector cells *in vitro* reported β-AR-mediated enhancement of the generation of alloreactive cytotoxic T lymphocytes and antibody-secreting cells [reviewed in 2, 13]. More recently, Sanders and colleagues have used highly purified lymphocyte populations to study T and B cells responses to β-AR stimulation. In co-cultures of antigen-specific B cells (the antigen-presenting cells and producers of antibody) with Th2 clones to provide help, β-AR stimulation increased antibody production [3]. Since Th2 clones do not express β-AR, B cell β-AR elicited the increased antibody production in this system. In subsequent experiments, they found that β-AR stimulation enhanced antibody production by up-regulating B cell accessory molecule expression and by increasing B cell responsiveness to interleukin (IL)-4 [23]. In contrast, when Th1 clones were exposed to β-agonists prior to antigen-induced activation, the number of antibody-forming cells decreased in association with reduced interferon (IFN)-γ production [3]. These findings suggest that β-AR stimulation inhibit Th1 responses when antigen is presented to them by B cells. However, Th1 responses also exhibit differential responsiveness to catecholamine stimulation, depending on a number of factors. For example, when Th1 clones were activated with anti-CD3 instead of antigen, IL-2 production was reduced, but IFN-γ production was unchanged in the presence of β-agonists [24]. When naïve CD4+ T cells were grown in Th1-promoting conditions, β-AR stimulation elicited increased IFN-γ production in an IL-12-dependent manner [25]. On the other hand, β-AR stimulation reduced monocyte- and dendritic cell-derived IL-12 production [26]. These results demonstrate that the type of immune cell involved and its activational or maturational state contribute to the complexity of crosstalk between catecholamines and the immune system. This complexity likely reflects the importance of the SNS in fine-tuning a response with the goal of maintaining immune system homeostasis.

GLUCOCORTICOIDS (GC), HPA AXIS, AND IMMUNITY

Source and Regulation of GC Secretion

Corticosteroids synthesized by the adrenal gland are mineralocorticoids and GC. Mineralocorticoids regulate fluid and electrolyte balance by affecting ion transport in the kidney. Cortisol, the primary circulating GC in most species (including humans), has many activities, including resistance to stress, regulation of intermediary metabolism, and immunosuppressive and anti-inflammatory effects. GC synthesis and secretion is

mainly regulated via the HPA axis. Corticotropin-releasing hormone (CRH) is secreted from the paraventricular nucleus (PVN) of the hypothalamus into the hypophyseal portal blood supply. CRH along with arginine vasopressin (AVP) stimulates adrenocorticotropin (ACTH) synthesis and release from the anterior pituitary gland. ACTH then circulates through the blood to the adrenal cortex, where it induces the expression and release of GC. Central circuits that impinge on PVN neurons and peripheral negative feedback mechanisms regulate the HPA axis.

GC Receptor Signaling Cells of the Immune System

GC exert most of their effects by ligand binding with cytoplasmic GC receptors (GR) after diffusing through the cell membrane. Upon binding of GC, the GR dissociates from a multi-protein complex [reviewed in 27] and the free GC-GR complex is imported into the nucleus. Inside the nucleus, the GC-GR complex binds to specific DNA sequences in the promoter regions of target genes, called GC response elements (GRE), to stimulate or inhibit target gene expression. There are two forms of human GR: GRα and GRβ. GRα is the classical GR that transactivates GRE. GRβ is a non-steroid-binding truncated form that does not bind to the ligand and is unable to transactivate GRE. A variety of GC target tissues expresses GRβ, although there is some controversy over the relative concentrations of GR isoforms and the function of GRβ. GRβ may act as a dominant negative modulator of the GRα isoform, and thereby contribute to the development of GC resistance.

Activation of the HPA Axis Following Immunization

Administration of Cytokines

Systemic administration of single proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, or IL-6, can directly stimulate the pituitary to release ACTH [reviewed in 28]. Treatment with CRH-specific antibodies, or specific CRH receptors antagonists, prevents cytokine-stimulated ACTH release, supporting a CRH-dependent mechanism. There is an AVP-dependent component to the ACTH response induced by IL-1β, as treatment with AVP antibodies also reduces this response, although not to the magnitude observed by treatment with CRH antibodies. This finding suggests a secondary role for AVP. Activation of PVN neurons, either directly or via their afferent pathways, may also result from the production of cytokine-induced intermediates such as prostaglandins (PGs) and catecholamines that may be produced by cells in the circumventricular organs, in the wall of the ventricles and/or within brain glial cells and astrocytes [see discussion in 28]. PGs are important for this response, since indomethacin or ibuprofen blocked the immediate ACTH response to these immune mediators particularly during the early part of the response. The route through which cytokines reach the brain influences the effects of PGs [28, 29]. The mechanisms responsible for the HPA response to cytokines administered i.p. may differ from those operative in other paradigms. Intravenous (i.v.), i.p., or intracerebroventricular (i.c.v.) injection of

IL-1β significantly increases ACTH levels, indicating that endogenous CRH mediates these responses. However, vagal afferents also mediate the brain-induced effects of i.p. injection of IL-1β, but not i.v. or i.c.v. routes of IL-1β, possibly by transmitting a stimulatory signal to the PVN.

Endotoxin and some proinflammatory cytokines activate NA circuits projecting to the hypothalamus [reviewed in 29]. Since catecholamines stimulate PVN neuronal activity, they are likely to participate in the HPA axis response to immune challenges. 6-OHDA lesioning studies and surgical transections of aminergic innervation support of this hypothesis, which for the most part indicate that these procedures interfere with cytokine-stimulated HPA activation [reviewed in 29]. Still, administration of adrenergic antagonists under some circumstances is without effect [reviewed in 29]. Discordance of these data may reflect the fact that ascending catecholaminergic pathways exert dual and opposite regulatory roles, and that the modulatory roles of peripheral and central catecholamines differ. Furthermore, the influence of catecholamines may depend on other factors, such as the route of administration, involvement of vagal afferents in mediating the cytokine effects on HPA activity, and possibly differences across time of the HPA axis response.

Administration of Endotoxin

Administration of endotoxin has been used to study immune-induced activation of the HPA axis under conditions that mimic the acute-phase response, the initial immune response to most antigens [reviewed in 29]. LPS also alters the production of hepatic proteins, plasma concentration of metals such as zinc and iron, and induces sickness behaviors—physiological changes that may affect HPA activity independent of the immune events in the strictest sense. Other complications making it difficult to interpret and compare these studies are that many studies use large doses of LPS, which may injure the blood-brain barrier and have little physiological relevance, doses of LPS are not reported in terms of the units injected, and commercially available endotoxins are not standardized. That being said, increased plasma and brain TNF-α, IL-1β, and IL-6 have been used as a means of assessing endocrine consequences of elevated levels of these proteins.

Endotoxemia from either centrally or peripherally injected LPS induces a significant increase in HPA activity. The route of LPS administration determines the pattern of PVN response [30]. In contrast to single cytokine administration, systemically injected LPS upregulates neuronal activity of the PVN and related areas [reviewed in 29], which contributes to increased release of ACTH. The same neurotransmitters involved in single cytokine-induced ACTH release are important for endotoxin effects on HPA activity, with the involvement of each being dose-dependent [28, 31]. Less clear is the involvement of individual cytokines in endotoxin-stimulated HPA activation, and is complicated by interactions between TNF-α, IL-1β, and IL-6 [31, 32]. Administration of antibodies against one of these cytokines, or attempts to correlate temporal changes in plasma cytokines and ACTH levels, have not provided solid convincing evidence that their rise in levels are necessary for the full HPA axis response [reviewed in 29].

Tissue Injury

Intramuscular injection of turpentine causes a biphasic HPA response [33]. The first phase corresponds to the stress of injection with no detectable changes in plasma IL-6 concentrations. However, the second phase coincides with an inflammatory response to the turpentine, a response accompanied by increased plasma ACTH, corticosterone, and IL-6 levels. CRH, AVP, and PGs play stimulatory roles in this model, whereas nitric oxide dampens the pituitary response [33]. IL-1β may be important for triggering the release of these signaling molecules, since mice lacking the gene for IL-1β have a blunted GC response to turpentine [34].

Nonimmune Stressors

Nonimmune stresses can increase circulating proinflammatory cytokine concentrations. Hemorrhage, and certain psychological and physical stresses all increase HPA activity through mechanisms that may include catecholamines and peripheral CRH [reviewed in 29]. However, in some experiments correlations between plasma ACTH and IL-6 levels in models of immune and nonimmune stresses are low. The role played by cytokines in the ACTH response to nonimmune stresses has not been established.

GC Enhance and Inhibit Immune Responses

GC regulate a wide variety of immune cell functions. GC modulate cytokine expression, adhesion molecule expression and immune cell trafficking, immune cell maturation and differentiation, expression of chemoattractants and cell migration, and production of inflammatory mediators and other inflammatory molecules [35]. At pharmacological concentrations, GC are routinely used as immunosuppressive therapeutic agents in many acute and chronic inflammatory and autoimmune diseases, in transplant patients and in the treatment of leukemias and lymphomas [reviewed in 29]..

The immunosuppressive activity of GC is mediated via multiple molecular targets within cells of the immune system [reviewed in 29]. For example, GC down-regulate cell surface adhesion molecules, including intercellular adhesion molecule-1 and E-selectin, inhibit CD40 ligand up-regulation on CD4+ T cells, interfere with transcription of the cytotoxic T lymphocyte serine protease granzyme B, and directly inhibit early T cell receptor (TCR) signaling events. However, inhibition of cytokine production is widely accepted as the most biologically relevant phenomenon contributing to GC-induced immunosuppression [36]. GC can inhibit the production of many cytokines, including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, granulocyte-macrophage colony-stimulating factor, TNF-α, and IFN-γ, and can also induce transforming growth factor-β production [reviewed in 29]. Most anti-inflammatory and immunomodulatory actions of GC are due to inhibition of the activity of transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) [37].

At physiological concentrations, GC do not suppress all immune system activity, but specifically regulate the immune response to achieve immune homeostasis. At

lower concentrations, GC cause a shift in immune responses from a proinflammatory cytokine profile of increased IL-1 and TNF-α to an anti-inflammatory cytokine pattern of increased IL-10 and IL-1 receptor antagonist [38]; thus, they tend to suppress cell-mediated and promote humoral immunity. As part of its permissive effects during an immune response, GC synergize the effects of IL-6 signaling [39]. These effects include up-regulation of IL-6 receptors and their signal transduction events and IL-1 receptor up-regulation. GC are a necessary prerequisite in the induction of the acute phase response [40]. The production of a variety of proteins and proteases during the inflammatory response is part of a complex defense mechanism to re-establish homeostasis. Initiation of the acute phase response requires synergism between cytokines and GC for the induction of several acute phase proteins [40].

Another important aspect of GC immunomodulatory activity is their effect on growth, differentiation, survival and redistribution of T lymphocytes. GC induce apoptosis in a number of cell types, including human osteoclast and osteoblast precursors, epithelial cells, eosinophils, thymocytes and differentiated T cells [reviewed in 29]. T cell apoptosis is part of a physiological process in the thymus that determines immune response development, and the expansion of T cell tumors. Different signals, including antigen/TCR interaction, are involved in regulating T cell death. In particular, co-accessory molecules, including CD4, CD28, CD44, CD2, and cytokines such as IL-2, IL-4 and IL-9, may be involved in regulating T cell apoptosis. GC critically regulates T cell apoptosis through their simultaneous activation of cell suicide and anti-death programs that partially determines the susceptibility or resistance of cells to apoptosis.

HOMEOSTATIC RESILIENCE OF NEURAL-IMMUNE INTERACTIONS

A significant factor in determining neural-immune interactions is the age of the host, because the homeostatic resilience of the immune, neuroendocrine, and sympathetic systems is not the same across life span. In very young and aged humans and animals, stress-induced immune changes tend to be more pronounced and longer lasting. This greater vulnerability appears to reflect the fact that many immune responses undergo developmental and plasticity changes, respectively, during infancy and in aging.

Environmental factors and experiences in pre- and post-natal life can profoundly affect the developmental programming of the SNS and HPA axis to alter neural and endocrine regulation of many physiological functions indefinitely. Because different cells and tissues are sensitive at different times, environmental challenges have distinct effects depending on both the challenge and its timing. Intrauterine and postnatal events, including maternal stress, drugs, sensory experiences, and environmental toxins can exert a formative influence over the maturation of the SNS and HPA, which begin developing during fetal life and continue well after birth.

Because these systems function to maintain homeostasis, it is reasonable to presume that environmentally induced programming of the SNS and HPA axis may be part of an adaptive mechanism to enhance offspring survival. It is also conceivable that altered developmental programming of the SNS and HPA may become misguided under certain

conditions (i.e., compromised pregnancy such as placental insufficiency, maternal stress, or nutrient restriction), or that an individual's environment may become drastically altered such that neuroendocrine programming becomes maladaptive. Under these conditions, programming homeostatic regulatory systems may contribute to the pathogenesis of certain diseases. Similarly, inflammatory mediators present during critical windows of early life development may influence the programming of various genes within the neuroendocrine-immune axis. The extent to which environmental toxins, synthetic drugs, or psychological, physical, and immunological stressors alter the program of SNS and neuroendocrine systems to affect immune regulation is not clear.

At the other end of life span, age-related decline in immune responses and dysregulated SNS and HPA activity create a second period of vulnerability to illnesses and chronic diseases. Age-related immune senescence causes an increase in morbidity and mortality from neoplasia, infectious disease, and autoimmune disorders. Altered central and peripheral regulation of autonomic and neuroendocrine functions contributes to reduced resilience of the immune system in aging. Even minor stressors such as small changes in housing conditions are sometimes sufficient to cause sustained shifts in lymphocyte responses in aged nonhuman primates [41]. In addition, the prevalence of chronic illness in old age superimposes pathological processes on normal aging that can be worsened by psychological factors or environmental events.

Sympathetic Modulation of Immunity in Aging and Disease

Aging influences qualitative and quantitative aspects of catecholamine interactions with the immune system. In aged Fischer 344 (F344) rats, splenic NA innervation and NE concentration is reduced [reviewed in 42]. SympX-induced changes in immune reactivity in aged rats were often equivalent to responses in sympathectomized young rats, suggesting that the diminished NA innervation in the aged F344 rat spleen is capable of signaling cells of the immune system. In contrast, in the aged mouse, splenic NA innervation was not altered, NE concentration was elevated, and SympX did not alter immune reactivity, a manipulation that reduced immune reactivity in young mice [42]. Altered lymphocyte β-AR signaling capacity may explain differential responses to SympX in aging. In aged mice and several rat strains whose splenic NA innervation is more resistant to the effects of aging, lymphocyte β-AR signaling is impaired [42], consistent with some reports in human peripheral blood mononuclear cells [reviewed in 42]. By contrast, lower splenic NE concentration in aged F344 rats was associated with increased sensitivity of spleen cells to β-AR stimulation [22]. These data suggest that in aging, strain and species differences the sensitivity of splenic lymphocytes to altered NE availability alters the immune responses to the SNS.

Similarly, under certain disease conditions, altered NA innervation and/or AR signaling capacity impairs sympathetic communication with cells of the immune system, influencing disease progression. Altered catecholamine communication with the immune system is evident in autoimmune diseases such as arthritis and multiple sclerosis [5–7] and in infectious diseases, such as leprosy and a mouse model of acquired immunodeficiency syndrome [15, 43, 44]. The impact of altered NA innervation of

lymphoid organs or lymphocyte signaling capacity in aging or in disease states has yet to be fully investigated. The evidence thus far suggests that such changes lead to a loss of homeostatic control of inflammatory and antigen-specific immune reactions, specifically a reduced capacity to limit inflammation and to respond to antigens.

Models of Altered HPA Activation: Aging and Disease

In aging, HPA activity rises in healthy individual, but often is blunted with chronic stress or disease. Overstimulation of the HPA axis increases circulating GC, suppresses immune responses and leads to enhanced susceptibility to infection, whereas under-stimulation lowers circulating GC and enhances susceptibility to inflammation [reviewed in 45]. HPA dysregulation may also occur at the receptor level, resulting in GC resistance and enhanced inflammation. Inbred rat strains, in which altered neuroendocrine responsiveness is associated with differential susceptibility and resistance to autoimmune/inflammatory disease, provide a genetically uniform system for studying neuroendocrine regulation of immunity. Inbred Lewis (LEW/N) rats are highly susceptible to development of a wide range of autoimmune diseases in response to a variety of antigenic stimuli. Fischer (F344/N) rats are more resistant to the same illnesses after exposure to the same dose of antigens or proinflammatory stimuli. These two strains manifest differences in HPA axis responsiveness. The inflammation-susceptible LEW/N rat exhibits a significantly delayed GC response to certain stimuli compared to inflammation-resistant F344/N rats [reviewed in 45]. Differences in the expression of hypothalamic CRH, proopiomelanocortin, corticosterone-binding globulin, and GC expression and activation occur in these two rat strains [reviewed in 45]. Disruptions of the HPA axis in inflammation-resistant animals, via genetic, surgical, pharmacological, or toxic interventions, are associated with enhanced susceptibility to, or severity of, inflammatory disease. Such interruptions can occur at the level of the hypothalamus, pituitary, or adrenal glands. Reconstituting the HPA axis in inflammation-susceptible animals, pharmacologically with GC or surgically by intracerebral fetal hypothalamic tissue transplantation, also attenuates inflammatory disease. In humans, the HPA axis response to stimulation with CRH, hypoglycemia, or psychological stresses is blunted in a variety of autoimmune diseases such as rheumatoid arthritis, in inflammatory diseases such as atopic dermatitis and other conditions such as fibromyalgia, and chronic fatigue syndrome [reviewed in 45]. In patients with rheumatoid arthritis, administration of GC decreases TNF-α release into the circulation. Conversely, chronic stimulation of the stress hormonal response with prolonged elevated GC in situations encountered by, for example, caregivers of Alzheimer's patients, students taking examinations, couples during marital conflicts, and army rangers undergoing extreme exercise is associated with an enhanced susceptibility to viral infection, prolonged wound healing, or decreased antibody production in response to vaccination.

Early Life Experiences in Developmental Programming of the SNS and HPA

Environmental factors during fetal or neonatal development can significantly affect the density of NA innervation, SNS and adrenal medullary basal activity, or their response to specific stressors. These changes persist into adulthood, possibly throughout life, becoming a permanent characteristic of the individual. One of the complications with studying environmental effects on SNS development is that the SNS is composed of multiple anatomically- and functionally-specific subunits, and that the postganglionic sympathetic neurons can differ among subunits with respect to neurophysiological characteristics. Because the programming of SNS function during development occurs on a regional rather than on a global basis, each subdivision of the SNS is likely to respond to a different set of environmental variables. This would mean, for example, that the structure and function of the sympathoadrenal system and sympathetic-immune circuits are unique to each individual, because they represent the sum total of environmental effects on these systems to which the individual was exposed during development. This also makes it difficult to extrapolate effects of one type of stressor on changes in SNS structure and function in one target tissue to other sympathetically-innervated targets. The effects of early life experiences and/or environmental exposures to drug or neurotoxins on the programming of sympathetic-immune regulation are largely unexplored. There are some studies demonstrating that early programming of the SNS by pre- and postnatal stressors such as temperature, maternal behavior, and nutritional deprivation can affect central autonomic sites and/or innervation of target tissues that are important for immune modulation [reviewed in 46]. The HPA axis also is susceptible to programming during fetal and neonatal development [reviewed in 47]. Many different manipulations in pre- and postnatal development can program the HPA axis in adult primates, guinea pigs, sheep and rats, including maternal stress, exposure to synthetic GC (sGC) and nutrient restriction prenatally, and neonatal handling, modified maternal behavior, exposure to sGC and infection postnatally.

The timing of HPA axis maturation relative to birth is highly species specific, and is closely linked to landmarks of brain development [48]. Maximal brain growth and a large proportion of neuroendocrine maturation occur *in utero* in animals that give birth to precocious young (e.g., sheep, guinea pigs, and primates) [48, 49]. However, in species that give birth to non-precocious young (e.g. rats, rabbits, and mice), neuroendocrine development mostly occurs postnatally [50]. Thus, the effect of pre- and perinatal manipulations on neuroendocrine development depends on the developmental stage relative to the manipulation and the species under study. Evaluating the effects of postnatal manipulations must consider changes in maternal–infant interactions, which also have major influences on HPA development and subsequent function [reviewed in 47].

Most prenatal stress studies have been performed in rats, with fewer studies in the primate, guinea pig and cow [reviewed in 47]. The prenatal stress paradigm typically involves maternal exposure to stress 1 to 3 times a day, either throughout or at selected time points during pregnancy. In rat pups, prenatal stress is generally linked with higher HPA responsiveness, and generally females are more susceptible to prenatal stress-induced programming than males [51]. However, there are conflicting reports that may result from differences in the time of day when blood samples were taken,

since prenatal stress induces a phase advance in the circadian rhythm of GC secretion [52]. In male offspring born to prenatally stressed mothers, most studies report no difference in basal HPA activity and reduced HPA responses to stress [53]; however, tremendous variability in HPA outcome exists, due to differences in the prenatal stress paradigms and variability in the methods used to activate HPA function in the offspring. Short-duration (30 minutes) of prenatal stress in late gestation enhances hypothalamic neuron differentiation in rat fetuses (both sexes). This is associated with CRH mRNA upregulation in the PVN of the hypothalamus and greater maturity of CRH neuron terminals in the hypothalamic median eminence [54]. In contrast, neurotoxic changes in fetal PVN occur with maternal stress of longer duration (240 minutes), but it is not known whether these effects persist into adult life.

Prenatal stress also affects GC feedback regulation of the brain. Adult female rats born to prenatally stressed mothers express fewer hippocampal GR and mineralocorticoid receptors (MR) compared with control offspring, consistent with heightened HPA activity [51, 52]. In studies where prenatal stress has no effect in male offspring, hippocampal GR binding is not affected [51]. When prenatal stress increases HPA activity in adult male offspring, expression of hippocampal MR is reduced [55]. Diverse experimental paradigms make many of these studies difficult to compare. Furthermore, the timing of the insult is crucial, particularly if data are to be compared between species. Interpretation of the data is also complicated because prenatal stress alters other aspects of maternal physiology. For example, repeated restraint reduces maternal food intake and causes weight loss, which can independently modify HPA function and behavior in her offspring [reviewed in 47]. Prenatal stress can also alter maternal behavior towards her offspring, having a major impact on offspring development.

The mechanism(s) by which prenatal stress programs HPA activity and behavior in offspring is/are not entirely clear. Stress changes many cardiovascular and endocrine parameters in the mother; endocrine parameters include increased ACTH, β-endorphin, GCs, and catecholamine secretion. The placenta forms a structural and biochemical barrier to many of these maternal factors. Alternatively, indirect effects on the fetus may consequently modify placental function. For example, catecholamines can constrict placental blood vessels and cause fetal hypoxia [56], which activates the fetal HPA axis [49]. Still, GC are a primary candidate for programming the fetal HPA axis during prenatal stress. Sustained increases or removal of GC during development [52; reviewed in 47] can permanently modify brain structure and function [reviewed in 47]. Greater GC transfer across the placenta of female compared with male fetuses may account for larger effects of prenatal stress in females [57]. Normally, access to maternal endogenous GC by the fetus is low because 11 β-hydroxysteroid dehydrogenase (11β-HSD) (particularly, placental 11β-HSD2) synthesized in the placenta inactivates GC [58]. Prenatal stress such as nutritional deficiencies can reduce 11β-HSD activity in several sites of men, pigs, and rats, causing changes in HPA activity [reviewed in 47]. Studies examining HPA programming using maternal adrenalectomy with replacement therapies suggest that maternal GC, or a GC-stimulated factor, pass to the fetus to mediate prenatal stress-induced changes in HPA function [59] by altering hippocampal GR and/or MR expression and central neurotransmitter systems to affect autonomic and limbic circuitry that regulate the HPA axis [reviewed in 47].

HPA/SNS Programming and Immunity

A variety of environmental factors alter the programming of the neuroendocrine-immune axis during early life, including: (1) neonatal handling stress, (2) maternal stress, deprivation and malnutrition, (3) neonatal cold stress, and (4) maternal, fetal, or neonatal treatment with certain hormones, neuropeptides, bacterial antigens, or pro-inflammatory cytokines [reviewed in 60]. Early interactions with mother and peers are necessary for normal behavioral and physiological development, including immunocompetence. For example, removal of juvenile monkeys from either the mother or another young juvenile transiently reduces lymphocyte proliferative responses [61], the number of circulating T cells, and NK cells activity for several days in the separated juvenile monkeys [62].

Isolation-reared monkeys have different immunological profiles. As adults, isolation-reared rhesus macaques have a lower ratio of Th to T-suppressor cells, an increased proportion of NK cells, and reduced survival rates compared with non-isolation-reared infants [63]. Environments in which infant monkeys are reared by humans in a nursery but routinely socialized with peers also have a long-lasting impact on immune development even though this form of rearing produces few, if any, behavioral anomalies [64]. Mitogen-induced lymphocyte responses were greater in 5-8-month-old nursery-reared rhesus macaques than mother-reared infants. Although levels were still elevated 1.5 years later, by 2.5 years of age only responses to concanavalin A were still significantly elevated [65]. Nursery-reared infants also had a significantly higher ratio of $CD4^+$ to $CD8^+$ T cells between 6 and 24 months of age and consistently lower NK cell activity than infants reared with their mothers [66]. Mitogen stimulation profiles in young monkeys reared by their mothers but weaned early (6 months of age) were intermediate between the profiles of nursery-reared and mother-reared monkeys until they were more than 1 year old, the age at which macaques are normally weaned from their mothers under free-ranging conditions [6, 7]. Nursery-rearing also affects other physiological set points. Nursery-reared infants reared with cloth surrogates or in continuous housing with peers showed lower basal cortisol levels at 14, 30, and 60 days of age than did mother-reared infants [6, 8]. Some of these effects on immune profiles are undoubtedly due to the psychological and physiological impact of rearing without regulatory input from the mother [41]. Collectively, these studies show that exposure of infant monkeys to psychosocial stressors affects immune function. It is not known whether altered immune functions from differences in rearing conditions increases the risk of disease.

One of the best studied immunological stressors on neuroendocrine programming is LPS. Similar to maternal sGC treatment, intra-amniotic injection of LPS (and IL-1β) during early and late ovine pregnancy stimulate inflammation in the fetal lung and lung development [reviewed in 60]. In adult rats challenged with LPS as neonates, HPA responsiveness is altered and presumed to be, at least partly, responsible for long-term effects on immune regulation. Neonatal LPS challenge can significant affect allergic sensitization, and subsequent Th2-mediated conditions such as hay fever, atopic dermatitis, and atopic asthma. Neonatal exposure to LPS attenuated LPS-induced fever

and hypothalamic cyclooxygenase-2 activity in adult rats. Systemic administration of LPS isolated from *Salmonella enteritidis* to neonatal Sprague-Dawley pups raised basal plasma GC concentrations and CRH and AVP mRNA expression in the PVN compared with the saline-treated pups [68]. These animals also exhibited a prolonged increase in plasma CG concentrations in response to auditory stress compared with the controls. When rats challenged with LPS during the neonatal period are challenged with *Mycobacterium butyricum* (complete Freund's adjuvant or CFA) to induce arthritis in adulthood, LPS-treated rats did not display a significant increase in hind paw volume, an effect associated with significantly higher plasma GC concentrations, than either saline-treated or neonatally handled rats. Since the LPS and handled treatment groups responded differently to challenge with CFA, other pathways independent of the HPA axis may be programmed during neonatal inflammatory stress [68]. Several rodent studies indicate that neonatal challenge with LPS leads to HPA hyper-responsiveness during adulthood, affecting responses to psychosocial and restraint stressors. In rats neonatally treated with LPS that have heightened HPA responsiveness to stress, NK cell activity was suppressed, and lung tumor colonization in response to MADB106 tumor cell challenge was enhanced [69]. Neonatal LPS-induced changes in immune function during adulthood may be attributed to reduced GR density in neural circuits that control HPA outflow, impairing GC feedback regulation with consequent HPA hyper-responsiveness to stress [68].

SUMMARY

Activating the SNS or HPA axis can enhance or inhibit immune reactivity depending on such factors as the magnitude and timing of sympathetic/HPA activation relative to immunization, the lymphocyte subsets participating in the response, age, and genetic background of the host. This complexity suggests that neural regulation of immunity is important in maintaining the balance between a rapid and effective response to an antigen while minimizing tissue destruction. Further work is needed to elucidate the mechanisms by which catecholamines and GC regulate immune function across life span, before we can apply this knowledge to the therapeutic treatment of human immune-related diseases, including chronic inflammatory diseases, cancer, neurodegenerative disorders, infectious diseases, and autoimmunity. The pre- and perinatal environment programs HPA and SNS function and associated behavior throughout life. The phenotype of HPA and SNS function following perinatal manipulation depends clearly on the timing, duration and intensity of the environmental manipulation, and gender. Studies are rapidly unraveling the mechanisms that underlie HPA programming, but information is limited on the mechanisms through which environmental manipulations alter SNS programming. A better understanding of these mechanisms could facilitate the development of therapeutic interventions focused at reversing the long-term consequences of experiencing an adverse intrauterine/neonatal environment that lead to pathophysiological diseases later in young and aging adults.

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29 Stress, Immune Function, and Resistance to Disease: Human and Rodent Models

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INTRODUCTION

Stress has been defined as a condition arising from events or environmental demands that exceed an individual's perceived ability to cope.[1,2] Pioneering work by Selye and others suggests that stress is associated with increases in the occurrence of various diseases in humans, including infectious diseases and cancer.[3,4] These studies led to the formation of the interdisciplinary field of psychoneuroimmunology (PNI) that focuses on the influence of the stress response on the interactions among the central nervous system (CNS), the endocrine system, and the immune system, and its impact on health. Several types of stressors in humans and rodent models have been utilized to show the diversity of effects that the various stressors can have on the immune system.[5–9] Studies have shown that although chronic stress (persisting for weeks, months, or years) generally has a negative effect on the immune system, acute stress (lasting from minutes to hours) can result in the up-regulation of some immune parameters thereby exacerbating clinical symptoms such as skin allergies and asthma. Studies have shown

that psychological stress can dysregulate immune responses by modulating the bi-directional communication between the nervous, endocrine, and immune systems, within this complex network.[10–12] This chapter provides a review of human studies and animal models that focuses on stress and its effects on the immune response against infectious agents, emphasizing the implications of these effects on resistance to disease.

Interactions among the Nervous, Endocrine, and Immune Systems

Psychological stress may influence the immune system by activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic-adrenal-medullary axis (SAM). The well-described innervation of primary and secondary lymphoid tissues by the autonomic nervous system also has been implicated in stress-related modulation of the immune response. These pathways operate by producing biological mediators that interact with and affect cellular components of the immune system.[13]

The factors involved in the interactions among the nervous, endocrine, and immune systems have been described. Stress-induced activation of the HPA axis influences the immune system by the release of neuroendocrine hormones from the pituitary gland, stimulating secretion of corticotrophin-releasing hormone (CRH) by the hypothalamus. CRH subsequently promotes the release of adrenocorticotropic hormone (ACTH) and corticosterone by the pituitary gland and the adrenal cortex, respectively. Lymphoid and myeloid cells express receptors for neuroendocrine hormones and neuropeptides; signals from the HPA axis bind to these receptors and up- or downregulate cellular activity. In addition, these cells have receptors for catecholamines (e.g., epinephrine and norepinephrine) that enable them to respond to signals from the SAM axis. These "stress" hormones can dysregulate immune responses, including synthesis of cytokines, sufficiently to increase the incidence of various diseases.

Stress-related immune dysregulation also involves the synthesis of hormones including ACTH, growth hormone, and prolactin.[14–17] Interestingly, it has also been demonstrated that various aspects of the immune response, for example., proliferation of B- and T-cells, cytokine production, antibody production, chemotaxis of monocytes and neutrophils, and natural killer (NK) cell cytotoxicity, can be affected by glucocorticoids (including cortisol) as well as peptides such as ACTH, CRH, endorphins, substance P, and somatostatin.[12,18]

In addition to the activation of the HPA and SAM axes, primary and secondary lymphoid organs, including bone marrow, thymus, spleen, and lymph nodes, are innervated by noradrenergic sympathetic and peptidergic nerve fibers of the autonomic nervous system.[19–21] The close association of these nerve terminals with immune cells facilitates direct neural-immune interaction through the formation of neuroeffector junctions. Norepinephrine, substance P and other neurotransmitters are released at these junctions and can subsequently affect immune cells in the immediate proximity (for example, the microenvironment of a lymph node), or at a distance, thereby modulating their activity. Collectively, stress hormones released by the CNS, HPA and SAM axes thus mediate changes in immune cell function and cell trafficking.

STRESS AND IMMUNE FUNCTION

STRESS-ASSOCIATED DYSREGULATION OF CYTOKINE PRODUCTION

Shifts in the Th1/Th2 cytokine balance have been shown to potentially affect health outcomes in individuals including the progression of infectious diseases (e.g., HIV infection) and autoimmune diseases (e.g., rheumatoid arthritis).[22] It has been shown that psychological stress brings about the dysregulation of this Th1/Th2 cytokine balance. For instance, observations by Marshall and co-workers[23] demonstrate that psychological stress associated with medical student exams can produce a shift in the Th1/Th2 cytokine balance, resulting in the prevalence of a Th2 response. We have confirmed these data and shown that the chronic stress of caregiving for a dementia patient is also associated with a Th1/Th2 shift. [24] This shift could be related to the downregulation of several immune measures we have observed in studies with healthy medical students. These include decreases in NK cell activity, the response of peripheral blood lymphocytes (PBLs) to mitogens, production of interferon-gamma (IFN-γ) by PBLs stimulated with Concanavalin A (Con A), antibody and virus specific T-cell responses to hepatitis B vaccination, and changes in the ability of the immune system to control the steady state expression of latent infections with Epstein Barr virus (EBV) and herpes simplex virus-1 (HSV-1).[25–29]

The stress-associated shift in Th1/Th2 cytokines observed in human subjects has also been demonstrated in restraint-stressed mice. Associated with the shift towards a Th2 response are a significant decrease in NK cell activity, decreased IFN-γ production by Con A-stimulated splenocytes, and a concomitant increase in serum corticosterone levels after 24 hours of restraint.[30] These observations are consistent with studies on the effects of restraint stress and the immune response to viral infection. Restraint-stressed mice exhibited a decline in NK cell activity and a decrease in the generation of virus-specific cytotoxic T lymphocytes to HSV-1 after primary infection, which resulted in an increase in the replication of the virus at the site of infection.[31,32]

STRESS-RELATED SUSCEPTIBILITY TO VIRAL INFECTIONS

Several studies have shown that psychological stress can cause significant changes in susceptibility to a viral infection(s). For example, Cohen and colleagues[2] determined the extent of stress-associated modulation of the immune response by inoculating healthy human subjects with one of five strains of live respiratory viruses. These authors observed an association between psychological stress and an increased risk for developing an acute respiratory infection. In addition, higher levels of psychological stress were also associated with a greater expression of illness (measured by more severe symptoms and greater mucus production) and an increase in IL-6 production by PBLs.[33] However, despite the strong association between psychological stress and the incidence of upper respiratory infection, it has been observed that only a fraction of individuals experiencing high levels of psychological stress actually develop the illness. Therefore, Cohen

and others further examined whether vulnerability to upper respiratory illness can be predicted by modulation of levels of the various components of the HPA axis, the SNS, and the immune response in individuals exposed to acute stressors. In a study of 115 students (age = 18 to 30 years), susceptibility to upper respiratory illness was shown to be associated with higher salivary cortisol levels (i.e., HPA axis activation) and lesser magnitude of immune responses (i.e., NK cytotoxicity and circulating $CD8^+$ cells) but were not associated with SNS markers of reactivity (i.e., blood pressure, heart rate, epinephrine, or norepinephrine).[34]

Effect of Stress on Reactivation of Latent Herpesviruses

Elucidating the mechanism of stress-related reactivation of human herpesviruses is of great interest for researchers and clinicians alike since the reactivation of these viruses in immune-compromised individuals can cause severe complications and death.[35] Of great interest to investigators in PNI is the effect of stress-induced immunomodulation on the reactivation of latent EBV and HSV-1. After primary infection by a herpesvirus, they latently infect a specific target cell(s) (e.g., for EBV these are B lymphocytes and nasopharyngeal epithelial cells) and persist for the duration of the host's life.[36] In healthy individuals, viral latency is efficiently maintained by the host's immune system, with the cellular immune response playing a major role.[37] However, these latent herpes viruses can be reactivated in individuals whose cellular immune response is in some way compromised. Examples are those observed in patients receiving organ transplants, in individuals affected by immunosuppressive diseases (e.g., AIDS) or in people who are experiencing stressful situations. It is believed that the loss of immunological control over viral latency is modulated by changes in the cellular immune response. Viral reactivation, results in the increased production of viral antigens which induces specific antibody production by memory B cells.

In order to further elucidate the factors involved in stress-associated reactivation of latent herpesviruses our laboratory utilized the paradigm of academic stress in medical students. When EBV antibody titers were compared in plasma samples obtained approximately one month prior to and then during several examination periods, it was observed that EBV IgG antibody titers to EBV-virus capsid antigen (VCA) were higher in samples obtained during exams, indicating a change in the control of the latent virus genome resulting in viral reactivation. We also found that examination stress reduced the memory EBV-specific cytotoxic T-cell response.[27] However, the one-to-one relationship between the change in antibody titer and the change in T-cell responses was not examined precluding the inference of any predictive nature of these factors as it relates to disease. Consistent with these results are data showing a decrease in the EBV-specific memory response of PBLs to several purified EBV polypeptides.[38] Proliferation of memory EBV-specific cytotoxic T-cells in blood samples obtained during an examination was downregulated compared to cells obtained from the pre-exam blood samples; PBLs from the students exhibited a lower T-cell proliferative response to 5 of 6 EBV polypeptides tested.[38]

Similar results were obtained in studies with Alzheimer's disease (AD) spousal

caregivers. Caregivers had higher EBV-VCA IgG antibody titers than matched controls.[39] In addition, AD caregivers exhibited significantly higher HSV-1 IgG antibody titers than matched controls, and a poorer HSV-1 specific memory T-cell response.[40]

The hypothesis that psychological stress exerts an effect on the control of latent herpesviruses is further supported by work by Esterling, Schneiderman and co-workers. These authors provided evidence for an association between emotional repression and the reactivation of latent EBV in 80 first-year undergraduate students.[41] They also examined the impact of two behavioral interventions (i.e., aerobic exercise and cognitive behavioral stress management) on virus latency and found changes in EBV-VCA IgG and human herpes virus-6 (HHV-6) antibody levels among 65 asymptomatic gay men. They found that both interventions helped buffer the impact of stress on latent EBV (measured by EBV-VCA antibody titers) and HHV-6.[42] They also found that control over viral latency was associated with differences in coping styles among 54 first-year undergraduate students.[43] Finally, in 76 healthy EBV seropositive undergraduates, they demonstrated that cognitive changes that took place during an emotional disclosure induction protocol can modulate control of EBV latency as measured by differences in EBV-VCA antibody titers.[44] These observations support the hypothesis that stress, both acute and chronic, can play a role in the modulation of herpesviruses latency.

There is substantial evidence to show that various kinds of stress could have different physiological outcomes with different patterns of virus reactivation. For example, the reactivation of EBV, HSV-1, and HHV-6 was studied in West Point cadets experiencing two different types of stressors, survival training (physical) and examination (psychological).[45] Blood samples were obtained before and after a 6-week training period referred to as "Beast Barracks," at a baseline about a month prior to final examinations, and finally during the week of final examinations. EBV-VCA IgG antibody titers remained unchanged in blood samples obtained prior to and immediately after Beast Barracks, suggesting physical stress did not have an impact on immune control of viral latency. However, EBV antibody titers were significantly higher in the blood samples obtained during examination week than at the baseline period before examination. None of the serum samples were positive for EBV-VCA IgM antibodies, indicating that the changes in antibody titers to EBV were not associated with recent EBV infections in the class. The antibody pattern to HSV-1 was somewhat similar to the antibody pattern to EBV except that the antibody titer to HSV-1 observed in the serum samples obtained during the final exam did not reach statistical significance. No significant changes in antibody titers to HSV-6 were found over the identical time periods, including the examination week. Thus, academic stress, but not survival training stress, modulated the steady-state expression of latent EBV (and perhaps HSV-1), resulting in the reactivation of the latent virus(es). The data provide additional evidence for the impact of different stressors on the steady-state expression of latent herpesviruses.

Consistent with this hypothesis are data from a study with mice. Social disruption of groups of 5 male BALB/c mice was shown to increase aggression among cohorts, activate the HPA axis, and reactivate latent HSV-1 in more than 40% of latently infected mice.[46] In contrast, restraint stress, which can down-regulate the innate and HSV-1 specific immune response, did not reactivate latent HSV-1.[47] HPA activation, as measured by serum corticosterone levels, was comparable using both types of stressors.

In spite of this, only social reorganization stress reactivated the latent virus. This study further supports the hypothesis that psychological stress is capable of reactivating latent herpesviruses, and shows that not all stressors produce the same health outcomes, implying that there are differences in physiological pathways associated with different types of stressors.

Stress-Related Effects on Immune Responses to Vaccinations

Several lines of evidence from human studies and animal models suggest that psychological stress can modulate cellular and humoral immune responses to vaccinations.[48,49] In order to test how psychological stress may affect an individual's ability to respond to a primary antigen (a vaccine or a virus), we inoculated 48 medical students with a recombinant hepatitis B (Hep B) vaccine.[28] The students were inoculated with the Hep B vaccine in a series of three injections over six months, administering the injections during a three-day examination period. Our results showed that medical students who experienced more stress and less social support exhibited a delay in seroconversion, and had a poorer antibody response to the Hep B vaccine after seroconversion at the 6-month time point post-inoculation. These students also showed a decreased virus-specific T-cell response to a Hep B viral protein *in vitro*. These results were confirmed in studies by Jabaaij and colleagues using a lower dosage of a recombinant Hep B vaccine.[50] However, no relationship between psychological stress and antibody production was observed when volunteers were inoculated with a higher dose of the vaccine, suggesting that the extent of stress-induced immunomodulation *in vivo* (for Hep B) may depend on antigen dose.[51] Cohen and others[52] observed that high trait negative affect (also known as neuroticism) and a diminished T-cell proliferative response to PHA were correlated with a lower antibody response in 84 healthy graduate students inoculated with standard course of three Hep B injections. This study suggests that individuals with high trait negative affect (who tend to be moody, nervous and easily stressed) have less protective immune responses.

In a similar study on AD caregivers inoculated with an influenza virus vaccine,[53] subjects that were vaccinated with a trivalent Fluzone vaccine, 32 caregivers (mean age = 73.12 ± 8.64 years) showed a poorer antibody response to the vaccine (defined by a four-fold increase in antibody titers), than the 32 well-matched controls that were immunized during the same time period. PBLs from these subjects also exhibited less IL-2 production when exposed to the vaccine *in vitro*, which is indicative of the virus specific T-cell response to the vaccine. In addition, PBLs obtained from caregivers produced less IL-1ß when stimulated with lipopolysaccharide (LPS) than PBLs from control subjects. We further showed that these deficits in immune responses persisted in former caregivers as well.[54] A similar study by Vedhara and colleagues using an influenza virus vaccine compared the antibody response of 50 elderly spousal caregivers of patients with dementia to 67 control subjects. Antibody titers were measured on the day of immunization, and at 1, 2, and 4 weeks after immunization. Caregivers were less likely to develop a four-fold increase in antibody response to any of the three influenza virus components of the vaccine. They also reported an inverse relationship

between the level of salivary cortisol and IgG antibody titers to one of the influenza virus strains in the vaccine.[55] Together, these studies suggest that psychological stress can modulate immune responses to viral antigens that could be sufficient to put individuals (particularly older individuals) at risk for developing infectious diseases. Our laboratory had previously shown that caregivers reported significantly more days of illness with upper respiratory tract infections.[39] This observation is significant since normal aging has been shown to be associated with immunosenescence which may make individuals more susceptible to stress-associated decreases in cellular immunity.[56]

The results obtained from the academic stress and older AD caregivers suggest that the tendency of a stress-induced shift in Th1 toward Th2 pattern of cytokine synthesis is not absolute, and that humoral immunity is also subject to suppression by stress. We conclude this based on results showing stress-induced changes in both the antibody and T-cell response components of the immune response.[54,57]

In a study of 52 older subjects, it was found that stress could also impact the antibody response to a pneumococcal bacterial vaccine.[57] We measured antibody titers of current caregivers (mean age = 68.09 ± 3.80 years), former caregivers (mean age = 72.46 ± 2.29 years), and control subjects (mean age = 69.54 ±1.69 years), just before vaccination, and at 2 weeks, 1 month, 3 months, and 6 months after vaccination. We found no group differences in the initial antibody response to the vaccine between these groups. However, mean antibody titers of current caregivers diminished significantly over the 6-month study, while antibody titers to the vaccine remained stable in both former caregivers and control subjects. Results implied that the IgG antibody patterns in the three groups over 6 months could be the result of either changes in the number of IgG-producing cells or the level of IgG being produced by the same number of cells. These data further support the evidence of health risks associated with dementia caregiving described above. In addition, these data provided the first evidence to show that chronic stress can inhibit the stability of the IgG antibody response to a bacterial vaccine.

Similar outcomes from studies using other vaccines have been observed. For example, Irwin and colleagues found that major depression was associated with a marked decline in varicela zoster virus (VZV)-specific cellular immunity, as measured by the ability of PBLs to proliferate in response to VZV antigens.[58] Additionally, in a study with 240 Israeli schoolgirls (mean age =12.4 ± 2 years) there was a correlation between psychological variables (i.e., internalizing, neuroticism, self-esteem, and post-vaccination fatigue syndrome) measured at baseline and antibody titers to a rubella vaccine assessed at 10.5 weeks post-immunization, but only in subjects that were seronegative at baseline.[59] For example, higher self-esteem predicted higher antibody titers to rubella virus following vaccination.

Recently, Miller and colleagues examined whether moderate stressors could modulate the antibody response to an influenza virus vaccination in 83 healthy young adults. These authors observed that subjects experiencing higher levels of stress exhibited poorer antibody responses to the New Caledonia strain of the vaccine. It is of interest that stress ratings assessed 2 days before the administration of the vaccine and on the day it was given were not associated with the antibody response. However, psychological stress experienced 10 days after vaccination was associated with the subjects' immune response. In addition, feelings of stress and loss of sleep seems to be

involved in the mechanism, resulting in the diminished humoral immune response. No association between salivary cortisol levels, alcohol consumption, physical activity, or cigarette smoking and immune response was found.[60]

Studies by Burns and colleagues examined whether mild chronic stress experienced by young, healthy adults results in an impaired immune response to influenza vaccination. Antibody titers and psychological status were assessed prior to vaccination and 5 weeks and 5 months later, with a four-fold increase in antibody against at least one viral strain considered as protective. Their data showed that subjects who did not present a four-fold increase in antibody levels reported significantly more stressful life events and perceived stress than those who had a four-fold increase in antibody levels 5 months after vaccination. This study further supports the idea that psychological stress can affect the long-term maintenance of antibody levels following vaccination even in young, healthy adults.[61,62]

Rodent models have been utilized to elucidate mechanisms of stress-related effects on immune function. Studies by Bonneau and colleagues examined the effects of acute maternal stress on antiviral immunity in the offspring.[63,64] Neonates are heavily dependent upon maternally derived, HSV-specific antibody for resistance to HSV infection. Previous studies have documented a decreased transfer of maternal IgG antibody and immunocompetence of the offspring following perinatal exposure to stress-induced corticosterone. However, it was demonstrated that the transplacental transfer of relatively high amounts of HSV-specific antibody is resilient to acute maternal stress thereby protecting neonatal mice against HSV-2-associated mortality. It was further shown that transplacentally acquired HSV-specific antibody declines rapidly in neonate serum such that by 7 days after birth only 10% of this antibody remains (which is still protective against HSV-associated mortality in 7-day-old mice). This overall kinetics of HSV-specific antibody decline was not affected by prenatal stress. In further studies, an immunization strategy that elicits low levels of HSV-specific antibody in maternal serum was used. Despite a stress-induced increase in corticosterone, the prenatal transfer and protective capacity of low amounts of HSV-specific antibody remained intact during acute maternal stress.[65]

Sheridan and colleagues used restraint stress to examine the effects of psychological stress on natural killer (NK) activity and its impact on influenza strain A/PR8 viral replication in mice. Their data showed that restraint stress delayed the recruitment of NK1.1+ cells into the lung parenchyma during infection, and resulted in lower gene expression of MIP-1α and MCP-1 (chemokines responsible for NK cell recruitment). Additionally, restraint stress suppressed the gene expression of IL-12 and IL-15, macrophage-derived cytokines involved in the response of NK cells. They hypothesized that the restraint stress-mediated reduction in NK cell numbers and function would enable viral replication to continue unchecked. This was supported by an enhanced viral replication observed in the lungs of restraint stressed animals. Interestingly, IFN-α and IFN-ß gene expression were elevated, presumably in response to the increased viral load in the stressed mice. Together, these data show that restraint stress suppressed expression of the cytokine genes involved in the recruitment and activation of NK cells during an experimental influenza viral infection resulting in diminished NK cell function and enhanced viral replication.[66]

Stress-related effects on the control of other viruses have also been described. For instance, Welsh and others have used the role of restraint stress in Theiler's virus infection in mice as a model for multiple sclerosis. It was shown that restraint stressed CBA mice had higher levels of mortality following infection with Theiler's virus. They proposed that this was due to high levels of stress-induced corticosterone, which resulted in decreased numbers of circulating lymphocytes, decreased inflammatory cell infiltrates into the brain and consequently decreased viral clearance from the CNS.[67] Restraint stressed mice also developed clinical signs of encephalitis, thymic atrophy, and adrenal hypertrophy. Decreased numbers of circulating lymphocytes and increased number of neutrophils were observed in the stressed mice. Stressed mice also had lower numbers of splenocytes, which correlated with the decreased numbers of lymphocytes in circulation. Restraint stress caused elevations in serum tumor necrosis factor alpha (TNF-α). Virus-induced NK cell cytotoxic activity was significantly reduced in restrained mice at one day after infection perhaps accounting for the reduced viral clearance from the CNS. These data suggest that stress-induced immunosuppression of cytolytic NK cell activity may account in part for the reduced ability to clear virus from the CNS and for the increased mortality observed in this model.[68]

Stress and Tumorigenesis

Results from both animal and human studies suggest that psychological factors may affect cancer development and progression.[69–71] Classic studies by Riley and colleagues have elegantly shown that a stress-associated decrease in cell-mediated immunity resulted in increased tumor development in rodents exposed to an oncogenic RNA virus. For example, exposure to physical stressors has been shown to result in an increase in incidence and severity of tumor development in mice inoculated with murine sarcoma virus.[72] The role of "immune surveillance" in cancer progression has been supported by data showing that subjects who are immunosuppressed have an increased risk for cancer, particularly patients with immunogenic tumors.[73] Studies have focused on the effects of psychological stressors on NK cell activity, as mediated by neuroendocrine hormones and cytokines, because of their important role in the surveillance of tumors.[74,75] There are a few studies that support that stress could affect the development of abnormal/tumor cells. One study in humans showed that psychological stressors can affect cellular DNA repair in PBLs. It was demonstrated that lymphocytes from 28 non-psychotic, non-medicated psychiatric patients exhibited a reduced ability for DNA repair after X-irradiation relative to age- and gender-matched controls. In addition, within this group, those patients with higher levels of depression showed significantly poorer DNA repair capability than their less depressed counterparts.[76] Another human study using the academic stress paradigm, on the other hand, observed an increased DNA repair capacity in PBLs from 12 of 16 first- or second-year medical students during a five-day examination period, compared to vacation period three weeks later.[77] The contradiction between these two studies can be explained by the difference in methodologies used. The assay for DNA repair in the first study involved the repair of DNA in PBLs after X-irradiation[76] while the study by Cohen and colleagues[77] measured the ability of PBLs

to repair DNA that was previously damaged by UV-irradiation and then transfected into cells. In addition, different subject populations (psychiatric patients in the former study, and examination stress in healthy medical students in the latter) may have led to the different outcomes. That different stressors can result in distinct physiological outcomes are supported by previously discussed studies on stress-induced viral reactivation in mice and humans.[45–47] While the two studies on DNA repair seem contradictory at first, they more importantly support the hypothesis that psychological stressors can affect DNA repair. The study by Cohen and co-workers further suggest an increased occurrence of DNA damage in response to stress.[77]

A higher mutation rate in response to stress is also supported by data from a study with rats showing that several different types of stress can induce sister chromatid exchange (SCE) in bone marrow cells.[78] The processes for repair or removal of damaged DNA are important parts of the initiation events for tumorigenesis, since faulty DNA repair has been associated with an increased incidence of cancer.[79]

In a further study supporting the hypothesis that psychological stress impairs the DNA repair process, it was observed that the levels of O^6-methyl guanine methyltransferase, a DNA repair enzyme induced by exposure to carcinogens, were significantly lower in splenic lymphocytes from rats exposed to rotational stress, as compared with splenic lymphocytes obtained from control rats.[80] Thus, consistent with the studies with human subjects, these data also suggest that stress may alter the DNA repair process and affect the persistence of damaged DNA, therefore increasing the chance of the generation of abnormal cells.

Another mechanism by which stressors can affect carcinogenesis is through the modulation of apoptosis. For example, work from our laboratory has shown a stress-related inhibition of apoptosis in human PBLs in an examination stress study with medical students.[81] Low concentrations of the tumor-promoting phorbol ester, 12-0-tetradeconoyl-phorbol-13-acetate (TPA), blocked ionizing radiation-induced apoptosis in PBLs. Academic stress enhanced this process.[81] Furthermore, there was an apparent inhibition of cellular DNA fragmentation, and a significant increase in total cellular DNA in surviving PBLs. Considering the data on the effects of psychological stress on DNA damage and repair, and apoptosis, it is important to note that if stress can modulate apoptosis, the survival of cells with abnormal levels of DNA could increase the risk of the development of malignant cells. The above data suggest that the effect of psychological stress on cancer initiation and progression may be through both stress-induced immunomodulation of tumor-specific T-cell responses and NK cell activity and direct effects on genomic DNA.

Recent data from our laboratory suggest that behavioral effects on more downstream events may contribute to another aspect of the process of tumorigenesis (i.e., tumor metastasis). Matrix metalloproteinases (MMPs) belong to a family of structurally-related zinc-dependent endopeptidases that, together, are capable of degrading all extracellular matrix (ECM) molecules.[82] These enzymes have been implicated in the degradation of the ECM during metastasis.[83,84] As several cytokines, including IL-1 and IL-8, have been implicated in the control of MMP expression,[85–89] it is conceivable that the stress-associated increase in incidence and/or progression of cancer is mediated (at least in part) by modulation of expression of MMPs and their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMP). We have recently shown that two

stress hormones, cortisol and norepinephrine, may play important roles in the modulation of MMP expression.[90]

The hypothesis that stress can modulate MMP expression is also supported by studies in mice. Using social isolation as a stressor, the mRNA levels of MMP-2, MMP-9, matrix-type matrix metalloproteinase-1 (MT1-MMP), and urokinase-type plasminogen activator were higher in the tumor and liver tissues of the isolated mice than in control mice.[91] Furthermore, a recent study has shown that restraint stress causes an increase in expression of the plasminogen activator inhibitor-1, another key player in the plasminogen/plasmin enzyme system in mice.[92] As these enzymes have been described to have functions besides their role in ECM remodeling,[93] studies on stress-related effects on MMP/TIMP balance have implications in the relationship between stress and cancer initiation and progression.

RODENT MODELS AND THE ELUCIDATION OF THE ROLE OF STRESS ON THE EXACERBATION OF DISEASE

A glucocorticoid-resistance model has been proposed to provide an explanation for how stress might influence diseases in which excessive inflammation is observed (e.g., allergies, autoimmune diseases, rheumatoid arthritis, and cardiovascular disease). In these cases, chronic stress diminishes the immune system's sensitivity to glucocorticoids that normally terminate the inflammatory response. For example, in a study of a group of 50 parents caring for a child undergoing treatment for pediatric cancer, whole blood of parents of cancer patients exhibited a lesser dexamethasone-dependent suppression of IL-6 production *in vitro* compared to parents of medically healthy children.[94]

The mechanism involved in this phenomenon of glucocorticoid resistance has been examined in mice. Using the social stress paradigm, Sheridan and colleagues showed that splenocytes from stressed animals showed enhanced survival and proliferative response to LPS, that, unlike splenocytes from control animals, were not suppressed by exogenous glucocorticoids (i.e., glucocorticoid resistance).[95] Further studies indicate that glucocorticoid resistance is not due to any modification at the DNA or RNA levels but is due to diminished glucocorticoid receptor function, particularly in $CD11^+$ monocytes/macrophages. This was associated with the inability of glucocorticoids to suppress the activity of NF-κB and the lack of nuclear translocation of the glucocorticoid receptor.[96] Further studies indicate that LPS-induced glucocorticoid-resistance involves a two-step process that is mediated by Toll-like receptor 4 (TLR4). TLRs are the mammalian homologues of the *Drosophila* Toll receptor and were found to play a role in recognition of bacterial antigens and have been implicated in the control of innate and adaptive immunity in vertebrates.[97]

DISCUSSION AND DIRECTIONS FOR FUTURE RESEARCH

The studies on stress-associated immunoregulation reviewed here are of great interest to both basic scientists and clinicians. Whereas these results implicate stress in the dysregulation of the cellular immune response in healthy individuals, of greater importance

is the implications of these observations for individuals whose cellular immune response is in some way compromised, as occurs in organ transplant patients, in individuals affected by AIDS, and in the elderly. It is important to note that small changes in immune parameters that we, and others, observed in the studies reviewed here are associated with the health outcomes. These relationships are similar to the relatively small changes in the immune response associated with immunotoxic xenobiotics.[98,99] We suggest that the field of PNI provides a literature that can be useful in designing studies to determine the health implications of immunotoxicant exposure that may produce mild to moderate immune dysregulation.[1,98]

Greater understanding of the signaling mechanisms that cause dysregulation of the cellular immune response will better enable the medical community to deal with problems that are caused by stress and depression, perhaps through stress intervention modalities that may help reduce the impact of stress on disease.[100–103] To this end, rodent stress models have been developed and successfully utilized for the elucidation of the mechanisms involved in stress-related changes in the immune system. Use of these models can overcome the sampling limitations that one encounters in human studies. For example, Pruett and colleagues have shown that serum corticosterone levels (expressed as the area under the corticosterone concentration vs. time curve (AUC)) can be used as an excellent predictor of the effects of restraint stress and chemical stressors on a variety of immunological parameters in the mouse spleen and thymus. However, since stress-related changes in immune responses in humans can only be assessed through blood samples, these authors further examined the utility of the AUC of circulating corticosterone level as a predictor of suppressed host immune function in mice. It was observed that among all blood parameters measured, only MHC II expression showed consistent quantitative associations with corticosterone AUC.[104] This further suggests that both human studies and rodent models are needed to fully understand the relationship between stress and health.

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30 Recreational Drugs, Immune Function, and Resistance to Infection

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INTRODUCTION AND HISTORICAL BACKGROUND

Widely used illegal drugs such as marijuana, cocaine, and opiates, including heroin, have been extensively studied for effects on host physiology, especially for effects on the immune system. In particular, recreational use of these drugs has aroused serious concerns about the consequences that drug abuse may have on resistance to infectious diseases. Numerous clinical studies have addressed the use of illegal drugs, susceptibility to infections and the mechanisms involved. In addition, excessive use of legal addictive psychoactive substances, such as alcohol and nicotine, correlates with major health problems, including hospitalization of alcoholics and heavy smokers for infectious diseases.

Studies concerning the effects of addictive drugs on immunocompetence have taken on greater urgency with the onset and explosive expansion of the worldwide pandemic of acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV). AIDS causes collapse of the immune system, rendering individuals highly susceptible to opportunistic microorganisms which normally would not cause life threatening infections.[1–2] Drugs of abuse have been suggested as possible co-factors in a more rapid progression of AIDS by altering susceptibility to infectious diseases.[3–7] Approximately one third of HIV-infected patients are intravenous drug abusers and it is believed that contaminated needles or equivalent paraphernalia spreads

the virus. Furthermore, AIDS patients often use other drugs such as marijuana, alcohol, and nicotine, which are now known to be immunosuppressive.

EFFECTS OF MARIJUANA COMPONENTS ON THE IMMUNE RESPONSE AND SUSCEPTIBILITY TO INFECTION

Cannabus sativa, also known as marijuana, is the most frequently used illicit drug in the United States. A significant percentage of individuals, especially those less than 20 to 25 years of age, are frequent users. Marijuana has been recognized for centuries as a therapeutic agent, in particular as an analgesic, muscle relaxant, appetite stimulator, and anti-convulsant. Chemically, marijuana contains over 400 compounds and more than 60 are psychoactive cannabinoids, with the major component being delta-9-tetrahydrocannabinol (THC).

This drug has marked immunomodulatory effects and can alter the normal function of T and B lymphocytes, NK cells and macrophages, both *in vivo* and *in vitro*, in humans and animals (Table 30.1).[9,10] Although the molecular and cellular mechanisms for these effects are not fully defined, it is believed that both receptor and non-receptor mechanisms are involved. Cannabinoid receptors (CBRs) are G-protein coupled 7-

TABLE 30.1
THC and Other Cannabinoids Induced Modulation of Immune Functions *in vivo* and *in vitro*

Mode of Administration	Activity	Effect
In Vivo		
Human	Lymphocyte proliferation	Decrease
	Anti-microbial activity	Decrease
	Cytokine production	Decrease
Mice	Serum Ig levels	Variable
	Cellular immunity	Decrease
	Anti-microbial activity	Decrease
	Humoral immunity	Decrease
	Apoptosis	Increase
	Cell signaling	Variable
In Vitro		
Human	Lymphocyte proliferation	Decrease
	NK cell activity	Decrease
	Neutrophil function	Decrease
	Cytokine production	Variable
Mice	Lymphocyte proliferation	Decrease
	NK cell activity	Decrease
	Antibody formation	Decrease
	IL-2 system receptors	Decrease
	Cytokine production	Variable
	Apoptosis	Increase
	Cell signaling	Variable

trans-membrane molecules; two receptors, CB1 and CB2, have been identified. The CB1 receptor is expressed in the brain and certain peripheral tissues and is responsible mainly for psychoactive neurologic effects, while CB2 receptors are located in the periphery, especially on immune cells. Identification of CBRs led to the identification of endogenous cannabinoids that bind these receptors; the majority of these compounds are eicosanoides.[9–14] The broad spectrum of effects on immune function mediated by THC binding to CB1 receptors in the CNS and CB2 receptor on immune cells results in decreased host resistance to bacterial and viral infections, shown by various studies with THC treated experimental animal models.[15–8] THC was first isolated and subsequently synthesized approximately three decades ago and it was shown that treatment of animals *in vivo* or human and animal lymphoid cells *in vitro* suppressed many immune functions, including lymphocyte proliferation, antibody formation and cytotoxic activity. However, some studies indicated that THC could enhance certain immune functions. For example, B cell proliferation occasionally increased with THC treatment at uM concentrations, as did production of chemokines such as MIP1a and IL-8. However, *in vitro* exposure to greater THC concentrations usually decreased cytokine levels. These results indicate that THC, as well as other cannabinoids, are actually immunomodulatory, affecting the immune response in both a negative and positive manner (Table 30.1).

In vivo studies showed that *in vitro* proliferation of lymphocytes isolated from marijuana smokers is suppressed, especially with heavy marijuana smoking, and that the relative proportion of lymphocyte subpopulations was also altered. Concentrations of serum IgG are decreased and IgE concentrations are increased in marijuana smokers. Furthermore, phagocytic and bactericidal activity of alveolar macrophages from heavy marijuana smokers are decreased. These effects translate into reduced host resistance following administration of cannabinoids, including THC, in both humans and animals (Table 30.2). Increased susceptibility has been demonstrated to opportunistic microbes including HIV, Herpes simplex virus, Friend leukemia virus *Listeria*, *Treponema pallidum, and Legionella.*

There are still major gaps in fully understanding the cellular and molecular mechanisms mediating effects on immunity and resistance to microbial infection.

TABLE 30.2
Cannabinoid Effects *in vivo* on Resistance to Infections

Infectious Agent	Host Species	Effect
HSV	Mice	Mortality
Listeria	Mice	Mortality
HSV	Human	Recurrence
HSV	Guinea pigs	Infection
FLV + HSV	Mice	Mortality
Staphylococci	Rats	Lung infection
Treponema pallidum	Rabbits	Progression
Legionella pneumophila	Mice	Mortality
Staphylococci	Rats	Mφ activity reduced
HIV	Human	Mortality risk

At least some of the cannabinoid-induced modulation of immune cells, and effects on host resistance to infection, are mediated directly by binding of cannabinoids to CBRs, particularly CBR2. Host immunity to microbes, however, involves many cell types, both immune and non-immune, as well as soluble factors including cytokines, chemokines, neurocytokines, and hormones related to the HP axis. It is therefore likely that a variety of cellular and molecular mechanisms whereby cannabinoids, including THC, affect immune function.

Detailed studies have been performed with the model opportunistic intracellular bacterium *Legionella pneumophila*, an ubiquitous intracellular microorganism known to be the etiologic agent of Legionnaire's disease.[17,18] Th1 cell mediated immune responses are crucial for resistance to and recovery from Legionella infection, whereas Th2 responses, important for humoral immunity, are nonprotective. THC pretreatment of mice infected with *Legionella* affects both innate and adaptive immunity. Mice given THC injections one day before and one day after sublethal challenge infection with *L. pneumophila* die of septic shock resulting from production of high levels of proinflammatory cytokines. THC rapidly induces production of proinflammatory cytokines as shown in CB2 transfected HL-60 cells stimulated with the cannabinoid agonist CP55,940. Furthermore, a single injection of THC in mice 18 hours before infection inhibits development of Th1 immunity. This involves both CB1 and CB2 receptors; Th1 cell development is inhibited by reduced production of IFNγ and IL-12 as well as reduced expression of IL-12Rβ2 mRNA. This cannabinoid-induced shift away from a protective Th1 response has been reported in other animal models involving THC treatment and tumor immunity.

OPIATE EFFECTS ON IMMUNITY AND SUSCEPTIBILITY TO INFECTIONS

Opiates comprise a collection of drugs derived from *Papaver somniferum*, including opium, morphine and heroin.[19] Opiates have had a major impact throughout history on mankind, both from abuse and from wars for control of opium. There is evidence that there was widespread poppy cultivation even in the Stone Age. The term "opium" is derived from the Greek word meaning "of sap" or "juice," since the drug is obtained from the juice of the poppy plant. In recent centuries, the addictive nature of opium was recognized. For example, in the early 1800s morphine, and later codeine, heroin, and other opium alkaloids, were synthesized from opium and often used medicinally. Nevertheless, during the late 1800s and early 1900s many physicians began to recognize infections as a serious complication of opiate addiction, and experiments conducted in the late 1890s showed that morphine altered phagocytosis and leukocyte trafficking in guinea pigs. It became quite evident in the early part of the last century that many common infections were associated with opiate use.[20–24]

It is now recognized that opiates reduce resistance to a variety of infectious agents in both humans and animals. These effects are summarized in Table 30.3 and Table 30.4 and discussed below. For example, pulmonary infections caused by mycobacteria, staphylococci, streptococci, *Hemophilus* and other common organisms are frequent among opium abusers.[23,24] Other infectious diseases caused by microbial pathogens in

TABLE 30.3
Opiates-Induced Modulation of Immune Functions *In Vivo* and *In Vitro*

Mode of Administration	Receptor Mediation	Immune Function	Activity
In vivo			
Rodents	+	Phagocytosis	Decrease
	+	Antibody production	Decrease
	+	Mitogen- proliferation	Decrease
	+	NK/ CTL Activity	Decrease
	+	Cytokine production	Decrease
	+	Serum IL-6 Levels	Increase
	NE*	LPS-induced sepsis	Increase
	+	IL-12	Increase
	+	Cutaneous hypersensitivity	Decrease
Humans	NE	Phagocytosis	Decrease
	NE	NK activity	Decrease
		Antibody-Dependent Cell Cytotoxicity (ADCC)	Decrease
Monkeys	NE	Chemotaxis	Decrease
	NE	PMN killing	Decrease
Pigs	NE	Cutaneous hypersensitivity	Decrease
In vitro			
Mice	+	Phagocytosis	Decrease
	+	Antibody Induction	Decrease
Humans	+	Chemotaxis	Decrease
	NE	Superoxide	Decrease
	+	Cytokine production	Variable
	+	Chemokine production	Variable
	+	Phagocytosis	Increase

*NE = Not examined

i.v. drug abusers include AIDS (HIV), endocarditis (*Staphylococcus, Enterococcus*, *Pseudomonas, Klebsiella, Serratia*, and *Candida*), cellulites (*Staphylococcus*, *Streptococcus, Hemophilus, Enterobacter, Pseudomonas, Klebsiella, Clostridia, Candida*, and others), hepatitis A, B, and C, sexually transmitted infectious diseases and musculoskeletal infections (S*taphylococcus* and *Pseudomonas*).

TABLE 30.4
Biologic Effect of Opiates on Microbial Infection

Microorganism	Host Species	Activity
Salmonella typhimurium	Mice	Gut colonization
Toxoplasma gondii	Mice	Lethality
Escherichia coli	Mice	Sepsis
HSV-1	Mice	Infection
FLV	Mice	Mortality
Candida albicans	Mice	Mortality
Swine Herpesvirus\ *Pasteurella multocida*	Pigs	Pneumonia

A large percentage of infections among i.v. heroin abusers is associated with the methods of injection and lifestyle practices which increase exposure to microbial pathogens.[24–26] Although the use of contaminated needles or other drug paraphernalia often exposes the user to infectious agents, there is strong evidence that opiate-induced immunosuppression may be a cofactor for microbial infections. For example, various studies support the view that intravenous use of opiates increases the risk of acquiring HIV and influences the outcome of HIV infection. It is noteworthy that mortality rates from infectious diseases among HIV-infected i.v. drug abusers has decreased by discontinuing drug abuse and such abatement correlates with a decrease in progression rate to AIDS.[26] Therefore, a correlation between increased intravenous opiate use, increased susceptibility to infection, and depressed immunity is apparent. However, whether this correlation is due to increased exposure to infectious pathogens by risky behaviors, to the immunosuppressive effects of opiates or to a combination of both is still uncertain.

Several opiate receptors have been identified on cells of the nervous systems of animals and humans, with mu (μ), kappa (κ), and gamma (γ) subtypes being predominant. These classical opiate receptors are G- protein coupled 7-transmembrane molecules.[27] Opiates predominantly affect immune responses directly by ligation of μ, κ, and γ opiate receptors, as well as non-classical opiate-like receptors, on immune cells and indirectly by binding to receptors on CNS cells. Studies conducted *in vitro* with opiate-treated immune cells demonstrated receptor-mediated reduced phagocytosis, chemotaxis and cytokine and chemokine production. These effects are linked to modulation of host resistance to bacterial, protozoan, viral and fungal infections using animal models, cell lines and primary cells.

While opiates are known to directly modulate host immunity, their effects on physiological function of nonspecific host mechanisms and altered immune responses play an important role in increased susceptibility to infection. Many effects are thought to act through the central nervous system, especially via the hypothalamus-pituitary axis (HPA) by stimulating the release of corticotrophin-releasing hormone and adrenocorticotropic hormone, thus increasing circulating levels of glucocorticoids and corticosterone, the end effectors of the HP axis.[28] These mediators have an important role in regulating cellular immune responses and are known to suppress many protective immune parameters. In addition to the effects of corticoids, immunosuppression by the autonomic nervous system also may occur. For example, NK cell activity in experimental animals is suppressed via opiate receptors following morphine injection into the lateral ventrical of the rat brain[29,30] and CNS pathways are likewise involved in opiate suppression of lymphocyte proliferation.[31,32] Increased production of the immunosuppressive cytokine TGF-β1 by morphine treated lymphocytes is another indirect method by which opiates suppress immunity.[33] Thus, opiates suppress immunity by both direct and indirect mechanisms involving receptors on both CNS and immune cells.

COCAINE AND INFECTIONS

Cocaine is derived from the coca plant *Erythroxylon coca*. This drug is a water soluble alkaloid that is readily absorbed through mucous membranes. Cocaine's activity is due

to effects, at least partially, through the sigma 1 receptor, a protein first proposed to be involved with morphine binding. Receptors are distributed throughout the brain and periphery of an individual, similar to classical opiate and cannabinoid receptors.[34,35] Various *in vivo* and *in vitro* studies have addressed cocaine-induced modulation of immune responses and infection (Table 30.5 and Table 30.6). However, most studies concerning cocaine infections have centered on HIV and progression to AIDS.

Immunologic studies on i.v. drug abusers with AIDS have linked abuse of cocaine, more than other drugs, to the increased incidence of HIV seroprevelance and progression of AIDS.[36] Cocaine increases HIV replication in peripheral blood mononuclear cells (PBMCs) *in vivo* (Table 30.6). Cocaine increased the numbers of HIV-infected PBMCs and viral load, as well as decreased the CD4:CD8 ratio, in human PBMCs implanted into severe combined immunodeficient mice.[37] Immunomodulation may be due to receptors located in the periphery, since cocaine-induced suppression of mitogen stimulated lymphoproliferation.[38] Because of the possible link to AIDS, there is increasing interest in understanding the mechanism(s) by which cocaine affects immunity and alters susceptibility to infectious diseases.

NICOTINE EFFECTS ON IMMUNITY AND RESISTANCE TO INFECTION

It is widely accepted that cigarette smoking is linked to community acquired pneumonia and is one of the major risk factors for respiratory infections.[39,40] Cigarette smoke is composed of two components, the vapor and particulate phase. The immunosuppressive effect of tobacco smoke is partly due to nicotine, which occurs in the particulate portion. Nicotine, as well as other immunotoxins in tobacco smoke, are thought to be respon-

TABLE 30.5
Cocaine-Induced Modulation of Immune Functions

Mode of Administration	Activity	Effect
In vivo		
Human	Anti-microbial activity	Decrease
	Cytokine production	Variable
Rodents	Lymphocyte proliferation	Decrease
	Antibody formation	Decrease
	Cellular hypersensitivity	Decrease
	Humoral immunity	Variable
	Cytokine production	Variable
In vitro		
Human	HIV replication	Increase
	Lymphocyte proliferation	Decrease
	Cytokine production	Decrease
Rodents	Cytokine production	Decrease
	Lymphocyte proliferation	Decrease
	NK cell activity	Decrease

TABLE 30.6
Cocaine-Induced Altered Susceptibility

Infectious Agent	Host Species	Effect
LP-BM5	Mice	Increase cryptosporidiosis
HIV	SCID mice implanted with human PBMCs	Increase HIV-infected PBL Increase virus load Decrease CD4:CD8

sible for inhibitory effects on immune responses. Nicotine is a small organic alkaloid synthesized by the tobacco plant and is recognized as the major addictive components of cigarettes. While its small molecular nature allows nicotine to cross directly through cell membranes, the primary biological effects are receptor mediated. Nicotine is an agonist for nicotinic acetylcholine receptors (nAChRs) present on cells of the nervous system as well as other cells throughout the body, including immune cells.[41] Neuronal AChRs are known to be upregulated in smokers.[42] Nicotine from cigarette smoke rapidly accumulates in the brain and increases dopamine transmission within the shell of the nucleus accumbens, the region of the brain associated with reward processing that is associated with the addictive properties of other drugs, including opiates, alcohol, and cannabinoids.

It is now recognized that nicotine and cigarette smoke can affect immunity (Table 30.7). There is increasing evidence that nicotine induces glucocorticoid release via the HPA and directly affects cells of the immune system through ligation of nAChRs receptors on CNS and immune cells. Nicotine also inhibited production of IL-6, TNFα and IL-12 in murine alveolar macrophages infected with *Legionella pneumophila* via specific interaction with the acetylcholine receptor.[43] Nicotine affects murine splenocyte production of Th1 and Th2 associated cytokines by interacting with AChRs. Chronic nicotine treatment of rats induces T cell anergy, depletes intracellular IP3-intracellular Ca2+ stores and inhibits antibody forming cell responses and lymphocyte proliferation,

TABLE 30.7
Effects of Nicotine and Cigarette Smoke on Host Resistance Mechanism

Host Species	Immune functions	Effect
In vivo		
Rats	Antibody forming cells	Decrease
	Intracellular Ca^{++} stores	Decrease
	Lymphocyte proliferation	Decrease
	T-cell anergy	Increase
	Anti-microbial activity	Decrease
In vitro		
Human	NK activity	Decrease
	Cytokine production	Decrease
Mice	Splenocyte proliferation	Decrease
	Cytokine production	Variable
	Anti-microbial activity	Decrease

contributing to suppressed protective immune responses to microbial pathogens in experimental animals.[46,47] Animals exposed to cigarette smoke in inhalation chambers evince increased susceptibility to infections with aerosolized bacteria or viruses.[48] In humans, smoking among HIV positive individuals has also been shown to increase susceptibility to infection.[49] The mechanism by which nicotine increases or alters susceptibility to infectious diseases is important and further studies of the nature of immunomodulatory effects of nicotine by interaction with specific receptors are warranted.

ALCOHOL MODULATION OF RESISTANCE TO INFECTIOUS DISEASES

Alcohol abusers are known to experience a variety of health problems, including decreased liver function and increased rates of infectious diseases including community acquired infection, such as pneumonias.[49–51] Moderate alcohol use (1 beer or 1 glass of wine or 1 mixed drink per day) is thought by some physicians to be beneficial, but excessive alcohol use is known to be detrimental to health. Alcohol, unlike other addictive drugs of abuse, does not appear to bind to a specific receptor. During the last few decades, it was found that alcohol has multiple effects on host immune responses to microbial pathogens (Table 30.8). Alcohol may deplete circulating lymphocyte populations and alter lymphoid organ architecture and immune function. Furthermore, studies

TABLE 30.8
Alcohol Effects on Immune Functions.

Host Species	Immune functions	Effect
In vivo		
Mice	Cytokine production	Decrease
	NK activity	Decrease
	IgA and IgG production	Decrease
	Apoptosis	Increase
	Cellular hypersensitivity	Decrease
Rat	TNFα production	Decrease
	Serum chemokines	Decrease
	TNFα processing	Decrease
	Alveolar nitric oxide	Decrease
	Chemokine production	Decrease
Humans	Monocyte proliferation	Decrease
In vitro		
Mice	Macrophage killing	Decrease
	Bactericidal capacity	Increase
	Cytokine production	Decrease
Humans	Macrophage killing	Decrease
	Cytokine production	Variable
	TNFα receptor	Decrease
	NFκB activation	Decrease
	T-cell proliferation	Decrease
Rhesus macaques	TNFα	Decrease

in both animals and humans have shown that, *in vitro* and *in vivo*, exposure to alcohol suppresses production of cytokines important in antimicrobial immunity, such as TNFα secreted by macrophages from rats and Rhesus macaques.[53–55] Suppression of TNFα is a post-transcriptional event and involves TNFα converting enzyme (TACE) mediated processing of TNFα.[56] Furthermore, alcohol inhibits LPS-induced $NF_{\kappa}\beta$ activation, a transcription factor for inflammatory cytokines.[57] Of particular interest are the reports that excessive alcohol use decreases Th1 cytokine responses that provide protection against certain infections and increases Th2 cytokines.[58,59] Administration of IL-12, a cytokine important for Th1 activity, attenuates suppressed cell mediated immune response in alcohol-consuming mice.

Rodents given alcohol orally show marked modulation of immune cell function and increased susceptibility to infection (Table 30.9). For example, ethanol treatment augments intracellular survival of *Mycobacterium avium* complex and compromises macrophage responses to cytokines.[60] Ethanol treatment also increases the growth of *L. pneumophila* in non-permissive macrophage cultures *in vitro*.[61] Extensive studies have also shown that immune cells from mice given alcohol and infected with various intracellular bacteria such as *Salmonella* or *Listeria* show increased susceptibility to these bacteria.[62] Excessive alcohol abuse has also been linked to increased viral infection (Table 30.9), including Herpesvirus infection in adolescent women.[63] Furthermore, experimental studies show that alcohol alters cytokine responses of mice infected with a retrovirus that causes a murine AIDS-like syndrome.[64] Besides the report that alcohol exacerbates opportunistic infections in the murine AIDS-like syndrome, increased opportunistic infections due to alcohol abuse is believed to correlate with AIDS progression.[65, 66] In addition, Hepatitis B virus infection has also been linked to chronic liver disease in alcoholics and animal studies show that alcohol enhances liver damage by activating CD8+ cells and increased apoptosis.[67,68] Thus, animal models and clinical studies suggest that alcohol abuse is clearly detrimental to the host and related to increased susceptibility to microbial infection.

TABLE 30.9
The Effects of Alcohol on Infection Susceptibility

Infectious Agent	Host Species	Effect
Listeria	Mice	Decrease infection
Salmonella	Mice	Decrease infection
Streptococcus	Mice	Decrease infection
Mycobacterium	Mice	Increase disease
Mycobacterium	Rats	Decrease bactericidal
LP-BM5 retrovirus	Mice	Increase disease
LP-BM5 retrovirus	Mice	Increase *C. parvum* infection
LP-BM5 retrovirus	Mice	Increase Coxsackievirus myocarditis
Herpesvirus	Guinea pigs	Increase viremia

DISCUSSION AND CONCLUSIONS

Clinical and experimental studies have established a relationship between the use of drugs of abuse and increased susceptibility to infectious diseases, including AIDS. These compounds may act indirectly by stimulating the HPA axis, resulting in glucocorticoid production and dysregulation of the immune system, or directly by ligation of specific receptors. Receptor mediated effects on immune responses and infection have been studied in detail for many years, at first with opiates and then successfully with cannabinoids and more recently with nicotine. It is evident that some common mechanisms for the immunomodulatory effects of drugs of abuse exist, especially effects on Th1/Th2 responses, either by inhibition of Th1- or elevation of Th2-associated cytokines.

The apparent causal correlation between intravenous drug abuse and HIV infection has led investigators to propose that immunomodulation mediated by drugs is an important factor contributing to progression of AIDS in i.v. drug abusers. Although it is not yet possible to determine a definitive cause and effect relationship from epidemiologic studies, there is a growing consensus among investigators that drug-induced immunomodulation is involved. Results of studies on immunosuppression by drugs of abuse support the view that increased susceptibility to opportunistic infectious pathogens is related to alteration of immune responsiveness by the drugs. And, while there is convincing evidence that behavioral and social practices connected with drug abuse contributes to increased exposure to infectious pathogens, many investigators believe that a combination of increased exposure to microbes and drug-induced immunomodulation contributes to heightened susceptibility to infectious pathogens. There is a general consensus by investigators, both clinical and basic scientists, that it is essential to determine in-depth mechanisms by which drugs of abuse, both illicit and legal, compromise immune responses in general, especially in concert with immunosuppressive viruses.

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Part IX

Allergy and Hypersensitivity

31 Allergy to Chemicals and Proteins: An Introduction

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INTRODUCTION

Allergic diseases are the sixth leading cause of chronic illness in the United States, affecting roughly 17% of the population, and costing the health care system about $18 billion annually [1]. These diseases create issues for toxicologists in both occupational and private settings. The factors affecting the induction and expression of allergies are complex and can involve a variety of immune responses. Genes, lifestyle, and environmental exposure all appear to have roles in the development of allergic disease. Everyday exposures including air pollutants, pesticides, drugs, household products, cosmetics, animal dander, occupational exposures, and even the food we eat, can all contribute to allergic disease.

Xenobiotics can affect allergic disease in one of two ways. They can act as allergens and elicit hypersensitivity responses, or they can modulate hypersensitivity responses to other allergens such as pollen or dust mite by either acting as adjuvants, enhancing the development or expression of hypersensitivity responses, or by down regulating responses to other allergens as has been shown with co-exposure to endotoxin [2]. Xenobiotics that act as allergens include certain proteins that can by themselves induce an immune response, and low molecular weight chemicals (known as haptens). Haptens are physically too small to induce a specific immune response, but are chemically reactive and covalently bind to larger molecules, usually a protein, to form chemical-protein moieties of sufficient size and foreigness to induce an immune response that is then hapten specific.

Hypersensitivity (allergy) is defined as humoral or cellular responses to an otherwise innocuous antigen, which can lead to tissue damage. Hypersensitivity reactions

are divided into four types (originally proposed by Gell and Coombs) based on immunologic mechanism (Table 31.1) [3]. Although this classification is useful to understand mechanisms, often hypersensitivity responses are mixed involving more than one type. In all cases, hypersensitivity responses develop in two stages. (1) Induction (acquisition of sensitization) requires a sufficient single or cumulative exposure dose of the sensitizing agent to induce immune responses (i.e., development of antigen specific antibody or clonal expansion of antigen specific T cells). Obvious symptoms are generally not observed at this stage. (2) Elicitation occurs in sensitized individuals upon subsequent exposure to the same antigen and results in adverse responses that include inflammation. The dose responses for these two stages are different although not entirely unrelated.

MECHANISMS OF IMMUNE-MEDIATED INJURY (HYPERSENSITIVITY)

Type I hypersensitivity is mediated by antigen specific cytophilic antibody (usually IgE) that binds to mast cells and basophils. Some individuals have a genetic predisposition to develop IgE to common allergens (atopy). These individuals, referred to as atopic, are more likely to develop allergic rhinitis and asthma than the general population. In a sensitized individual, upon subsequent exposure, the allergen binds to cell-bound antibodies and cross-links IgE molecules, causing the release of mediators such as histamine and slow-reacting substance of anaphylaxis (SRS-A). These mediators cause vasodilation and leakage of fluid into the tissues, plus sensory nerve stimulation (leading to itching, sneezing and cough). Type I is also called immediate-type hypersensitivity because reactions can occur within minutes after exposure of a previously sensitized individual to the offending antigen. Type I reactions include immediate asthmatic responses to allergen, allergic rhinitis (hay fever), atopic dermatitis (eczema), and acute urticaria (hives). The most severe form is systemic anaphylaxis (e.g., in response to a bee sting), which involves a multisystem response that can result in severe airway obstruction and cardiovascular collapse leading to anaphylactic shock and potentially death.

Type II hypersensitivity is the result of antibody-mediated cytotoxicity that occurs when antibodies (IgG or IgM) are generated to cell surface antigens or haptens bound to the cell surface. Once antibodies bind to antigen on the cell surface they can activate the complement system and/or cytotoxic cells leading to lysis of the target cell. Frequently, red blood cells are the target, as in the case of adverse drug reactions, an incompatible blood transfusion or Rh blood incompatibility between mother and child. The basement membrane of the kidney or lung may also be a target. This type of damage can result from drug treatments with penicillin, quinidine, quinine, or acetaminophen. It is thought that these drugs interact with the cell membrane such that the immune system detects “foreign” antigens on the cell surface.

Type III reactions are the result of antigen-antibody (IgG or IgM) complexes that accumulate in tissues or the circulation, activate macrophages and the complement system, and trigger the influx of granulocytes and lymphocytes (inflammation). Examples include an Arthrus reaction when soluble antigen is injected into the skin of a sensitized individual and serum sickness, which occurs 7 to 10 days following the administration

of therapeutic horse anti-serum (used in pre-antibiotic days to treat some infections). Farmer's lung, a pneumonitis caused by molds has been attributed to both Type III and Type IV hypersensitivity responses.

Unlike the preceding three types, Type IV reactions are mediated by activated T cells rather than antibodies. Much has been learned about T cells since the four hypersensitivity classifications were originally proposed. As a result, the Type IV responses can now be divided into three subtypes, mediated by different populations of T cells, $CD4^+$ T helper (Th) 1 and Th2 cells, and $CD8^+$ cells (Table 31.1). Allergic contact dermatitis is attributed primarily to Th1 cells. These $CD4^+$ cells recognize modified extracellular proteins presented in the context of major histocompatibility complex (MHC) II molecules and activate macrophages, which release a variety of cytokines and chemokines, leading to inflammation characterized by the influx of neutrophils. $CD8^+$ T cells are cytotoxic and attack cells bearing modified intracellular proteins which are presented on the cell surface in the context of MHC I antigens. Allergic contact dermatitis to poison ivy, for example, occurs through this mechanism. These reactions generally occur 24 to 48 hours after exposure in a previously sensitized individual and are thus referred to as delayed-type hypersensitivity.Th2 cells facilitate the antibody class switch to IgE and mobilize and activate eosinophils and mast cells. Chronic responses in allergic asthma are, in part, attributable to Th2 cells.

There is evidence that chemicals can cause allergic disease based on each of these mechanisms [4]. These four mechanisms are not mutually exclusive; hence, more than one of these responses may be involved in reactions to a given allergen. Also, the resulting pathologies, particularly those caused by Type III and some Type IV responses may appear very similar although the mechanisms leading to the effect are different. The remainder of this chapter will focus on hypersensitivity problems that are most commonly encountered by toxicologists.

ALLERGIC CONTACT DERMATITIS

As the protective barrier between the body and the external environment, the skin is exposed to potentially damaging chemicals on a daily basis. It has been estimated that approximately 80,000 chemicals are in common use worldwide [5] and new products are being continuously introduced. Chemical production for most countries increased in 2004 with an increase in the total production index for all chemicals of 2.9% in the United States, 4.3% in the United Kingdom, and 6.8% in Canada [6]. As new products are being developed and chemical production increases, toxicologists are faced with the need to develop improved methods for evaluating the potential of these chemicals to induce contact dermatitis.

Skin diseases are the second most common form of occupational disease with contact dermatitis accounting for 10 to 15% of all occupational illnesses with an annual associated cost of at least $1 billion [7]. Although irritant dermatitis accounts for the majority of cases of contact dermatitis, studies have reported that contact allergies are relevant in between 20 to 50% of contact dermatitis cases [8]. In 2003 there were 49 cases of occupational skin disease reported for every 100,000 workers in private

TABLE 31.1
Mechanisms of Immune Mediated Injury (Hypersensitivity)

Type	Antigen	Induction (initial exposure to antigen)	Elicitation (re-exposure to antigen)	Example
I immediate	Soluble	Clonal expansion B cells Cyto-philic antibody (IgE) generated; binds to mast cells	Antigen binds to cell bound antibody; cross-links receptors, causing release of mediators	Anaphylactic response to bee sting; immediate response in allergic asthma
II cytolytic	Cell-associated	Clonal expansion B cells; IgM, IgG generated.	Ig binds to cell bound antigen; in the presence of complement and/or activated macrophages cell lysis occurs	Rh factor incompatability, hemolytic anemeia in reaction to drugs
III	Soluble	Clonal expansion of B cells; IgM, IgG generated	Recall Ig response; antigen antibody complexes form in some tissues leading to activation of macrophages and inflammation	Glomerular nephritis, Serum sick-ness, endocarditis acute hypersensi-tivity pneumonitis
IV Delayed, cell mediated				
Th1	Soluble	Antigen presented by MHC II; Clonal expansion CD4Th1	Th1 cells activated, release cytokines, activate macrophages, inflammation	Tuberculin Rx, contact dermatitis, berylliosis
Th2	soluble	Antigen presented by MHC II; Clonal expansion CD4 Th2	Th2 cells activated; Help for IgE production; eosinophil and mast cell activation	Chronic allergic rhinitis and asthma
CD8	Cell associated	Antigen presented by MHC I; clonal expansion of CD8 cells	Activation of cytotoxic (CD-8) T cells	Contact dermatitis (poison ivy); Chronic hypersensitivity pneumonitis

industry alone (U.S. Bureau of Labor Statistics) and it is believed that occupational skin disease is severely underreported and that the morbidity associated with workplace chemical exposure is much higher.

Unlike other types of chemically induced hypersensitivity, where sensitization may occur following several routes of exposure, exposure leading to allergic contact dermatitis (a Type IV response) is limited to skin exposure. The induction phase of contact hypersensitivity is initiated upon first exposure to a low molecular weight hapten that penetrates the stratum corneum and covalently binds to a protein. The now antigenic hapten-protein conjugate is endocytosed by Langerhans' cells (LC), specialized bone-marrow derived dendritic cells. Under the influence of epidermal (primarily keratinocyte and LC) cytokines, the expression of adhesion molecules on LC is altered and their morphology changes from dendritic to a more rounded appearance. LC process antigen as they migrate through afferent lymphatics to the paracortical region of the draining lymph node where they present the modified antigen to CD4+ T cells (or CD8 cells if the chemical alters intracellular proteins and is presented in the context of MHC I). Antigen specific T cells then undergo clonal expansion generating memory and effector cells. These cells then exit the lymph nodes through efferent lymphatics, and travel through the circulation to the skin (Figure 31.1a). At this phase an individual is considered "sensitized" but may not show any clinical signs resulting from chemical exposure.

The elicitation of a contact hypersensitivity response occurs when a sensitized individual is re-exposed to the antigen. The initial steps are identical to those in the

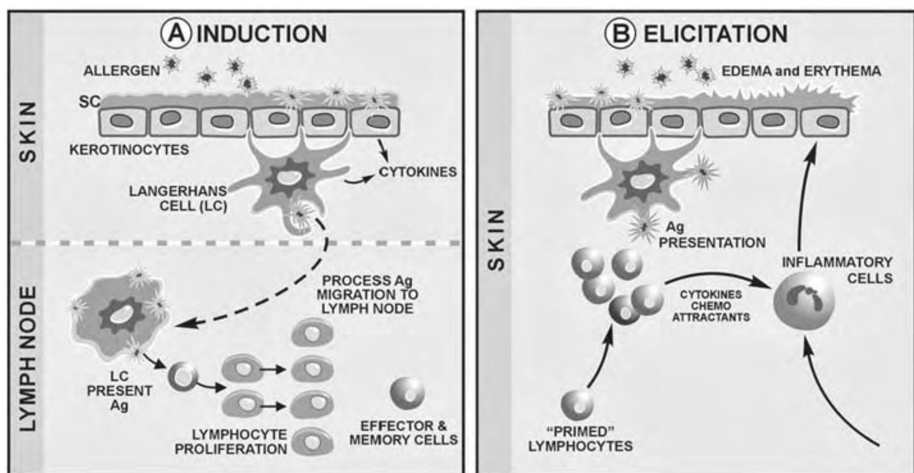

FIGURE 31.1 Schematic of allergic contact dermatitis (ACD): Chemical enters stratum corneum (SC) of skin, covalently binds to protein and is taken up by Langerhans cells (LC). Influenced by cytokines from LC and kerotinocytes, LC process antigen (Ag), migrate to the lymph node, and present Ag to lymphocytes, which then undergo clonal expansion. Memory and effector lymphocytes migrate to skin. On elicitation the process repeats, but Ag presentation takes place in skin, activated lymphocytes release pro-inflammatory cytokine, and inflammation characterized by erythema and edema occurs. See text for description.

induction phase where the hapten penetrates the skin, binds protein, and is picked up and processed by LC. At this stage antigen presentation to memory and effector T cells can take place in the skin as well as in the lymph nodes. Once again clonal expansion takes place and effector CD4 T cells release pro-inflammatory cytokines and chemotractants for other inflammatory cells including macrophages and neutrophils (Figure 31.1b). CD8 cells induce inflammation by killing the eliciting cell and by the release of proinflammatory cytokines.

Numerous factors may affect the development of allergic contact dermatitis including age, sex, genetics, and exposure conditions; these will be discussed in more detail in a subsequent chapter in this section. It appears that the relationship between age and sensitization correlates closely with exposure, and that post-infancy children are as susceptible to developing contact allergy as are adults [9]. This susceptibility appears to decline in old age. As is the case with autoimmunity, the prevalence of contact allergy has been reported to be higher among females than males, and experimental studies have shown that at a given sensitizing dose, a higher percentage of females become sensitized compared to males and the response to challenge is more pronounced [10]. Although a genetic basis for susceptibility to Type IV hypersensitivity to beryllium in the lung has been demonstrated [11], a genetic basis for susceptibility to sensitization with contact allergens is less clear. Evaluation of genetic susceptibility to nickel, one of the most well studied metal allergens, has failed to show a clear association with HLA [12, 13]. Studies have shown that most normal individuals will become sensitized to potent sensitizers when exposed to a sufficient concentration of chemical.

Exposure conditions appear to play the most important role in the induction and elicitation of contact hypersensitivity. These conditions involve factors inherent to the individual, including thickness of the exposed skin and skin condition at the exposure site. External factors including the vehicle, occlusive or non-occlusive conditions, temperature and humidity, and exposure to sunlight may also influence the development of contact allergy.

Differentiation of irritant and allergic contact dermatitis can be challenging, and a more comprehensive discussion of this topic can be found later in chapter 32. Irritation is a nonspecific inflammatory reaction as opposed to inflammation driven by adaptive, antigen-specific immune responses to an allergen. Clinical signs are similar and a thorough history including onset and progression of disease and exposure history are critical because reactions to contact allergens generally grow worse with progressive exposures whereas irritant responses remain the same. Patch testing for allergenicity is often required to make a definitive diagnosis, and although standard allergen panels are available for such testing, exposure history may lead to testing of alternative chemicals, products, or extracts.

Numerous testing strategies are available to toxicologists for the evaluation of the potential for chemicals to induce contact sensitization and as the biology of contact sensitization has become better understood more mechanistically based tests have been developed. Historically, guinea pig models have been used. These methods rely on the induction of sensitization in animals followed by the subjective evaluation of erythema and edema following test article challenge at a naïve site. Two of the most commonly used methods are the Buehler test [14] and the Guinea Pig Maximization Test [15].

The Buehler test utilizes exposure under an occluded patch for the induction of sensitization whereas in addition to exposure under occlusion, induction in the Guinea Pig Maximization Test also involves intradermal injection of the test article and the use of adjuvant. Because these tests evaluate the elicitation of a response in previously sensitized animals, an animal can be sensitized with one chemical and challenged at a naïve site with a related chemical to evaluate cross-reactivity. These tests provide sensitive and reproducible results and have been the mainstay of predictive testing for allergic contact hypersensitivity for decades. Limitations of the assays include their subjective endpoint, difficulty in testing irritant or colored substances and the use of large numbers (generally 20 per group) of animals.

In recent years, additional testing methods have been developed using mouse models. Like the guinea pig assays, the Mouse Ear Swelling Test involves sensitization of animals followed by the evaluation of the elicitation phase of the response. In this assay, mice are sensitized by topical unoccluded exposure to the shaved flank followed by topical challenge on the ear with the evaluation of ear swelling as an indication of a contact hypersensitivity response [16]. The mouse Local Lymph Node assay has undergone extensive development and validation and was the first assay to be peer reviewed by the Inter-Agency Coordinating Committee on the Validation of Alternative Test Methods [17, 18]. This assay evaluates only the induction (sensitization) phase of contact hypersensitivity and uses quantitation of ^{3}H-thymidine incorporation into proliferating draining lymph node cells to evaluate the sensitization potential of chemicals. Advantages over the guinea pig assay include the use of a quantitative endpoint, acquisition of dose response data, reduction in the number of animals used, lack of interference when testing colored materials, and a reduction in time on study and animal distress. By evaluating the dose response, this assay provides data that can be used to calculate an EC3 (the chemical exposure concentration required to induce a three-fold increase in stimulation over control) which can then be used to rank the potency of the chemical [18].

Efforts are underway to develop *in vitro* and computer models for hazard identification of contact sensitizers in order to reduce or replace animal usage. Given that dendritic cells (DC), one of the first cells modulated following chemical exposure, have been shown to be critical for the initiation of contact sensitization, *in vitro* methods have focused on this cell. Studies which have evaluated changes in DC surface markers and cytokine production following exposure to chemical irritants or sensitizers in cell culture have shown promise [19]. The development of dermal sensitization is a complex process and is dependant on properties of the chemical as well as the biology of the exposed individual. These factors pose challenges in the development of quantitative structure-activity relationships for the identification of chemical sensitizers. Programs such as Computer Automated Structure Evaluation (CASE: Multicased, Beachwood, OH) and Multiple Computer Automated Structure Evaluation (Multicase; Multicase) evaluate sensitization potential based on the comparison of the structure of a previously untested chemical with structural alerts found in known chemical sensitizers. In the Toxicity Prediction by Computer Assisted Technology (Topkat: Accelrys, San Diego, CA) chemicals with sensitization data derived from guinea pig assays were used to compute a variety of molecular characteristic and then discriminate analysis was used

to identify relevant characteristics and build the predictive model. Due to the incorporation of an optimum prediction space algorithm into the model, one can be assured that predictions are only made for chemicals within the model domain. The expert system, DEREK for Windows (LHASA Limited, University of Leeds, Leeds, U.K.) uses a rules based system dependant on the mechanistic understanding of the process of sensitization to identify links between chemical structure and sensitization potential. Data used is generated in guinea pigs, mice and humans. For a chemical with unknown sensitizing potential, investigators are provided with a responsible structural motif and a degree of confidence that the chemical is a sensitizer. For a review of quantitative structure activity relationships (QSAR) in the prediction of skin sensitization see Rodford and colleagues [20].

More detailed information on methods to predict the sensitization potential of chemicals can be found in chapter 34.

RESPIRATORY ALLERGY

Although any of the four types of immune mediated injury can occur in the lung as a result of chemical exposure, those that cause rhinitis and asthma via IgE- and Th2-cell-mediated responses are of particular concern. The incidence of atopic disease in general, and asthma in particular, has increased dramatically over the last several decades in the United States and other industrialized nations as a result of ill-defined changes in living conditions in modern Western society [21, 22]. Because this increase is too dramatic to be explained by genetic changes in the population, it is assumed that changes in environment, lifestyle, and/or medical practices must somehow have contributed to the increase. Several recent studies suggest that the prevalence of asthma may be stabilizing, or even beginning to decline in some areas [23–25], at least with respect to certain measures of disease prevalence [26]. Because reasons for the original increases in asthma prevalence remain unclear, an explanation for this apparent stabilization of asthma prevalence reported in some studies also remains elusive. In addition to the high prevalence of asthma in the general population, work-related asthma has become the most frequently diagnosed occupational respiratory illness [27]. An estimated 8 to 20 million workers are exposed to asthmagens [21] in the United States.

In sensitized asthmatic individuals, antigen challenge generally causes a Type I (IgE-mediated) immediate hypersensitivity response by release of preformed mediators, including histamine, and prostaglandins, which are responsible for bronchoconstriction and increased vascular permeability. Between 2 and 8 hours after the immediate response, asthmatics experience a more severe and prolonged (late phase) reaction that is characterized by mucus hyper-secretion, bronchoconstriction, airway hyperresponsiveness to a variety of nonspecific stimuli (e.g., histamine, methacholine), and airway inflammation characterized by eosinophils. This later response is driven by leukotrienes, chemokines and cytokines synthesized by activated mast cells and Th2 cells. Both proteins and haptens have been associated with these types of reactions.

Using animal models, whole body plethysmography has been used to assess both early and late phase airway responses in guinea pigs, rats and mice [2, 28–32]. However,

this approach (somewhat akin to the use of host resistance models to assess immune suppression) does not lend itself to routine toxicity testing due to cost and technical complexity.

For protein allergens, such as detergent enzymes, animal dander, and biotechnology products, induction of cytophilic antibodies in experimental animals (IgE in mouse and rat and IgG1 in guinea pigs) following intratracheal exposure has been used successfully for both hazard identification and to develop dose response data. The best example comes from the detergent industry using guinea pigs and mice [33–35]). In these studies, guinea pigs and, more recently, mice have been used to establish the relative potency of different enzymes based on the dose response following intratracheal instillation. The endpoint measured was serum cytophilic antibody. Subtilisin was chosen as the reference allergen because the American Conference of Government and Industrial Hygienists developed a threshold limit value in the workplace for subtilisin A of 60 ng protein/m^3 based on historic human data. By comparing new enzymes to this reference allergen it was possible to use animal cytophilic antibody responses (IgE and IgG1) to set safe exposure levels for humans [36].

Low molecular weight allergens, including diisocyanates, acid anhydrides, some reactive dyes, and platinum salts present more of a problem. Whereas there is no doubt that IgE antibody plays an important role in respiratory allergy to proteins, there is some debate about the relevance of IgE antibody for the development of occupational asthma in response to these chemical allergens. Although there is evidence that all known chemical respiratory allergens induce specific IgE in some symptomatic subjects, about half of subjects with allergy and asthma associated with diisocyanates do not exhibit this response and have late onset responses in the absence of an immediate response [37–40]. A small portion of subjects with asthma induced by acid anhydrides also exhibit late onset responses (associated with IgG) in the absence of immediate responses[41, 42]. For this reason there has been some debate about the role of IgE in diisocyanate associated asthma.

Evaluation of structure activity relationships has generally been used as the first step in hazard identification because these compounds must be reactive enough to haptenate a larger molecule, usually a host protein. Low molecular weight sensitizers are typically electrophiles, or proelectrophiles, capable of reacting with hydroxyl, amino, and thiol functionalities on proteins [43]. This reactivity can also be tested *in vitro* by mixing the chemical with a protein such as albumin. Sarlo and Clark proposed a tier approach to testing that included four steps: (1) structure activity assessment, (2) assessment of *in vitro* reactivity, (3) assessing cytophilic antibody following respiratory exposure, and finally (4) whole body plethysmography following repeated inhalation exposures [44].

Another tiered approach is based on the notion that low molecular weight chemicals that cause occupational asthma are a subset of a larger group of chemical sensitizers that yield positive results in animal tests for allergic contact dermatitis [45]. Thus, the first step in a tiered approach for identifying chemicals likely to be associated with allergic asthma would be a positive LLNA [45]. The credibility of this approach has been improved by the recent demonstration that dermal exposure can result in sensitization of the respiratory tract [46, 47]. Building on these studies, it has been suggested that

immune responses after dermal exposure might be used to predict whether IgE or Th2 responses would be likely to result from any exposure to a chemical and hence predict the potential to induce allergic asthma. Several possibilities have been suggested for the second tier of testing. One suggested tier two test is the mouse IgE test that bases respiratory hazard identification on elevated total serum IgE levels following dermal exposure to a chemical, relative to total IgE induced by vehicle or a chemical thought not to induce allergic asthma [48, 49]. Another possibility for tier two, cytokine profiling, bases hazard identification on induction of Th2 cytokines in the draining lymph node [45, 50] compared to appropriate negative and vehicle controls. Yet another approach evaluates the local production of IgE in draining lymph nodes following dermal chemical exposure by quantitating IgE bound to CD23 on B cells using flow cytometry [51]. None of these assays has been widely used or validated and to date their application has been limited to hazard identification. Respiratory allergy and occupational asthma and hazard identification for chemical allergens are discussed in more detail in chapters 33 and 34.

Although chemicals that induce asthma associated with IgE and Th2 immune responses have received much attention, chemical sensitization of the lung can occur via delayed type, Th1 mechanisms. The only well-studied example of this type of chemical sensitization is chronic beryllium (Be) disease (CBD), characterized by granulomas and mononuclear cell infiltration in the lung, which results from inhaling airborne Be, most likely in a relatively insoluble form. The latency period for CBD ranges from 1 to 40 years, with an incidence in exposed populations from 1-10%. The Be-specific lymphocyte proliferation test is a diagnostic tool used to confirm CBE. A patient's blood or lung lymphocytes are placed in cell culture with soluble Be and cell proliferation is assessed [52]. This serves as a reminder that lymphocyte proliferation, whether Th1 or Th2 can lead to lung sensitization and disease.

ADJUVANT EFFECTS OF AIR POLLUTANTS

In animal asthma models (including rodents and a nonhuman primate model), air pollutants have been shown to act as adjuvants when combined with experimental allergens, such as ovalbumin or dust mite allergen, resulting in enhanced IgE production, and increased allergic inflammation and airway hyperreactivity [53–57]. The hypothesis is that cytokines and other mediators produced by macrophages and epithelial cells as a result of pollutant exposure create an environment that favors the Th2 response associated with allergy [58]. Introduction of an allergen into such an environment might well result in more robust sensitization than would occur in the absence of air pollutant exposure. Human clinical studies have also demonstrated the adjuvant effects of diesel exhaust particles with respect to allergic (Th2) immune responses [59, 60]. Additionally, results from the five prospective epidemiology studies that support a modest increase in risk of asthma associated with air pollution have been reviewed recently [61]. Whereas there has been evidence for some time that air pollutants exacerbate existing asthma, these studies provide the first evidence that air pollutants may also contribute to the induction of new cases of asthma. An adjuvant effect on allergic sensitization is one

mechanism that could account for this increased risk of asthma and models have been proposed for extrapolation from rodent data to human effects [62].

FOOD ALLERGY

Food allergy is a relatively new concern for toxicologists. The application of biotechnology to food production, particularly genetic modification to confer pest resistance or increase nutritional value, has created the need to consider the potential that novel proteins introduced into the food supply could induce food allergies. Estimates of the prevalence of food allergies vary depending on the study. Approximately 6 to 8% of children suffer from food allergy during their first 3 years of life [63], Many of these children then go on to develop tolerance, and the prevalence of food allergy in adults is approximately 3% [64]. Only a few foods are known to cause the vast majority of food allergies. In children reactions are most commonly caused by eggs, peanuts, milk, soy, and wheat, whereas the most common reactions for adult are to shellfish, fish, tree nuts, and peanuts [65]. The introduction of new foods into a population by conventional methods (e.g., importation of kiwi) demonstrates the possibility that additional foods could be added to this list [66].

True food allergy is distinct from food intolerance in that the latter does not involve immune mechanisms although the symptoms generated may be similar. Food allergies can be caused by IgE-mediated or non-IgE mediated mechanisms although IgE-mediated events are the most common and have generated the most attention. IgE-mediated food allergies can cause symptoms in the skin (acute urticaria/angioedema and atopic dermatitis), the upper and lower respiratory tract, and the gastrointestinal tract. Typically symptoms develop within minutes of ingesting the food. In addition, IgE-mediated reactions to food allergens have been reported to be the leading cause of anaphylaxis seen in emergency departments in the United States. Typically, these patients have been asthmatics who unknowingly ingested the responsible food allergen and failed to recognize the initial symptoms. Anaphylactic shock has also been associated with exercise 2 to 4 hours after the ingestion of certain foods [65]. The skin prick test is typically used to diagnose Ig-E mediated food allergies, however, double-blind placebo-controlled, food challenge tests remain the gold standard for diagnosis [63].

Mechanisms underlying non-IgE mediated food allergy include immune complex formation and activation of lymphocytes. As with IgE-mediated responses, manifestations can be in the skin, gut, or respiratory tract; however, these reactions take several hours to days to develop [65].

The study of food allergy in general and efforts to develop methods to assess the potential allergenic risk of any particular protein have been hampered by lack of a good animal model. Feeding laboratory rodents antigens they have not previously encountered generally produces a state of antigen-specific unresponsiveness known as oral tolerance [67]. This phenomenon is preferentially directed against IgE and delayed-type hypersensitivity responses and can be adoptively transferred to other animals by transplanting T cells (probably CD25+CD4+ regulatory T cells). Tolerance induction is genetically determined and high sensitivity is co-inherited with low-IgE responder-

phenotype, such that 1,000 to 10,000-fold greater allergen exposure is required to tolerize high IgE responders. Tolerance induction also appears to be a function of age in that allergen exposure of neonates primes the individual for subsequent T cell reactivity rather than tolerance, presumably due to delayed postnatal maturation of one or more key elements of mucosal immune function that are rate-limiting in inducing tolerance. Whereas these rodent studies provide interesting insights into susceptibility factors for the development of food allergy and have also spawned studies on the potential therapeutic uses of oral tolerance to prevent autoimmune reactions, these findings present a significant road block in developing an animal model to test proteins for the potential to induce food allergy.

At the present time, the evaluation of the risk of allergenicity posed by proteins relies on the source of the gene from which the protein is derived (i.e., is derived from a known source of allergens), and its physical and chemical properties [65]. These properties include its structural relationship to other known protein allergens, and its resistance to degradation by proteases and acid encountered in the stomach. Resistance to degradation would protect a protein from digestion and allow for greater absorption in the gut and hence greater likelihood of presentation to the immune system.

SUMMARY

Allergic diseases are an important cause of chronic illness. Xenobiotics can cause or exacerbate allergic diseases via a number of mechanisms. This chapter reviews these mechanisms and provides a brief overview of the problems toxicologists most often encounter with respect to allergic disease, including allergic contact dermatitis, respiratory allergy, adjuvant effects of air pollutants, and food allergy. Subsequent chapters provide more in-depth discussions of these topics.

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32 Allergic Contact Dermatitis to Chemicals: Immunological and Clinical Aspects

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INTRODUCTION

The purpose of this chapter is to provide a general overview of the immunobiology of allergic contact dermatitis (ACD), along with review of the clinical aspects associated with the disease. Other detailed reviews covering the biology of ACD are available elsewhere.[1–3] A variety of characteristics determine whether a chemical can function as a contact sensitizer (or allergen), including the ability to penetrate into the skin, react with proteins, and be recognized as antigenic by immune cells. This review focuses primarily on the requirements for the acquisition of skin sensitization (the induction phase of contact allergy). The elicitation of allergic contact dermatitis in a previously sensitized subject has rather different requirements.[1–7] In immunological terms, the induction phase of skin sensitization can be summarized as follows. Several important

changes in the skin are provoked following topical exposure to a contact allergen, including the induction or altered expression of various chemokines and cytokines that together orchestrate the development of cutaneous immune responses. A proportion of epidermal Langerhans cells (LC), some of which bear antigen, is induced to migrate, via afferent lymphatics, to draining lymph nodes where they accumulate as mature immunostimulatory dendritic cells (DC). Antigen is presented to responsive allergen-specific T lymphocytes, [8,9] resulting in activation and selective clonal expansion of this population. The individual is now sensitized and is able to respond more aggressively and in an accelerated manner to the same chemical if encountered subsequently at the same or a different skin site. Clinically, ACD, which is a Type IV, delayed or cell-mediated, immune reaction, is characterized by erythema, edema, vesiculation, and pruritus. Although not a life-threatening condition, ACD can be very debilitating in severe cases and, when contracted in the workplace, may necessitate loss of or a change in work assignment or location.

IMMUNOBIOLOGY OF ALLERGIC CONTACT DERMATITIS

Skin Penetration and Access to Viable Epidermis

The first potential obstacle for a contact sensitizing chemical to negotiate is the *stratum corneum*. For a chemical to initiate an immune response in the skin it must cross this barrier in order to gain access to the viable epidermis where the cellular sentinels of the adaptive immune system (LC) reside. Under normal circumstances, unless the barrier function of the skin has been compromised by trauma or disease, skin penetration will be determined by the physicochemical properties of the chemical. One parameter that is of particular importance is the octanol/water partition coefficient (P or log P), a measure of the lipophilicity of the chemical. The higher the log P value, the more lipophilic the material and in general, lipophilic compounds penetrate the skin more easily than do hydrophilic materials. For various homologous series of chemicals, including phenols and alcohols, a good correlation exists between log P and absorption through skin. For structurally unrelated chemicals, however, permeability does not necessarily correlate directly with log P, because other properties such as molecular weight and melting point (and charge?) may also play a role.[10] There is also a relationship between log P and skin sensitization for structurally related chemicals, with, for example, the relatively lipophilic alkylated aldehydes (high log P) exhibiting marked contact sensitization potential whereas the hydrophilic hydroxyaldehyde oxidation products displayed lower log P values and reduced contact allergenic potential.[11]

In a recent published database of 211 compounds evaluated in the local lymph node assay (LLNA), a test for contact sensitizing potential, the range of Log $K_{octanol/water}$ ($K_{O/W}$) values ranged from < -3 to > 5 for the allergens in the database.[12] The majority of the allergens (92.3%), however, had Log $K_{O/W}$ values in the range of ≥ -1 to 3 and ≥ 3 to 5. This is not surprising since chemicals found in these ranges are known to be very good to good skin penetrants, respectively.[13–23] The non-allergens represented in the database had a similar log $K_{O/W}$ distribution as do the allergens. A similar range was evident for the Log Kp values for both the allergens and non-allergens.

It has also been postulated that the molecular size of chemicals is an important determinant of contact sensitization potential (the so-called 500 Dalton rule). The suggestion is that there is an upper size limit of approximately 500 Dalton for molecules that can pass through the *stratum corneum*, based on the observation that most topically applied pharmaceuticals (both those used for dermatotherapy and in transdermal drug delivery systems) have a molecular mass of less than 500 Dalton.[18] Furthermore, all components of the routine patch test series advised by the International Contact Dermatitis Research Group for the diagnosis of contact allergy that is composed of the most common skin sensitizers are less than 463 Dalton.[18] The only exception to this rule is neomycin sulphate, that has a molecular weight of 712. However, this molecule is a dimer of two neamine molecules (each with a molecular weight of 322 Dalton), and it may be that the monomer is the sensitizing agent. In the database of 211 compounds evaluated in the LLNA, all of the allergens and non-allergens have molecular weights (MW) below 500 Daltons.[12] The MW distribution of the allergens shows that majority are in 100 to 300 range (79.3%). It is generally believed that chemical allergens have low molecular weights (< 500 D) and a Log $K_{O/W}$ of >1 that is thought to favor the penetration of the chemical across the lipid-rich stratum corneum.[19]

Protein Reactivity of Haptens and Pro-Haptens

A further key property of contact sensitizing chemicals is protein reactivity, or metabolism to a protein reactive species. In their native state, low molecular weight chemicals (or "haptens") are unable to induce immune responses. Immune recognition requires the formation of a larger complex between the chemical and a protein. This theory of covalent interaction between skin proteins and chemical sensitizers (the so-called electrophilic theory) was postulated first by Landsteiner and Jacobs in 1936[20] and has since been extended and refined by others.[21–23] However, the relationship between electrophilic activity and skin sensitization potential is not absolute. Chemical allergens (or their metabolites) have electrophilic properties and are able to react with various nucleophiles to form covalent bonds. Nucleophilic centers are rich in electrons and therefore are partially negatively charged, while electrophilic centers, deficient in electrons, are partially positively charged. The main mechanisms for the formation of covalent bonds involved in contact allergy can be grouped into three main categories: nucleophilic substitutions, on either a saturated (e.g., alkyl halides and epoxides) or unsaturated center (aromatic halides or esters), and nucleophilic additions (e.g., carbonyl derivations and α,β-unsaturated systems).[24] In proteins, the side chains of many amino acids contain electron-rich groups capable of reacting with allergens. Lysine and cysteine are those most often cited, but other amino acids containing nucleophilic heteroatoms, such as histidine, methionine, and tyrosine can react with electrophiles.[24] Thus, electrophilic allergens have the capability to react with nucleophilic amino acids in proteins, to form extremely strong covalent bonds resulting in the formation of a stable immunogen capable of triggering a T lymphocyte response and the acquisition of skin sensitization. Some types of chemical allergens that contain electrophilic centers include: acylating agents, alkylating and arylating agents, Michael electrophiles, aldehydes and precursors, and free radical generators. It is the understanding of these

types of chemical reactions types that lead to the development of DEREK (Deduction of Estimated Risk from Existing Knowledge), an expert system designed to identify skin sensitization alerts.[25]

As stated, some materials (prohaptens) may be converted chemically, often by oxidation, to protein-reactive haptens; examples include limonene and colophony.[26–27] In addition, xenobiotic metabolizing enzymes in the skin, which is now recognized as an important site of extrahepatic metabolism, can convert prohaptens to electrophilic species as a result of detoxification of the parent molecule.[28–29] An example of the role that metabolism can play in the development of contact sensitization is the activation of cinammic alcohol to the presumed allergen cinnamic aldehyde. Cinnamic alcohol is not itself protein-reactive and yet it is a known human allergen and is a constituent of the European Standard Test Allergen Fragrance Mix. The presence of protein-bound cinnamaldehyde has been detected in skin treated with cinammic alcohol using immunohistochemical techniques; this moiety is a potent contact allergen and is presumably formed by the action of cutaneous alcohol dehydrogenase, providing for a potential mechanism for sensitization to cinammic aldehyde.[28–29] The involvement of reactive intermediates in skin sensitization has been demonstrated with other chemicals, such as the formation of benzoquinonediimine from azo hair dyes[30] and orthoquinone from isoeugenol.[31] The most common environmental skin allergy in North America is caused by exposure to poison ivy. The sensitizing chemicals found in poison ivy and poison oak belong to a family of catechols known as urushiols. Urushiol is a prohapten and must be oxidized to become antigenic. The enzymes involved in this two electron oxidation have not been identified but it is the orthquinone that is thought to be the reactive chemical entity.[32] Eugenol and isoeugenol are considered also to be prohaptens. Interestingly, their metabolism appears to be unique such that the metabolites that bind and generate an immune response are distinct in that individuals sensitized to these materials do not cross-react.[33] It is important to note that Schmidt and Chung[34] have challenged the established view that certain compounds such as nitrohalobenze compounds (e.g., 2,4-dinitrochlorobenzene, DNCB) react with protein via an aromatic nucleosubstitution mechanism. They have proposed that for these compounds, and possibly others, a prohapten mechanism is involved where the chemicals are activated by NADPH-dependent reductases in the skin that generate free-radical and closed-shell metabolites which are then capable of protein reactivity. If this holds true, many chemical allergens that were previously thought not to undergo biotransformation in the skin would apparently require this step prior to their reacting with protein and initiating skin sensitization.

In recent years there has been considerable new information obtained on how the immune system recognizes haptens or prohaptens at a chemical level.[35–36] T lymphocytes detect haptens as structural entities, attached covalently or by molecular complexes to self-peptides anchored in binding grooves of major histocompatibility antigens (MHC). It is proposed that generation of antigenic determinants during skin sensitization with reactive chemical allergens may be the result of at least four different mechanisms: (1) modification of soluble proteins that may be endocytosed by LC and presented as hapten-modified peptides on MHC class II after intracellular processing, (2) a similar process for modified membrane proteins (non-MHC) of LC, (3) direct binding of the hapten to

peptides already associated with class II (or class I) MHC molecules on the surface of LC surface, (4) or, the reactive chemicals might penetrate the plasma membrane and modify cytoplasmic proteins which then will be processed and presented by LC.[35]

For effective sensitization, a chemical must therefore be inherently protein-reactive or must be converted in the skin to a protein-reactive metabolite. Chemicals that are unable to associate effectively with proteins will fail to stimulate a cutaneous immune response. For those chemical contact allergens that require metabolism to a protein reactive species, it is possible that genetic differences in metabolism may play a role in the differential susceptibility of individuals to the development of contact hypersensitivity responses to these materials.

Cutaneous Inflammation and Local Trauma

It is assumed that the hapten-protein conjugates formed as described above will be internalized and processed by epidermal LC. Following activation, these cells are stimulated to leave the epidermis and migrate to draining lymph nodes, providing a mechanism for transporting antigenic signals from the skin to the regional lymph nodes. As part of the process of migration, LC undergo functional maturation such that they lose the ability to process antigen and acquire instead the characteristics of antigen-presenting DC. The mobilization and maturation of LC are orchestrated by epidermal cytokines and chemokines, the downstream effects of which (for example, changes in adhesion molecule expression) facilitate the movement of LC from the epidermis, their migration across the basement membrane and their later localization within the paracortical regions of regional lymph nodes.[37–39] The response of LC to contact allergens is impaired or inhibited, and the development of skin sensitization is compromised, if the necessary cytokine signals are unavailable.[38,39,40,41] Cytokines known to influence LC function, and to be required for optimal contact sensitization, include interleukin (IL) 1β, tumor necrosis factor α (TNF-α) and granulocyte/macrophage colony-stimulating factor (GM-CSF). These three cytokines act in concert on LC during their transit to the local lymph node to effect their functional maturation into immunostimulatory DC.[42,43] In addition, IL-1β and TNF-α (together with IL-18) provide mandatory signals for the mobilization of LC.[40,41,44,45] These cytokines must therefore be available locally at relevant concentrations for the normal development of skin sensitization. Other cytokines such as IL-10 play a down-regulatory role in the skin in both the induction and elicitation phases of contact sensitization by inhibiting LC cell migration and accessory function and the production of inflammatory cytokines including interferon γ (IFN-γ).[46–48]

In many instances it appears that topical administration of a contact allergen alone is sufficient to trigger the induction or upregulation of those cytokines necessary for the effective acquisition of sensitization. Under these conditions of exposure, the chemical allergen itself causes sufficient cutaneous inflammation and irritation and hence the production of proinflammatory cytokines by skin cells. Chemical allergens that do not provoke the level of trauma necessary to provoke proinflammatory changes may fail to induce cytokine responses. The ability of physical inflammation to augment contact sensitization responses in human subjects was documented as long ago as 1966, prior

to any understanding of the relevant biological mechanisms.[49] More recent studies have demonstrated that individuals who are patch test positive (in this case, to colophony) have a lower threshold of sensitivity to the skin irritant sodium lauryl sulfate (SLS) than patch test negative matched controls suggesting increased susceptibility to allergy in these subjects.[50] Further evidence of this relationship between irritation and sensitization derives from studies performed in mice with DNCB, a potent contact allergen that is also a skin irritant at high concentrations. The ability of DNCB to induce in mice draining lymph node activation following topical application was measured with or without the coadministration of SLS. At high (irritant) doses of DNCB, SLS did not impact on the levels of immune activation induced by the allergen. However, at lower (non-irritant) concentrations of DNCB, responses were augmented by SLS. The interpretation is that topical exposure to comparatively high levels of DNCB provides both a sensitizing signal and sufficient trauma to provoke optimal proinflammatory cytokine production. Following exposure to lower (and less irritant) levels of the allergen, insufficient levels of inflammation are provoked and optimal immune activation requires the provision of an exogenous inflammatory stimulus (supplied in this instance by SLS).[51]

In summary, therefore, it is our view that for the optimal acquisition of skin sensitization a certain level of skin irritation or skin trauma will be required, and that chemicals that fail to trigger sufficient local cytokine production may (in the absence of any additional exogenous proinflammatory stimulus) be unable to realise their full potential as allergens. Given that the chemical matrix in which a chemical is experienced on the skin can impact on both penetration and proinflammatory activity, it comes as no surprise that the vehicle in which a contact allergen is delivered to the skin can have important influences on sensitizing activity.[52–54] For example, it has been demonstrated that the sensitizing potential of the skin sensitizing fluorochrome fluorescein isothiocyanate (FITC), measured as a function of induced proliferative responses in the draining lymph node, was augmented substantially by the addition of dibutyl phthalate (DBP).[52] *In vitro* skin absorption studies indicated that DBP was associated with a small increase in percutaneous absorption of FITC, but more importantly, DBP treatment resulted in a marked increase in the frequency of lymph node DC bearing detectable antigen. In other experiments, coadministration of the skin irritant SLS with suboptimal concentrations of the contact allergen DNCB augmented proliferative responses as described above by provision of danger signals, under conditions where no impact on the efficiency of skin absorption was observed.[51,53] Although it is clear that the vehicle matrix can have important effects on skin sensitizing potency by a variety of mechanisms, not all allergens are affected similarly, thus it is not possible at present to predict in a generic way the likely impact of formulation without recourse to direct testing.[54–56]

Immune Recognition

The induction of skin sensitization and the subsequent elicitation of allergic contact dermatitis are dependent on the development of hapten-specific T lymphocytes. The

inducing hapten is presented to responsive T lymphocytes in skin draining lymph nodes by antigen-presenting cells. The T lymphocytes recognize the hapten as a structural entity attached to self peptides anchored within the binding grooves of MHC determinants (both MHC Class I and MHC Class II) displayed by the antigen presenting cells.[57,58] These cells either derive directly from antigen-bearing LC that have migrated from the skin, or are resident DC that have acquired antigen from LC arriving in the lymph nodes. The draining lymph nodes become activated, characterized by an increase in node weight and total cellularity, T cell activation and proliferation, and the production of various cytokines. The importance of peripheral lymph nodes for the acquisition of contact sensitization has been confirmed recently by the observation that lymphotoxin-α-deficient mice that lack lymph nodes fail to develop skin sensitization.[59]

It appears that the vigor of T lymphocyte responses to contact allergen is determined by a series of quantitative inter-dependent biological relationships. The effectiveness of LC migration from the epidermis (and thus the amount of antigen reaching the node) correlates with the dose of chemical experienced. Further, the vigor of T lymphocyte proliferation is dependent upon the extent of DC accumulation in draining lymph nodes.[60] Finally, there is evidence also that the magnitude of T lymphocyte proliferative responses (equivalent to the degree of clonal expansion) that occurs in the induction phase of contact sensitization in turn correlates with the extent to which sensitization is acquired and the vigor of reactions provoked during the elicitation phase.[61] The assumption is that cell turnover in the induction phase will control the frequency of various specific effector cells that are responsible for eliciting allergic contact reactions following subsequent encounter with the inducing allergen.[62] Given these relationships, it is perhaps not unexpected that the threshold concentration of chemical that is necessary to provoke lymphocyte proliferation in mice has been shown to correlate with relative sensitizing potency. Thus, the dose of chemical required to stimulate a threshold (threefold increase compared with concurrent controls) level of thymidine incorporation in the murine LLNA relates to what is known of the differential ability of agents to cause allergic contact dermatitis among humans.[63,64]

The second phase of skin sensitization, called elicitation, occurs upon a subsequent encounter with the inducing hapten. As in the induction phase, the chemical enters the epidermis and is processed by LC. But during elicitation, the protein-bound hapten is presented by the LC, and by other cells resident in the skin, to memory T cells which have percolated into the skin. Upon recognition of the antigen, the memory T cells become activated and produce a number of pro-inflammatory cytokines which trigger an inflammatory response, resulting in the clinical signs associated with allergic contact dermatitis: erythema, edema, vesiculation, and pruritus. It is important to distinguish between the induction and elicitation phases of ACD. The induction phase represents the initial exposures that eventually lead to an immune response of sufficient magnitude such that the individual is considered to be sensitized. The elicitation phase, then, represents dermal exposures in already-sensitized individuals such that the exposure results in a cutaneous allergic reaction with clinical manifestations (e.g., erythema, edema, vesiculation, pruritus). While both induction and elicitation are dose-dependent phenomena that exhibit thresholds, fewer studies have been conducted examining elicitation thresholds.

CLINICAL ASPECTS OF ALLERGIC CONTACT DERMATITIS

CLINICAL PRESENTATION

Contact dermatitis—both irritant and allergic—is an inflammatory skin condition caused by skin contact with an exogenous agent or agents, with or without a concurrent exposure to a contributory physical agent, such as ultraviolet light. Contact dermatitis can result from a nonimmunologic reaction to chemical irritants (irritant contact dermatitis) or from an immunologic reaction to allergens (allergic contact dermatitis). Irritant contact dermatitis is a cutaneous inflammation resulting in most circumstances from a direct cytotoxic effect of a chemical or physical agent. As emphasized in this chapter, allergic contact dermatitis is a type IV, delayed or cell-mediated, immune reaction. A variety of chemical databases are currently available which provide information on over 150,000 chemical substances, many of which are identified as irritants, but only 3,700 of those chemicals have been identified as allergens and used in skin patch testing.[82] These allergens are mostly confined to small-molecular-weight chemicals that act as haptens, and based upon skin patch studies usually only a small proportion of people become sensitized, which is most likely due to their exposure to the allergen.

In allergic contact dermatitis, the skin initially turns erythematous and can develop edema, papules, and small, oozing vesicles. After several days, crusts and scales form. Stinging, burning, and pruritus may accompany the skin lesions. With no further contact with the etiologic agent, the dermatitis usually disappears in 1 to 3 weeks. With chronic exposure, deep fissures, scaling, and hyperpigmentation can occur. Exposed areas of the skin, such as hands and forearms, which have the greatest contact with allergens, are most commonly affected. If the agent gets on clothing, it can induce dermatitis at areas of greatest contact, such as thighs, upper back, armpits, and feet. Dusts can produce dermatitis at areas where the dust accumulates and is held in contact with the skin, such as under the collar and belt line, at the tops of socks or shoes, and in flexural areas, such as the antecubital and popliteal fossae. Mists can produce a dermatitis on the face and anterior neck. Allergens can be transferred to remote areas of the body, such as the trunk or genitalia, by unwashed hands or from areas of accumulation, such as under rings or interdigital areas. It is often impossible to distinguish clinically irritant contact from allergic contact dermatitis, as both can have a similar appearance and both can be clinically evident as an acute, subacute, or chronic condition.

PUBLIC HEALTH IMPORTANCE

Measures of the public health importance of a disease include the absolute number of cases, the incidence rate, the prevalence (rate), the economic impact of the disease, and the prognosis and preventability of the disease.[65] Contact dermatitis is the most common occupational and environmental skin disease. Epidemiologic data show that contact dermatitis comprises 90 to 95% of all occupational skin diseases.

Specific national data sources on allergic contact dermatitis are limited. In the United States, data from the National Ambulatory Medical Care Survey, a national

probability sample survey of nonfederal office-based physicians, showed that in 2002 skin rash was the principal reason for 11.8 million patient visits—1.3% of all visits for that year.[66] Based upon previous surveys, it is estimated that approximately 50% of these visits would have had a diagnosis of contact dermatitis or other eczemas.

In 1988, the National Health Interview Survey (NHIS) included an Occupational Health Supplement, which included questions on dermatitis. The survey consisted of personal interviews of people in randomly selected households. For 30,074 people participating in the NHIS, the period prevalence for all forms of dermatitis was 11.2% and for contact dermatitis was 2.8%. Projecting these results to the U.S. working population resulted in an estimate of 13.7 million people with dermatitis and 3.1 million people with contact dermatitis.[67]

More information is available on the public health impact of occupational contact dermatitis. Specific national occupational disease and illness data are available from the U.S. Bureau of Labor Statistics (BLS), which conducts annual surveys of approximately 180,000 employers selected to represent all private industries in the United States.[68] All occupational skin diseases or disorders, including allergic contact dermatitis, are tabulated in this survey. BLS data show that occupational skin diseases accounted for a consistent 30 to 45% of all cases of occupational illnesses from the 1970s through the mid-1980s, and in recent years accounted for 15% of all occupational illness.[68] The decline in this proportion may be partially related to an increase seen in disorders associated with repeated trauma.

In 2003, BLS estimated 43,400 cases of occupational skin diseases or disorders in the U.S. workforce.[68] However, because of BLS survey limitations, it has been estimated that the number of actual occupational skin diseases may be on the order of 10 to 50 times higher than that reported by the BLS.[69] This increase would potentially raise the number of occupational skin disease cases to between 400,000 and 2 million per year. In 2003, BLS data showed an annual incidence rate of 49 cases per 100,000 workers.[68]

The economic impact of a disease can be measured by the direct costs of medical care and workers' compensation or disability payments, and the indirect costs associated with lost workdays and loss of productivity. In 1984, the estimated annual direct and indirect costs of occupational skin diseases exceeded $22 million.[69] However, considering that the actual annual incidence may be 10 to 50 times greater than reported in the BLS data, the total annual cost of occupational skin diseases in 1984 may have ranged from $222 million to $1 billion.[69]

The recent Burden of Skin Disease study, a joint project of the American Academy of Dermatology Association and the Society of Investigative Dermatology, found that the third most common skin ailment was contact dermatitis. According to this study, 72 million Americans had contact dermatitis in 2004 which resulted in $1.4 billion in direct costs and almost $500 million in indirect costs due to lost productivity.[83] A review of 1993 BLS data showed that of 60,200 cases of occupational skin diseases, 12,613 (21%) resulted in one or more days away from work.[70] The mean time away from work was 3 days, but 17% lost workday cases had over 11 days away from work. Of those with days away from work, 70% had a diagnosis of dermatitis. In 2001, of the 38,900 skin disease cases, 6,051 (16%) resulted in days away from work, with a median of 3 days lost.[68] Of these, 78% had dermatitis. A study of 235 Canadian workers with oc-

cupational skin diseases showed that 35% had been away from work for greater than 1 month, 14% between 1 week and 1 month, 17% less than 1 week, and 33% did not lose workdays because of the skin condition.[71]

Studies on the prognosis of occupational contact dermatitis point out that primary prevention is very important. For example, of 555 patients completing a follow-up questionnaire 2 to 3 years after diagnosis, only 26% of the women with contact dermatitis had complete healing (22% had continual symptoms and 52% had recurring symptoms) and only 31% of the men had complete healing (29% had continual symptoms, 40% had recurring symptoms).[72] A telephone survey of 235 occupational skin disease patients, conducted a mean of 4 years after diagnosis, showed that 40% had continuing dermatitis, although of this group, 76% reported an improvement in their skin condition.[71] Outcomes may or may not be influenced by leaving the dermatitis-provoking job. In addition, many skin disorders, including contact dermatitis, have been shown to have a significant impact on quality of life.[73]

Diagnosis and Overview of Key Skin Allergens

The most frequent causes of allergic contact dermatitis in the United States include plants (poison ivy, poison oak, and poison sumac), metallic salts, organic dyes, plastic resins, rubber additives, and germicides.[74] The most common skin patch test allergens found to be positive in patients along with potential sources of exposure are shown in Table 32.1.[75] In patients with occupational contact dermatitis who were skin patch tested, the common allergens included carba mix, thiuram mix, formaldehyde, epoxy resin, and nickel.[76]

The environmental cause or work-relatedness of allergic contact dermatitis may be difficult to prove. The accuracy of the diagnosis is related to the skill level, experience, and knowledge of the medical professional who makes the diagnosis and confirms the relationship with environmental or workplace exposures. Guidelines are available for assessing the work-relatedness of dermatitis, but even with these the diagnosis may be difficult.[77] The diagnosis is based on the medical history and the history of environmental and occupational exposures as well as the physical findings. The importance of the patient's history of exposures and disease onset is clear. Standardized questionnaires for surveying work-related skin diseases are available and can be helpful in the workplace.[78] In many instances, allergic contact dermatitis can be confirmed by skin patch tests using specific standardized allergens or, in some circumstances, by provocation tests with nonirritating dilutions of industrial contactants. Skin patch tests should only be conducted by health care professionals trained in conducting and interpreting the tests. Skin patch tests should never be conducted with unknown substances.

Prevention

Strategies in the prevention of allergic contact dermatitis include:

TABLE 32.1
North American Contact Dermatitis Group Patch Test Results, 1998 to 2000.[11] Prevalence of 20 most common positive reactions (n varies from 5770 to 5835)

Test Substance	Common sources	% Positive
Nickel sulfate 2.5%	Metals, jewelry	16.2
Balsam of Peru 25%	Perfumes, creams	12.3
Neomycin	Creams, lotions	11.5
Fragrance mix 8%	Toiletries, scented products	10.9
Thimerosal 0.1%	Cosmetics, cleansers	10.8
Sodium gold thiosulfate 0.5%	Jewelry, dental products	10.5
Formaldehyde 1% aqs	Fabrics, skincare products	9.2
Quaternium-15 2%	Cosmetics, sunscreens	9.2
Bacitracin 20%	Ointments, creams	9.2
Cobalt chloride 1%	Metals, jewelry	7.6
Methyldibromo glutaronitrile phenoxyethanol 2.5%	Biocides, skincare products	6.0
Potassium dichromate 0.25%	Cement, leather	5.8
Ethyleneurea melamine formaldehyde resin 5%	Textiles	5.0
p-Phenylenediamine 1%	Hair dyes, leather	4.9
Carba mix 30%	Rubber, pesticides	4.8
Thiuram 1%	Rubber, pesticides	4.7
Propylene glycol 30% aqs	Cosmetics, topical meds	3.7
Cinnamic aldehyde 1%	Fragrances, flavorings	3.6
Methyldibromo glutaronitrile phenoxyethanol 0.4%	Biocides, skincare products	3.5
Amidoamine 0.1% aqs	Shampoos, liquid soap	3.4

- Identifying allergens.
- Substituting chemicals that are not allergenic or less allergenic.
- Establishing engineering controls to reduce exposure.
- Utilizing personal protective equipment (PPE), such as gloves and special clothing.
- Emphasizing personal and occupational hygiene.
- Establishing educational programs to increase awareness in the workplace.[79]

Chemical changes in industrial materials have proved to be beneficial. For example, the addition of ferrous sulfate to cement to reduce the hexavalent chromium content was effective in reducing occupational allergic contact dermatitis in Europe. The use of PPE must be considered carefully since it may actually create problems by occluding irritants or allergens or by directly irritating the skin. Similarly, the excessive pursuit of personal hygiene in the workplace may actually lead to misuse of soaps and detergents and resulting irritant contact dermatitis. The effectiveness of gloves depends on the

specific exposures and the types of gloves used. The effectiveness of barrier creams is controversial since there are limited data on the protective nature of these topical products during actual working conditions involving high-risk exposures. Individuals with occupational skin diseases should be protected from exposures to presumed causes or exacerbators of the disease. In some cases of allergic contact dermatitis, workers may have to be reassigned to areas where exposure is minimized or nonexistent.

CONCLUSIONS

It is possible to identify various factors that confer on chemicals the ability to induce skin sensitization and allergic contact dermatitis. These include the capacity to gain access to the viable epidermis across the *stratum corneum*, to associate stably with host proteins, to provoke a certain degree of proinflammatory cytokine production by skin cells, and to be recognized by specific T lymphocytes. The effectiveness with which these requirements are met, and possibly other properties of the chemical that influence the vigor of induced immune responses, together with the extent of exposure, will dictate the degree to which sensitization is achieved.

Allergic contact dermatitis can be a common disease, often has a poor prognosis, and results in a noteworthy economic impact. Skin diseases such as allergic contact dermatitis are amenable to public health interventions. The U.S. Public Health Service goal for 2010, as stated in its Healthy People 2010: National Health Promotion and Disease Prevention Objectives, is to reduce national occupational skin disorders or diseases to an incidence of no more than 46 per 100,000 full-time workers.[80] Both irritant and allergic contact dermatitis are considered priority research areas, as outlined in the National Occupational Research Agenda, introduced in 1996 by the U.S. National Institute for Occupational Safety and Health (NIOSH).[81] Increased knowledge and awareness of environmental and occupational skin diseases by health care professionals will assist in achieving the national public health goals.

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33 Respiratory Allergy and Occupational Asthma

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INTRODUCTION

Asthma is a disease characterized by inflammation in the airways, reversible airflow obstruction and bronchial hyper-reactivity. Occupational asthma (OA) is generally defined as asthma that has a causal relationship to exposures in the work environment. Various surveys estimate that 3 to 20% of adult onset asthma is related to work and that 4% of adult asthma is made worse by work [1–3]. Surveys conducted in Europe indicate that there are 12 to 170 new cases of OA/million workers/year. Numbers in the United States range from 29 to 710 cases/million workers/year [2]. An estimate of 200 to 300 agents has been causally associated with OA but only about 8 to 12 are responsible for the majority of OA cases reported globally [4]. OA can be associated with exposure to irritants or sensitizing substances that are categorized into high molecular weight (HMW) and low molecular weight (LMW) agents. The HMW agents are usually proteins while the LMW agents are chemicals, metals and drugs. Immune mediated mechanisms are associated with OA to the sensitizing substances since there is a latent period between exposure and onset of disease, there is memory, specificity and in many cases demonstration of an immune response. OA can occur upon exposure to irritants via exacerbation of pre-existing asthma in a worker or induction of asthma. This chapter will focus on OA caused by exposure to HMW and LMW sensitizing agents, mediating their effect through immune mechanisms.

OCCUPATIONAL ASTHMAGENS

It has been estimated that 200 to 300 agents have been identified as inducers of occupational asthma [4]. The LMW agents can be broadly classified by chemical class such as isocyanates, anhydrides, metals, dyes, amines, drugs, acrylates and other compounds. The HMW agents can be broadly classified as animal protein, plant protein and microbial byproducts. Table 33.1 shows a partial listing of LMW and HMW sensitizers along with the workplace environments where these agents can be encountered.

MECHANISM OF IMMUNE MEDIATED OCCUPATIONAL ASTHMA

Respiratory hypersensitivity is an adverse reaction in the respiratory tract driven by immune mechanisms such as IgE antibody mediated allergic responses. Other less well understood mechanisms that have an immune component are also involved in respiratory hypersensitivity. OA is one outcome of respiratory hypersensitivity. Respiratory hypersensitivity and OA to proteins are primarily mediated by IgE antibody with subsequent inflammatory cell infiltrates. This same mechanism is responsible for OA to specific LMW chemicals such as the acid anhydrides and platinum salts. However, the role for IgE mediated responses in OA to other LMW chemicals such as the isocyanates and plicatic acid is poorly defined and other mechanisms may be responsible.

The development of respiratory hypersensitivity requires an induction phase where exposures to the sensitizer lead to an interaction with immune cells and the eventual development of specific effecter immune molecules (e.g., antibodies) and cells (e.g., T lymphocytes) [5]. The induction phase can require months to years of exposure before there is a detectable immune response and/or onset of symptoms typical of respiratory hypersensitivity, including asthma. Classic IgE mediated responses have been described as Th2 cell dominant responses. A subset of CD4+ T cells known as Th2 cells push the immune response to the development of IgE and IgG4 antibodies in humans along with secretion of cytokines that attract and activate inflammatory cells such as eosinophils.

Upon inhalation of protein allergen, the protein comes in contact with bronchial epithelial cells and dendritic cells. Investigative studies suggest that these interactions in the lung can impact the progression of the immune response in the lymph nodes [6]. The dendritic cells internalize the protein, travel to the draining lymph nodes, mature where they become proficient at processing the protein into peptides and presenting these peptides via MHC Class II complex to CD4+ T lymphocytes. Those T cells that have receptors that can bind to the peptide will differentiate and proliferate, secreting cytokines that promote further T cell expansion. These cells will also interact with B cells that have receptors that recognize the protein. Activation of the transcription factor GATA-3 leads to the differentiation of T cells to Th2 cells [7]. These cells will secrete cytokines such as IL-4 and IL-13 and upon binding to B cells via CD40 (and other surface receptor-ligand interactions), push the B cells to undergo class switching and produce IgE antibody. This antibody binds to specific regions on the protein allergen. IL-4 signals B and T cells through activation of signal transducer and activator of transcription 6 or STAT6. STAT6 activation contributes to the continued differentiation

TABLE 33.1
Examples of HMW and LMW Agents that Cause Immune-Mediated OA

Agent	Job/Industry
Isocyanates:	
• Toluene diisocyanate (TDI) • Diphenylmethane diisocyante	• Plastics, adhesives, foams, automotive
Anhydrides:	
• Phthalic anhydride • Trimellitic anhydride	• Plastics, resins, paint, polymers
Amines:	
• Ethylenediamine • Piperazine	• Rubber, photography, paint, dyes
Metals:	
• Hexacholorplatinate • Chromium salts	• Mining • Welding
Dyes:	
• Remazol black b • Paraphenylaminediamine	• Textile • Hairdressers
Drugs:	
• Chlorhexidine • Ampicillin	• Medical • Pharmaceuticals
Microbial sources/proteins:	
• *Bacillus* enzymes • *Aspergillus* amylase	• Detergent industry • Baking industry
Insect sources/proteins:	
• Storage mites • Spider mites • Mealworms	• Farmers, granary workers • Greenhouse • Bait
Animal sources/proteins:	
• Rat/mouse urinary proteins • Shellfish tropomyosin • Pancreatin extract (enzymes)	• Laboratory animal handlers • Seafood processing, fishermen • Pharmaceutical
Plant sources/proteins:	
• Latex (various hevien all ergens) • Psyllium (Isphagula) • Papain • Soybean	• Medical, dental • Medical, nursing home • Meat processing • Dockworkers, farmers, granary workers

of T cells into Th2 cells. This same process occurs for certain LMW chemicals. These chemicals function as haptens and bind to carrier molecules (usually endogenous protein) to form a chemical-carrier complex that is large enough to be recognized by the immune system and induce a cascade of events leading to IgE.

The allergen-specific IgE antibody binds to the high affinity Fc receptor for IgE on the surface of tissue mast cells and circulating basophils. IgE can also be found in

the circulation but the half-life is shorter as compared to cell bound antibody. Once the IgE antibody has been produced, the individual is considered to be sensitized. This is not a disease state but it does raise the risk for the individual to develop symptoms of respiratory hypersensitivity.

The disease stage occurs when re-exposure leads to binding of the protein allergen to pre-formed IgE antibody on the surface of mast cells and basophils [5]. If adjacent IgE bind to the allergen, they will cluster on the surface of the cell, triggering a cascade of events that leads to the release of pro-inflammatory mediators from granules inside these cells. These mediators include histamine and chemotactic and pro-inflammatory molecules such as neutral proteases and proteoglycans. In addition, metabolism of arachidonic acid to pro-inflammatory prostaglandins and leukotrienes occurs. Mediator release causes the symptoms of respiratory hypersensitivity that range in severity from rhinitis to asthma to anaphylactic shock. These symptoms, including asthma, can occur within a few minutes of exposure to the protein. This is defined as the immediate-onset reaction and is clearly linked to IgE antibody. For some individuals and for some sensitizing agents a severe reaction can occur between 2 and 8 hours after the immediate response. This is often characterized by mucus hypersecretion, bronchoconstriction, airway hyper-reactivity to a variety of nonspecific stimuli and airway inflammation characterized by infiltration of eosinophils and lymphocytes. This response is defined as the late-phase response. The late-phase response is not IgE mediated, but is linked to the pro-inflammatory effects of the various mediators. Late-phase responses are thought to involve both CD4+ and CD8+ T lymphocytes. In some cases, these reactions lead to chronic inflammation in the tissue (with an accumulation of eosinophils), characteristic of chronic asthma.

Why certain individuals develop Th2 mediated responses with the generation of IgE antibody and why some proteins but not all will induce this response is not well understood. Atopy, defined as the propensity to develop Th2 mediated diseases such as allergic asthma has a genetic component. Multiple genes are involved in the induction and development of asthma and polymorphisms among these genes may affect the severity of disease. Other personal risk factors such as smoking, obesity and barrier abnormalities may also play a role. Not all proteins or chemicals will induce Th2 mediated immune responses so the nature of the inducing agent is also important. For example, many of the prevalent environmental protein allergens have hydrolytic activity [8]. The interaction of these proteins in the lung environment may impact the differentiation of T cells to Th2 cells. Food allergens are less prone to digestion by stimulated gastric juice as compared to other proteins [9]. These observations suggest that the physical-chemical characteristics of the protein can be important to the development of Th2 dominant immune responses.

Certain LMW agents will cause OA via a poorly defined mechanism. Only about 20% of workers with OA to toluene diisocyanate (TDI) have IgE detectable to TDI indicating that IgE antibody may be more an indicator of exposure rather than a mediator of the disease [10]. Similar data exist for workers with asthma caused by plicatic acid from western red cedar [11]. The inability to detect IgE antibody in the majority of these workers may be based in technical issues such as the nature of the chemical-protein

carrier molecule used to detect the antibody. Conversely, other immune mechanisms may be responsible for the asthma. Analysis of induced sputum and bronchial washings and biopsies indicate that OA to isocyanates may be mediated by cellular responses since there is an increase number of CD25+ cells, activated eosinophils and an increase in circulating CD8+ T lymphocytes [12]. Similarly, increases in T lymphocytes and eosinophils were found in the bronchial mucosa of workers with western red cedar OA. *In vitro* studies showed that TDI can form adducts with intracellular glutathione, leading to a depletion of thiols [13]. Thiol depletion can have several effects on immune reactivity. Using cell culture, investigators showed that oxidized glutathione inhibited the conjugation of hexamethylene isocyanate (HDI) to albumin whereas conjugation was not effected by reduced glutathione [14]. Several investigators have shown that depletion of thiols, especially in antigen presenting cells, will favor the development of Th2 responses [15,16]. Another study showed that workers with certain polymorphisms in glutathione had an increase risk of TDI asthma [17]. Taken together these data support an immunological mechanism behind isocyanate asthma.

Since the mechanism of OA to certain LMW chemicals is not well defined, there are hurdles to diagnosis, management and treatment. For example, investigative work done in guinea pigs and mice indicate that TDI can affect airway sensory nerves, altering neuropeptides such as substance P [18]. Plicatic acid from western red cedar can cause direct histamine release as well as fix complement [19]. These are non-immunological effects that can lead to symptoms that mimic immune mediated responses. When examining a worker population, investigators need to be aware that some cases of OA may be due to immune and non-immune mechanisms.

DIAGNOSIS OF OCCUPATIONAL ASTHMA

Clinicians agree that diagnosis of OA requires demonstration of a direct causal relationship between the onset of asthma symptoms with the work environment and not to elements encountered outside the workplace.

The first step in diagnosing OA is confirmation of asthma. Asthma in adults can be confirmed/diagnosed via measurements of ventilatory and lung functions (reversible airflow limitations and bronchial hyper responsiveness to pharmacological agents such as methacholine) using a spirometer. These tests can detect, differentiate, and diagnose various obstructive or restrictive lung diseases such as asthma and chronic obstructive pulmonary disease (COPD), respectively (20). One caveat is that an individual with OA can still have normal ventilatory and lung functions if spirometry is performed long after exposure to the work environment has occurred such that recovery from the offending agent is achieved. If asthma has been diagnosed, understanding work history and reported symptoms at and away from work can shed light on the nature of the disease and/or suggest its association with OA. This is an imperfect tool for diagnosis. A survey of 79,204 asthmatic members of a health maintenance organization (HMO) in the United States revealed that only 15% of general practitioners investigated the work-relatedness of asthma symptoms [21]. In addition, some individuals develop asthma symptoms at

night (night waking with wheezing/coughing, dyspnoea), further contributing to difficulty in diagnosing OA as patients and doctors miss the association between a late-phase response which can occur 2 to 8 hours after exposure at the workplace.

To further diagnose the work-relatedness of asthma, serial monitoring of peak expiratory flow (PEF) and/or forced expiratory volume in one second (FEV1) with a portable spirometer can be conducted at work and at home. Ideally, the worker should take several measurements during the work period and after work and on days off for at least two weeks. A chart of PEF and/or FEV1 values vs. days at work or away from work will generally show low PEF and/or FEV1 values at work with higher values on days away from work for a worker with OA. It is easy to train individuals to use peak flow meters but compliance of the worker to diligent monitoring is important for a valid and accurate assessment of OA.

Skin prick test (SPT) and analysis of blood serum for specific IgE antibodies can also be performed to address the immunological component of the asthmatic symptoms and determine if the individual is sensitized to the suspected occupational allergen. These tests are generally useful for HMW allergens but not for all LMW allergens. Most LMW allergens are small compounds such as chemicals, drugs, and metals and some do not consistently induce an IgE antibody-mediated response. Although these tests show that the worker has been sensitized to a particular workplace allergen, it does not directly imply the onset of asthma symptoms to that particular workplace agent as other non-immunological responsive agent in the work environment can induce the asthmatic response.

The gold standard for confirming the diagnosis of occupational asthma is a specific inhalation challenge test (SIC). This bronchial provocation test exposes the individual to the workplace environment and the suspected asthmagen in recreated working conditions or to the asthmagen specifically at sub-irritating concentrations. Investigators have used several approaches to generating atmospheres containing the suspected asthmagen. Pepys used a chamber where the patient poured lactose powder from one pan to another to create airborne dust [22]. The suspected asthmagen would be added to the powder at increasing levels until the patient experienced a significant drop in pulmonary function. A positive SIC, measured as bronchial hyperreactivity to the occupational agent minutes or even hours after challenge, meets the criteria of OA. In addition, a decrease in the concentration of methacholine needed to elicit a change in pulmonary function from pre- to post-challenge is another indication of the relationship between OA and the suspected asthmagen. Due to the late-phase response that can occur in some individuals, the test should be conducted for 24 hours and the patient should be under close observation. Because of the risk involved with this test it should be done in specialized centers by trained personnel that can intervene if a severe airway response occurs.

SIC are not performed all the time as some clinicians don't have access to these specialized centers or none are available. For some sensitizers, a challenge test is not needed since other information is available and sufficient to prove OA. For example, if an allergen has been identified in the workplace and the worker is sensitized to the allergen and has asthma symptoms at work that resolve or improve away from work then the causal relationship can be ascertained and diagnosis of OA be reported.

PERSONAL RISK FACTORS ASSOCIATED WITH DEVELOPMENT OF OCCUPATIONAL ASTHMA

Many epidemiological studies have been conducted to understand the environmental and personal risk factors associated with asthma and OA [23,24]. The risk factors can vary depending upon the nature of the sensitizing agents. Personal risk factors such as smoking status, atopy and HLA Class II phenotype have been linked to OA to some HMW and LMW sensitizers. Atopy is a risk factor for development of OA to some HMW substances but not to LMW chemicals. This has been demonstrated in the baking industry [25], among lab animal handlers [26], in the seafood industry [27], and among workers exposed to green coffee dust [28]. Smoking status has been identified as a risk factor for certain HMW and LMW chemicals. Smokers were at higher risk of developing OA to platinum salts versus non-smokers [29,30]. A similar finding was made among anhydride workers and workers exposed to green coffee dust [28,31]. It is less clear of the relationship between smoking status and OA among workers exposed to isocyanates [32].

Genetic factors have been studied for certain sensitizing agents. Studies among isocyanate workers showed a relationship between development of isocyanate induced asthma and the HLA-DQB1*0503 allele [33]. The aspartic acid residue at position 57 was determined to be critical [34,35]. These same studies showed a negative association between isocyanate asthma and the HLA-DQB1*0501 allele [36]. Like isocyanates, the HLA-DQB1*0501 allele had a negative relationship with asthma to western red cedar. The HLA-DQB1*0302 and *0603 alleles were positively associated with western red cedar asthma. The significant association between HLA class II alleles and OA caused by isocyanate and western red cedar point to an immunological mechanism.

Similar studies showed a positive association between HLA alleles and development of IgE antibody. Sensitized platinum workers were DR3+ and DR6- while non-sensitized workers were DR3- and DR6+ [37]. The linkage between HLA phenotype and development of IgE antibody was greatest among the low exposure group. A second study showed a relationship between HLA DR3 and IgE to inhaled anhydrides [38]. A study among detergent workers showed a positive association between HLA-DR4+ and IgE antibody to the serine protease derived from *Bacillus licheniformis* [39].

Upper airway symptoms such as rhinitis can be a risk factor for asthma [40,41]. A survey of work related rhinitis and asthma among Finnish workers showed an increase risk of OA following development of occupational rhinitis [42]. The mean interval of time between notification of rhinitis and occurrence of asthma was 37 months with a range of 1 to 138 months. This same relationship between rhinitis and asthma was seen in a reference population but at a lower rate. Gautrin's survey of 769 students beginning work in industries bringing them into contact with HMW allergens showed that pre-existing rhinitis and conjunctivitis was significantly associated with the development of OA [43]. In addition, students with bronchial hyperreactivity prior to starting their careers also had a higher risk of developing OA. Surveys of seafood workers also showed that upper airway symptoms preceded the onset of asthma [27]. However, it is important to point out that not all studies on OA found that rhinitis was a risk factor. An additional study on student apprentices showed that only 20% of those with OA

developed rhinitis prior to the onset of asthma [44]. The onset of rhinitis and asthma was the same in 80% of the studied population.

Other personal risk factors such as increased body mass index are being considered as to whether they are important to asthma [45,46]. Whether emerging risk factors, along with the general increase in atopic diseases from the mid-1960s to the present [47] will have an impact on development and incidence of OA remain to be determined.

EXPOSURE-RESPONSE RELATIONSHIPS

Exposure is the most significant risk factor for the development of OA to both HMW and LMW sensitizers. Studies have shown that the greater the intensity of exposure, the greater the risk of developing allergic antibody and OA [43]. As the pool of workers with IgE antibody to an occupational sensitizer increases, so does the risk for development of occupational allergy and asthma among these workers. Laboratory animal workers sensitized to animal allergens were 4 times more likely to develop chest symptoms as compared to their non-sensitized counterparts [48]. Evaluation of various worker populations have shown that the numbers of workers that develop IgE antibody to the occupational allergen decrease with decrease in exposure followed by a concomitant decrease in the incidence and prevalence of OA. This has been most apparent in the detergent enzyme industry where 20-plus years of prospective medical monitoring has proven this maxim [49,50].

The development of IgE antibody along with the development of asthma or allergy symptoms can require several years of exposure. Medical monitoring for sensitization among detergent workers exposed to enzymes indicates that the development of IgE antibody can be detected 6 months after starting employment. The development of IgE antibody to organic anhydrides required a mean of 8.8 months [51]. Onset of anhydride-induced symptoms became apparent within 2 years of exposure. Similar findings were demonstrated for workers exposed to seafood allergens [27], laboratory animal allergens [26], latex [52], isocyanates [53], anhydrides and allergens encountered in the baking industry [25].

It has been technically challenging to measure exposure in the workplace. Collection of air samples over extended periods of time may be needed to accurately detect and measure the sensitizing agent in the air. These sampling methods also smooth out peaks in exposure. This is one reason why it has been difficult to quantitatively describe exposure response relationships for occupational sensitization and asthma. However, division of job tasks into high, medium and low categories of exposure and/or description of intensity of exposure based on duration and job task has allowed investigators to show a dose-response relationship for sensitization and development of OA. Studies provide support that there are thresholds of exposure for sensitization and development of symptoms. Studies among laboratory animal workers have shown that those individuals working more than 52 hours/week with rats or mice had a 2.5-fold increase risk in sensitization as compared to individuals working 16 hours/week or less [54]. It was estimated that the risk of developing allergic antibody to platinum salt increased 1.13 fold with every increment in exposure of 1 ug/m^3 [55]. Twenty-plus years of prospective

medical monitoring of detergent workers exposed to microbial enzymes showed that workers with IgE antibody to the enzymes could still work with these materials and not develop allergy or asthma symptoms [50]. When exposure led to incidence rates of sensitization greater than 3% per year, there was a higher risk of allergy symptoms. Higher incidence rates of sensitization are linked to greater intensities of exposure. These data indicate that the threshold of exposure for symptoms caused by enzymes is higher than the threshold of exposure for sensitization. Similarly, seafood workers sensitized to fish/shellfish allergens were at greater risk of developing allergy symptoms and asthma when in jobs that afforded greater duration of exposure [27].

Inhalation of HMW sensitizing agents is the primary route of exposure for sensitization and elicitation of asthma [56]. Once sensitized, it is possible to elicit allergic reactions upon other routes of exposure including via the skin. Protein contact dermatitis has been described for many occupations where there is contact with protein sources including natural rubber latex [57], animals and insects [27,58], food [59], and other agents [60]. Airborne contact dermatitis to proteins and chemical has also been described as a rare event [61–64]. Experimental work in mice has shown that it is possible to induce a Th2 response to HMW proteins such as ovalbumin and latex following prolonged exposures to compromised (abraded or occluded) skin [65–68]. Inhalation of LMW sensitizing agents is also a significant route of exposure however experimental data from animal models suggest that skin contact can also induce sensitization [69]. It is difficult to assess from the epidemiology data if skin contact in the workplace can also lead to sensitization to LMW agents. Like HMW agents, skin contact with LMW agents can elicit allergic symptoms among sensitized workers. One study suggests that skin contact to MDI in a sensitized worker led to airway symptoms [70]. The findings from the animal studies indicate that caution should be taken and workers minimize their skin contact with both HMW and LMW sensitizing agents.

SOCIOECONOMIC IMPACT OF OCCUPATIONAL ASTHMA

Occupational asthma has adverse social and economic impacts both on the employee and the employer. In the United States, the cost of occupational asthma in 1996 was estimated to be 1.1 billion dollars which included costs such as hospital stays, physician visits, asthma medication, medical insurance administration, and lost earnings [71]. These costs are incurred by both employer and employee. In 1999, the economic cost of occupational asthma in the United States was reported to be 1.5 billion dollars [72].

In a recent study of a United States employer, the annual per capita employer expenditures for an asthmatic worker was approximately 2.5 times higher than the control non-asthmatic worker ($5,385 vs. $2,121, respectively) [73]. Factoring in disability claims, total cost to the employer was approximately 3 times higher for the asthmatic worker than the control ($14,827 vs. $5,280, respectively).

Workers afflicted with OA, may suffer multiple hardships including lower wages due to transfer to other jobs, reduction in job responsibility, lost work days, lack of productivity, and long term unemployment. Quality and satisfaction of life can also be affected if there is development and/or worsening of ongoing asthma, exacerbation

of nocturnal asthma symptoms and a lack of job satisfaction. A study of 112 workers previously diagnosed with OA one year earlier, 32% still remained employed at the same job and exposed to the asthmagen while 68% left their previous jobs, thus removing themselves from further exposure [74]. The group that remained exposed to the asthmagen suffered more asthma symptoms and took more asthma medication such as inhaled steroids than the group that was removed from exposure. However, the group that remained exposed to the asthmagen reported less of a reduction in annual income compared to the latter group (a median loss of annual income of 35% compared to 50%, respectively). These findings were also reported in a cohort study of 25 patients with OA to both HMW and LMW allergens [75]. In another follow-up study that surveyed 209 workers that had been diagnosed with OA 1 to 5 years earlier 72% of the workers were still employed at the time of the survey with the remainder being unemployed (25%) or retired (3%) [76]. Fifty-five percent of the employed workers still worked for the same company but only 19% had the same job while 44% had the same job with better protection and 37% had different jobs within the same company. Seventy-five percent of the workers that left their former company reported that it was because of OA. Overall, 46% of the OA cohort in the study reported loss of income. Similar to the earlier studies, people that stayed with the same company suffered less economic loss than the people that left their former company (19% reporting loss of 19% of income compared to 84% reporting loss of income of 50%, respectively). In a longer term follow-up study of people diagnosed with diisocynate-mediated OA (10 years after being diagnosed with OA) 44% were still employed, 14% unemployed, and 41% retired [77]. Eighty percent of the unemployed and retired say it was because of OA. Thirty-four percent of the workers were not satisfied with their life situation. Fifty-seven percent were unsatisfied due to asthma/respiratory symptoms, 25% because of unemployment, 12.5% because of poor economic situation, 8% because of low compensation and the remainder for various miscellaneous reasons.

MANAGEMENT OF OCCUPATIONAL ASTHMA

Clinical experience dictates that workers with OA should be removed from exposure to facilitate their recovery. However, only about 25% of removed workers regain normal lung function [78]. Approximately 20 to 56% of workers removed due to isocyanate OA were clear of symptoms upon re-exposure to these chemicals [79]. Lemiere's study of 15 subjects with OA to a variety of HMW and LMW sensitizers showed that 60% had decreased but persistent bronchial hyper responsiveness 2 to 6 years after removal from exposure [80]. Therefore, preventing OA by controlling exposure, minimizing or preventing sensitization and preventing the onset of upper airway symptoms is a viable approach to keeping the workforce healthy.

Important to any management program is understanding if the materials being used in the workplace can function as occupational allergens. Various animal models have been used but none have been through the rigors of validation to show that they are predictive across a range of compounds. Most occupational allergens have been identified via clinical assessment. Education of the entire workforce is important since knowing that an allergen is being used is one step in the process of personal protection.

Pre-employment screening of potential workers for risk factors associated with occupational asthma is not viable from a legal/ethical stand or from a practical point of view. Newman-Taylor estimated that seven atopic individuals would have to be denied employment in order to eliminate one case of OA [81]. Since asthma is a complex disease, focus on one risk factor (e.g., atopy) will have little impact on prevention. Investigators have suggested that this can lead to a false sense of control with the opportunity for greater disease [82].

There are a few examples of how proactive exposure control programs have been able to eliminate or greatly reduce OA in certain industries. In the health care industry, allergy and OA to latex protein is a prevalent disease. Minimizing exposure to latex by removal of the allergen (e.g., switching to the use of non-latex gloves or low allergen, non-powdered latex gloves) has helped to reduce exposure to latex proteins and subsequently reduce the number of people with symptoms [83,84].

In Canada, legislation requires air monitoring of isocyanate levels and medical monitoring of exposed workers as part of an exposure control program. This has led to a decrease in the number of isocyanate induced compensated medical cases [85]. In addition, the program allowed for faster detection of isocyanate-induced OA cases, leading to earlier removal of symptomatic workers and remediation of the workplace. Gannon and colleagues observed that of the workers removed from exposure to a workplace allergen those that had been diagnosed significantly earlier after the onset of their first symptom and removed sooner had a better prognosis of fewer and less severe asthma symptoms [74].

A comprehensive program was developed by the detergent industry to manage OA to enzymes. Changes in formulation to reduce aerosolization of detergent and enzyme dust, changes in engineering to minimize mechanical actions that lead to aerosolization, improved ventilation and filtration, access to personal protective equipment, alteration of clean up procedures, air monitoring and medical monitoring have all contributed to significant decreases in sensitization to enzymes and OA is now a rare event [49]. Overreliance on one parameter (e.g., formulation) without adequate attention to the others had led to onset of OA in isolated manufacturing sites (86).

SUMMARY

OA is a prevalent disease in the workplace with significant impact on health and quality of life. Management of OA is based on a multidisciplinary approach including methods to identify allergens, use of appropriate exposure control methods, education of the workforce and surveillance (air levels and medical).

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34 Chemical Allergy: Hazard Identification, Hazard Characterization, and Risk Assessment

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INTRODUCTION

Chemical allergy is the commonest manifestation of immunotoxicity, which is defined as the adverse health effects that result from the interaction of xenobiotics with the immune system. In the context of environmental and occupational health, the two forms of chemical allergy that are of the greatest importance are allergic contact dermatitis (ACD) or skin sensitization and respiratory allergic hypersensitivity resulting from sensitization of the respiratory tract. The former is considered usually to be a delayed type hypersensitivity (DTH) reaction in which T lymphocytes play a central role [1–2]. There are many hundreds of chemicals that have been identified as having the potential to cause chemical contact allergy [3] and various methods have been available for some decades for the identification of potential contact sensitizers. Historically, guinea pigs were the species of choice for the assessment of skin sensitization, the approach being

to examine the ability of test chemicals to elicit challenge-induced cutaneous reactions in previously exposed animals [4]. In recent years, a detailed appreciation of the events associated with the induction phase of skin sensitization has facilitated the development of an alternative strategy: the murine local lymph node assay (LLNA) [5–7]. The LLNA is based upon the observation that the initiation phase of contact sensitization is dependent upon the stimulation of lymphocyte proliferative responses in the lymph node draining the site of topical exposure [8]. In this assay, activity is measured as a function of the vigor of proliferative responses elicited in draining lymph nodes following topical application of the test chemical [5–8].

Fewer chemicals have been identified as causative agents of chemical respiratory allergy; such include acid anhydrides, isocyanates, some platinum salts, certain reactive dyes, and chloramine-T [9–10]. Nevertheless, respiratory hypersensitivity is a significant health problem with important socioeconomic consequences [11], with diagnosis resulting usually in a requirement to avoid exposure and hence increased likelihood of periods of unemployment [12–13]. In contrast to the situation for contact sensitization testing, there are at present no well validated or widely accepted methods for the predictive identification and characterization of chemical respiratory allergens. A major constraint to the development of such tests has been continuing uncertainty regarding the relevant immunobiological mechanisms. Whereas immediate type hypersensitivity responses and asthma to protein allergens are generally acknowledged to be dependent on IgE-mediated mechanisms, there is no such consensus regarding chemical respiratory hypersensitivity [14–15]. Despite this uncertainty, there is increasing evidence that allergic sensitization of the respiratory tract will be favored by the induction of a polarized type 2 immune response, conditions that facilitate the development and expression of immediate type hypersensitivity reactions, including respiratory sensitization [15–16]. Analogous with testing for contact sensitization, toxicological investigations of chemical respiratory allergy were initially conducted using the guinea pig, primarily because it is possible in this species to elicit and measure with relative ease challenge-induced pulmonary reactions that resemble in some ways the clinical manifestations of human allergic asthma [17–19]. With the increased understanding of the immunobiology of chemical respiratory allergy have come new opportunities for the development of tests for the identification of potential respiratory allergens: tests that do not rely upon the elicitation of clinical manifestations of respiratory distress.

This chapter will review the application of these methods for the hazard identification and characterization of chemical allergens and, where appropriate, for the measurement of relative potency in the context of risk assessment.

IMMUNOBIOLOGY OF CHEMICAL ALLERGY

Before consideration of the available methods, what is known of the immunobiological mechanisms of both forms of chemical allergy will be reviewed briefly. The development of immune responses, including allergic responses, is directed by the activity of $CD4^+$ T helper (Th) cell subpopulations and their cytokine products. Two phenotypes

of Th cell, designated Th1 and Th2, predominate in the mature immune response and are characterized by their selective cytokine secretion profiles [20–22]. Th1 cells express preferentially interferon γ (IFN-γ), interleukin (IL)-2 and tumor necrosis factor β (TNF-β) whereas only Th2 cells express IL-4, IL-5, IL-6, IL-10 and IL-13. These functionally distinct subpopulations of Th cells allow the host to tailor the quality of immune responses to challenge that is most appropriate to the nature of the immunological insult. Cell-mediated immunity, necessary to confer host resistance to viral challenge for example, is provided by Th1 cells. In contrast, Th2 cells provide for immunity against multicellular parasites, producing cytokines that promote humoral immune function and the costimulation and differentiation of B lymphocytes [23–24]. The existence of functional subpopulations of Th cells also provides a mechanism for the development of different qualities of allergic disease. It has been demonstrated that the Th1 cytokine IFN-γ facilitates DTH responses and that adoptive transfer of Th1 cell clones alone is sufficient to transfer DTH reactions to naïve recipients [25–26]. In contrast, treatment with the type 2 cytokine IL-4 was sufficient to reverse established DTH reactions [27]. Thus, chemical contact allergy, a DTH reaction, is likely to be favored by the induction of Th1 cells. Conversely, immediate type hypersensitivity reactions are promoted by Th2 cells and their products. For example, IL-5 and IL-10 are growth and differentiation factors for mast cells and eosinophils and stimulate the recruitment of eosinophils, cells which play important roles in the elicitation of the clinical manifestations of the allergic response [16, 28]. Importantly, IFN-γ and IL-4 (type 1 and type 2 cytokines, respectively) exert mutually antagonistic effects on IgE antibody responses, with IL-4 essential for the induction and maintenance of IgE antibody responses and IFN-γ exerting an inhibitory effect [29–31]. Th1 and Th2 cells, with their divergent patterns of cytokine production, develop from a common precursor cell, designated Th0 [32–33]. Th0 cells have the potential to express both Th1 and Th2 type cytokines, but with time or repeated exposure differentiate into Th1 or Th2 cells with their polarized cytokine secretion patterns. This process is controlled largely by positive feedback and negative cross-regulation by Th1 and Th2 cell products [28].

It has recently become apparent that in addition to Th cell subpopulations, there exist similar subpopulations of $CD8^+$ T cytotoxic (Tc) cells that may also play a role in the development of allergic diseases. Two populations, designated Tc1 and Tc2, have been described that are analogous with Th1 and Th2 cells with respect to their selective cytokine secretion patterns [23]. There is increasing evidence that IFN-γ-producing Tc1-type cells play an important role in the mediation and/or regulation of contact hypersensitivity reactions [34]. Until recently, in common with DTH reactions to complex protein or cellular antigens, ACD was considered to be mediated by $CD4^+$ IFN-γ producing effector cells. It is now apparent that $CD8^+$ (Tc1-type) effector cells also contribute to the development and elicitation of ACD to chemical allergens [35-36]. However, there is limited evidence of a physiological role for Tc2-type cells in immediate type hypersensitivity reactions [16, 37].

In summary, chemical contact sensitization will be favored by the selective activation of Th1 and Tc1 cells, whereas immediate type hypersensitivity reactions such as chemical respiratory hypersensitivity are apparently associated with the preferential activation of Th2 (and possibly Tc2) type cells.

HAZARD IDENTIFICATION

Contact Sensitization

For some decades, there have been available guinea pig tests for the prospective identification of chemicals with the potential to cause contact sensitization, including the occluded patch text of Buehler and the guinea pig maximization test first described by Magnuson and Kligman [4, 38–39]. In these assays, sensitization potential is assessed as a function of the incidence of challenge-induced cutaneous reactions in previously-sensitized animals. Such assays served a very useful purpose but were not without their limitations, including a need to challenge the animals at sub-irritant doses, an inability to test colored compounds and some animal welfare concerns regarding the use of restraint and adjuvant. These reasons, coupled with significant advances in our understanding of the immunobiological mechanisms of contact allergy led to the development of an alternative assay; the murine LLNA. This assay has now been validated and adopted formally by various regulatory agencies in the United States and the European Union (EU) for use as a stand-alone alternative for the purposes of hazard identification (that is, a method that can be used not only for the identification of skin sensitization hazard, but also for confirmation of the absence of such hazard) [40–44].

Local Lymph Node Assay

The LLNA measures the ability of topically applied chemicals to induce proliferative responses in lymph nodes draining the site of exposure; that is, sensitizing activity is assessed as a function of events occurring in the induction, rather than of the elicitation, phase of sensitization [6]. This endpoint is relevant for the evaluation of skin sensitizing potential as the activation and clonal expansion of allergen-responsive T lymphocytes is the central event in the acquisition of skin sensitization, with the vigor of lymph node cell proliferative responses correlating with the extent of sensitization [8]. In the standard assay, CBA strain mice receive repeated topical applications of the test chemical (or of the relevant vehicle control) on the dorsum of both ears. Proliferation in the (draining) auricular lymph node is measured as a function of *in vivo* incorporation of tritiated thymdine (^{3}H-TdR) or ^{135}I-deoxyuridine [5, 45]. There is currently some debate about the need for concurrent positive controls for the LLNA, or whether such controls should be conducted at regular (6 monthly) intervals [46]. If it is necessary to include a positive control, then the most suitable chemical allergen for this purpose is hexyl cinnamic aldehyde (HCA) [47–48].

For each concentration of chemical tested in the LLNA, a stimulation index (SI) is derived using the value obtained with the concurrent vehicle control as the denominator. Chemicals that induce an SI of three or more are defined as skin sensitizers. This threshold for positivity was based initially on empirical observations in investigations of both contact allergens and non-sensitizing chemicals [5]. Subsequently, a retrospective statistical analysis of results obtained in the LLNA with a wider range of chemicals (130) confirmed that an SI value of 3 does indeed provide the most appropriate basis

for discriminating between skin sensitizers and non-allergens [49]. The LLNA has been compared extensively with guinea pig predictive tests and human contact sensitization data [6, 50–57] and in the context of both national and international inter-laboratory trials [5, 45, 50, 58–61]. These investigations demonstrated that equivalent results are generated in independent laboratories using a wide variety of chemical allergens and non-sensitizing chemicals. In addition, the sensitivity, selectivity and overall accuracy of the LLNA were found to be comparable with, or better than, commonly used guinea pig tests. As with other toxicological tests, the potential for false positive results needs to be considered. There have been reports that some irritants, particularly the non-sensitizing skin irritant sodium lauryl sulfate (SLS), elicit weak positive responses in the LLNA [6, 45, 62]. However, the majority of non-sensitizing skin irritants fail to cause positive responses in the LLNA [59-60, 63–64]. Furthermore, various criteria can be used to exclude false positives, including possession of irritant activity, lack of a structural alert and weak positivity at high doses only [63].

Chemical Respiratory Allergy

Historically, guinea pigs were the species of choice for the assessment of respiratory sensitizing potential and much has been achieved using these methods. Such methods vary in detail, but common to most of them is the fact that sensitizing activity is measured as a function of inhalation challenge-induced pulmonary reactions in animals sensitized previously to free chemical or to hapten-protein conjugate, via one of several routes of exposure [17–19, 65–67]. The measurement of pulmonary challenge reactions in sensitized guinea pigs permits, in theory at least, consideration of dose-response relationships in the elicitation of respiratory hypersensitivity which may be of use in the risk assessment process. However, such methods have been found to lack sensitivity and it has often proven difficult to elicit a positive bronchoconstrictive response, particularly to some isocyanates [68]. Furthermore, the number of materials which has been examined is rather limited, with the majority of the work performed with two classes of chemicals, the diisocyanates and the acid anhydrides. For these and other reasons there has been a growing interest in the development of alternative methods, particularly those in which chemicals are assessed as a function of immune responses induced in rodents.

Rodent IgE Tests

Over the past decade, there have been considerable advances in our understanding of the immunobiological mechanisms that result in the quality of immune response necessary for the induction of chemical respiratory allergy. Experiments designed to characterize immune responses in mice to chemical sensitizers have demonstrated that different classes of chemical allergen stimulate the development of qualitatively discrete immune responses consistent with the selective emergence of functional subpopulations of T lymphocytes [16]. Thus, topical exposure of BALB/c strain mice to chemical contact allergens such as 2,4-dinitrochlorobenzene (DNCB) results in the induction of

lymphocyte proliferation, IgG antibody production and the elicitation of delayed type cutaneous hypersensitivity reactions (measured as a function of challenge-induced increases in ear thickness) in the absence of detectable IgE responses [69–71]. In contrast, exposure of mice to chemical respiratory allergens such as trimellitic anhydride (TMA) was associated also with the appearance of specific IgE antibody and increases in the total serum concentration of IgE [69–70] and the elicitation of immediate (1 hour) cutaneous hypersensitivity reactions [71]. The observation that topical exposure of mice to chemicals that are known or suspected to be human respiratory allergens provokes a significant increase in the total serum concentration of IgE forms the basis of the mouse IgE test for the prospective identification of chemical respiratory allergens [71–75].

The following protocol was developed: groups of animals are exposed topically to chemical bilaterally on the shaved flanks and 7 days later on the dorsum of both ears. Fourteen days after the initiation of exposure, serum samples are prepared, the concentration of IgE in these samples measured using a sandwich enzyme-linked immunosorbant assay [ELISA] calibrated with a monoclonal IgE standard and the results recorded as IgE concentration in µg/ml [71]. Where possible, application concentrations are selected that are positive in the LLNA, demonstrating that the chemical is immunogenic via this route of exposure. If a chemical fails to stimulate increases in serum IgE at application concentrations that are LLNA positive, then it is possible to conclude that the chemical is inherently immunogenic via this route of exposure, but the response provoked is not of the quality necessary to cause a detectable increase in IgE production. Using this protocol, it was demonstrated that various respiratory sensitizers (including TMA and certain diisocyanates) stimulated dose-related increases in total serum IgE concentration compared with concurrent vehicle-treated control values [71–74]. No similar changes were observed when mice were exposed for comparative purposes in an identical manner to immunogenic concentrations of the contact allergens DNCB, oxazolone, eugenol, isoeugenol, or formaldehyde [72, 73, 75].

Although this method shows some promise as a predictive method, it has become apparent that variability in constitutive levels of serum IgE can influence the performance of the test. The reason for this variability remains unclear, but it is evidently not treatment-related. For example, in a recent inter-laboratory evaluation of the mouse IgE test, variations in IgE levels among treatment groups and untreated (naïve) mice were recorded [76]. However, in four out of the five participating laboratories TMA was still clearly distinguishable from DNCB with respect to induced increases in total serum IgE [76]. In additional experiments, induced IgE responses in BALB/c strain mice were compared with those provoked in mice of C57BL/6 strain. Both strains of mice displayed TMA-induced increases in serum IgE. However, C57BL/6 mice exhibited much more stable serum IgE concentrations and lower constitutive levels of this immunoglobulin, suggesting that this strain may be more suitable for the conduct of the mouse IgE test [76]. Furthermore, a successful mouse IgE test was reported recently using mice of the F1 hybrid of BALB/c and C57BL/6 strain mice (B6C3F1) [77]. Although the mouse IgE test shows some promise, additional investigations are required for optimization of this test for routine application for the prospective identification of chemical respiratory sensitizers.

Other investigators have considered the use of an alternative species, the Brown

Norway (BN) rat, for the analysis of induced changes in serum IgE concentration [78–80]. The BN rat is analogous to the BALB/c mouse with respect to its predisposition towards the development of IgE responses. Serial bleeds can be taken in this species, allowing prescreening of animals and the kinetics of the induction of IgE responses to be assessed. Initial results were encouraging, with relatively stable constitutive levels of serum IgE observed that were unaffected by treatment with vehicle or with the contact allergen DNCB or the non-sensitizing skin irritant methyl salicylate [78–80]. In contrast, exposure to TMA resulted in a significant increase in the concentration of total serum IgE in the majority of experiments. There were some inter-batch differences in the responsiveness of BN rats to TMA, and in a minority of experiments there was no significant increase in IgE following exposure to TMA [80]. These experiments suggest that it is prudent to incorporate a concurrent positive (TMA) control in such tests. Additional experiments have been conducted using a wider range of chemical respiratory sensitizers including selected isocyanates [81]. Although topical exposure of BN rats to TMA stimulated robust increases in total serum IgE, the other respiratory allergens either failed to induce IgE or induced only transient and relatively weak changes in serum IgE. These data suggest that the rat IgE test in its current format is insufficiently sensitive for the routine identification of all potential chemical respiratory allergens.

HAZARD CHARACTERIZATION

An alternative approach to hazard identification of chemical respiratory allergens is a method that can be applied to hazard characterization of chemical allergens; cytokine fingerprinting, a method for characterizing the quality of immune response that a chemical is able to elicit in mice that is able to distinguish between contact allergens and chemicals that are able to cause sensitization of the respiratory tract. This method is based on the observation that chemical allergens induce in mice of discrete forms of immune response that are characterized by selective cytokine secretion patterns which are consistent with the evolution of preferential Th cell (and Tc cell) responses [37, 81–85]. The induction of cytokine expression *per se* is a reflection of the overall immunogenicity of a chemical, whereas the selectivity of cytokine production is a function of the quality of immune response provoked and, as a consequence, is indicative of the type of allergic response which will develop subsequently.

CYTOKINE FINGERPRINTING: MEASUREMENT OF PROTEIN

In the most common configuration of the method, groups of BALB/c strain mice are exposed repeatedly by topical application to the test chemical over a period of 14 days. In common with the IgE test, application concentrations are selected on the basis of immunogenicity (LLNA positive) when administered by this route. Concurrent control animals are treated with either the reference respiratory allergen TMA, or the reference contact allergen, DNCB. One day following the final exposure, mice are sacrificed and the draining auricular lymph nodes excised and pooled for each experimental group.

A single cell suspension of LNC is prepared under aseptic conditions and cultured for various periods of time. The production by LNC of type 1cytokines (such as IFN-γ and IL-12) and type 2 cytokines (such as IL-4, IL-5, IL-10 and IL-13) is measured using cytokine specific ELISAs or cytokine microarrays. It is possible to measure the spontaneous production of IL-5, IL-10, IL-12, IL-13 and IFN-γ by LNC without restimulation *in vitro*. However, IL-4 appears to be produced in comparatively small amounts and to induce detectable levels of this cytokine an additional stimulus is required: concanavalin A (con A), a T lymphocyte mitogen. Culture of LNC derived from mice treated with chemical respiratory sensitizer with con A will stimulate the production of detectable levels of IL-4. Treatment with the same mitogen of LNC derived from naïve or vehicle-treated mice fails to induce measurable IL-4 secretion [86].

Investigations have been conducted using a range of additional chemical respiratory allergens, including isocyanates, platinum salts, glutaraldehyde, and various acid anhydrides. In each case, a preferential type 2 cytokine expression profile was recorded [86]. Using the same exposure protocol as that used for the chemical respiratory allergens described above, treatment with allergens such as 2,4-dinitrofluorobenzene (DNFB), isoeugenol and hexyl cinnamic aldehyde (contact allergens that apparently lack respiratory sensitizing activity) stimulates instead a selective type 1 cytokine secretion pattern [86]. These data demonstrate that for a variety of chemical allergens, the measurement of induced secretion of IFN-γ and IL-12 provide preferential markers of type 1 responses, whereas IL-4, IL-5, IL-10 and IL-13 can be used as markers of selective type 2 responses. The observation that different classes of chemical allergen stimulate divergent immune responses at the level of cytokine expression has been confirmed by other investigators [87–89]. In apparent conflict with these data are reports that contact allergens such as DNCB and oxazolone can stimulate type 2 cytokine expression [91]. The important point here is that the divergent cytokine secretion profiles provoked by different classes of chemical allergen are selective, not absolute. It is the balance between Th1 and Th2 cell activation and cytokine products that determines the nature of the developing immune response. Thus, the observation by Ulrich and colleagues [90] that contact allergens such as DNCB stimulated measurable IL-4 expression is not inconsistent with the results reported herein, as spontaneous expression of type 2 cytokines was also detected after treatment with DNCB, albeit at much lower levels than those stimulated by TMA [82].

Cytokine Fingerprinting: Measurement of Message

Cytokine profiling has also been measured as a function of changes in cytokine mRNA expression using either reverse transcription polymerase chain reaction (RT-PCR) [87, 91–93] or ribonuclease protection assay (RPA) [94–97]. Measurement of cytokine transcripts by RT-PCR revealed that prolonged exposure to TMA induced increased levels of IL-4 mRNA expression compared with treatment with DNCB [87, 92–93]. However, expression of the type 1 cytokine IFN-γ by DNCB-activated LNC was variable and failed to discriminate between contact and respiratory allergens [87, 91, 93). A similar profile was observed for freshly isolated tissue analyzed by RPA. This somewhat less

sensitive technique detected preferential type 2 cytokine expression following prolonged treatment with the respiratory allergens TMA and various diisocyanates [94–97). However, a selective type 1 cytokine mRNA phenotype, particularly with respect to IFN-γ, was not induced by treatment with the contact allergens DNCB or DNFB [95–97). The reasons for the failure to detect increased mRNA for IFN-γ despite robust secretion of this cytokine are presently unclear, although it would appear that production of this cytokine by draining LNC is controlled mainly at the level of secretion. With respect to characterization of sensitizing hazard, the judicious view is that cytokine profiling by RPA or RT-PCR may identify those chemicals with respiratory sensitizing potential as a function of induced type 2 cytokine mRNA expression, but are inappropriate for the identification of contact allergens. More experience is required with a wider range of respiratory sensitizers before the sensitivity and selectivity of these methods versus measurement of protein secretion can be assessed.

RISK ASSESSMENT

The identification of potential hazard is an important first step in any toxicological evaluation. For accurate assessment of risks to human health, however, it is necessary to integrate information on the likely conditions and extent of exposure with an understanding of potency. At present there is insufficient understanding of relative potency of chemical respiratory allergens to permit such analyses. For chemical contact allergens, however, considerable progress has been made in the application of data from the LLNA to the risk assessment process [98–100]. For contact sensitivity, potency is defined as a function of the amount of chemical required for the acquisition of skin sensitization, with the important parameter in this respect being the amount of chemical experienced per unit area of skin [101]. The approach adopted with LLNA data is to derive mathematically by linear interpolation an EC3 value (Estimated Concentration of chemical required to provoke an SI of 3); the minimum amount of chemical that is required to induce in the assay a threshold positive response [102]. EC3 values are expressed in terms of total amount of chemical applied (most commonly as a percentage but also as a molar value) or amount of chemical applied per unit area of skin. The correlation between skin sensitizing potency of chemical allergens measured in the LLNA (EC3 values) and what is known of their relative activity in humans has been investigated. There was found to be a very close correlation between clinical assessments of potency and EC3 values; the chemicals considered to be the most active contact allergens in humans having the lowest EC3 values (relatively small amounts of compound required to induce a threshold positive response) [103–104]. Chemical allergens have been shown to differ by several orders of magnitude in terms of their relative potency and therefore with respect to EC3 values [103–104]. The view currently is that classification schemes should be predicated on no less than 10-fold differences in EC3 values; one such recent scheme derives from a recent European Centre for Ecotoxicology and Toxicology Task Force [100]. The recommendation was that 4 categories would be used, based on percentage concentration EC3 values, and identified by the descriptors: *Extreme, Strong, Moderate,* and *Weak.* The suggestion was that the scheme should distinguish between

contact allergens on the basis of 10-fold differences in potency as follows: Extreme, EC3 < 0.1; Strong, EC3 > 0.1 to < 1; Moderate, EC3 > 1 to <1 0; Weak, <1 0 to > 100.

Critical to conducting a sound skin sensitization risk assessment is having a thorough understanding of the anticipated consumer exposure to the ingredient, as well as knowledge of the allergenic potency of the chemical and dose response characteristics. The importance of exposure and potency estimation in a skin sensitization quantitative risk assessment has been described in recent publications highlighting the application of this process to the evaluation of new and existing chemicals [105–109]. Key steps of the quantitative risk assessment process are determination of known safe benchmarks, application of sensitization assessment factors and calculation of consumer exposure through normal product use. Using these parameters, an acceptable exposure level can be calculated and compared with the consumer exposure level. The ratio of the acceptable exposure level to the consumer exposure level must be favorable to support the safe use of the skin sensitizer. This approach has been valuable for conducting both retrospective and prospective skin sensitization risk assessments.

CONCLUSIONS

Over the past few decades, there has been a substantial increase in our understanding of the cellular and molecular events through which sensitization to chemical allergens is acquired and the mechanisms that result in different qualities of allergic response. In parallel, considerable progress has been made towards the development of methods for the identification, characterization and risk assessment of chemical allergens. For chemical contact allergy, improved methods for skin sensitization hazard identification are available and such have been applied successfully to the risk assessment process. Although there are as yet no validated or widely applied methods for the identification of chemical respiratory allergens, there are methods which show some promise, including cytokine profiling. It is anticipated that the progress to date will provide the foundations for the further development and refinement of methods for the characterization of chemical allergens.

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35 Food Allergy: Immunological Aspects and Approaches to Safety Assessment

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INTRODUCTION

Food allergy, by definition, describes the adverse health effects that result from the elicitation of allergic reactions to dietary proteins. This does not necessarily imply, however, that allergic sensitization is invariably acquired via dietary exposure to proteins. As will be discussed later in this chapter, sensitization to food proteins may develop following encounter via other routes of exposure.

An important point is that food allergy can be, and should be, distinguished from other forms of food intolerance where the pathogenesis is non-immunological in nature.

In recent years food allergy has assumed greater significance for the practicing toxicologist, primarily with respect to the need for effective assessment of the allergenic potential of novel foods, and in particular foods derived from genetically modified crop plants. This area of immunotoxicology has attracted considerable interest, and detailed

reviews of the main issues are available elsewhere [1–11]. It is the purpose of this chapter to review briefly basic clinical and immunological aspects of food allergy, and to consider some of the approaches available for identification of allergenic hazards.

FOOD ALLERGY: GENERAL AND CLINICAL ASPECTS

True food allergy is an important and not uncommon disease [12–14]. Estimates vary, but the prevalence of food allergy among adults in Western Europe and the United States is believed to be no less than 1%, with some more recent analyses suggesting figures approaching 3%. The prevalence among children is somewhat higher and in the region of 5 to 6% [15, 16]. The disparity between these figures reflects the fact that food allergy in children not infrequently resolves with increasing age, the best example being allergy to cows' milk. Allergy to other foods, notably peanuts and tree nuts, is commonly life-long, but not invariably so, and the development of clinical tolerance has been found here also [12, 17, 18]. The evidence is that, in common with other forms of atopic allergic disease, the prevalence of food allergy in Europe and the United States has been increasing significantly during recent years [19, 20].

In Western Europe and the United States, most cases of food allergy are associated with a relatively limited range of produce, the most common causes being cows' milk, hens' eggs, peanuts, tree nuts, wheat, soybeans, fish, and shellfish [21–24]. It is clear that the extent of allergy associated with particular foodstuffs is related to established dietary habits and preferences, the introduction of new foods into national diets, the ways in which food is prepared, and probably the age at which a particular food is first experienced in the diet [25–30]. The important observation with respect to the theme of this chapter is that changes in dietary habits, and the introduction of new foods into diets, can result in the appearance, and increasing incidence, of allergy to the novel food; an illustrative example being the growing importance of allergy to kiwi fruit in the U.K. [29].

The clinical symptoms of food allergy vary. They can include nausea and vomiting, abdominal pain, distension, flatulence, and diarrhea, although other organ systems may be involved, such as the respiratory tract (allergic rhinitis and asthma), and the skin (atopic dermatitis, acute urticaria, and angioedema) [2]. Occasionally severe systemic (anaphylactic) reactions may be elicited and these can prove fatal [2, 31, 32]. Although various novel therapeutic approaches are currently being explored, no effective treatments for food allergy are presently available. The disease is managed by avoidance of relevant produce [3, 33].

IMMUNOLOGY OF FOOD ALLERGY

Food allergy by definition is dependent upon the induction of a specific immune response, and this needs to be of a vigor and quality necessary for the acquisition of sensitization, and the subsequent elicitation of an allergic reaction following encounter with the relevant food protein. Although cell-mediated immune responses are known

to play important roles in some forms of food allergy (e.g., cows' milk—enteropathy, and coeliac disease), it is most commonly IgE antibody mediated effector mechanisms that are implicated. Sensitization is characterized here by the induction of a specific IgE antibody response. These antibodies distribute systemically and associate, via specialized plasma membrane receptors (FcεR), with tissue mast cells. The subject is now sensitized. If there is subsequent exposure to the same protein, or to an immunologically cross-reactive protein, then mast cell-associated IgE will be cross-linked causing degranulation and the release of pre-formed and newly synthesized mediators that will act in concert to provoke the local and/or systemic inflammatory reactions that drive the symptoms of food allergy.

The initiation and maintenance of IgE antibody responses are regulated by, and dependent upon, the generation of T lymphocyte responses of the correct (Th2-type) phenotype. These cells produce type 2 cytokines (including interleukins (IL) 4, 5, and 13) that collectively facilitate IgE antibody production, and that participate importantly in the elicitation of IgE-mediated allergic reactions [34–36].

At a rather simplistic level it is possible to view the induction of IgE antibody responses to food proteins, and the development of clinical food allergy, as a breakdown of immunological tolerance [37–41]. The latter is best regarded in operational terms as a process whereby dietary exposure to foreign proteins can be tolerated, insofar as potentially tissue damaging allergic responses are not induced. This does not imply, however, that oral tolerance to food proteins is necessarily an absolute phenomenon, and a more accurate description is immunological hypo-responsiveness. Thus, oral tolerance is selective, and although IgE antibody responses and cell-mediated immunity are markedly down-regulated, IgG antibody is much less affected. For this reason IgG antibodies to food proteins experienced in the diet are routinely identified in subjects with no history of food allergy [42]. Indeed, it may be that the elaboration of IgG antibody responses to dietary proteins serves some useful purpose in providing a mechanism for clearing proteins or peptides from the circulation that have been absorbed inappropriately from the gastrointestinal (GI) tract.

In summary, therefore, the ability to tolerate dietary exposure to food proteins does not demand the suppression of all immune responses, but rather the avoidance or selective inhibition of immune responses of the quality required for IgE antibody production and allergic sensitization. Against this background an intriguing question relates to the nature of factors that influence the loss or failure of immunological tolerance and acquisition of allergic sensitization to food proteins, and which underlie inter-individual differences in susceptibility to food allergy [43].

FACTORS AFFECTING THE ACQUISITION OF FOOD ALLERGY

One important variable appears to be conditions of exposure, and this embraces considerations of the potential impact of the level and duration of exposure, the age of first exposure (and whether there has been opportunity for exposure *in utero* or during lactation), and the matrix in which ingested food proteins were first experienced [44–47]. One additional aspect of exposure merits attention and that is the route through

which protein is encountered, or at least first encountered. There is no *a priori* reason to assume that the acquisition of allergic sensitization to food proteins is achieved only following exposure via the GI tract. Adaptive immune function is systemic in nature, and there is evidence that inhalation or dermal exposure to food proteins, or to other proteins cross-reactive with food proteins, can result in sensitization for food allergy. One manifestation of the development of sensitization via inhalation exposure is the oral allergy syndrome (OAS). The disease is characterized by IgE antibody-mediated reactions in the oropharyngeal mucosa provoked by local contact with food proteins; the foods frequently associated with OAS being fruit and vegetables that contain labile proteins with epitopes that are cross-reactive with those expressed by pollen (such as birch pollen) proteins. The evidence is that in OAS sensitization to food proteins is achieved via inhalation exposure to pollen, rather than by dietary contact with the food protein itself (because the latter is insufficiently stable to survive for sufficient time with the hostile environment of the GI tract to elicit an immune response) [48, 49].

Currently, there is considerable interest in the possibility that an important route of exposure for the development of food allergy is via skin contact with proteins [50–54]. The relevance of the skin in this context is not simply that it represents another route via which systemic sensitization might be achieved, but rather that the stimulation of cutaneous immune responses to protein allergens may impair or subvert establishment of oral tolerance following subsequent dietary exposure to the same protein. The possibility is that the route of exposure through which a food allergen is first experienced may have a long-lasting impact on the characteristics of immune responses to that protein.

Although the conditions, route, and timing of exposure appear collectively to have a pivotal influence on whether and to what extent sensitization to a particular food protein will be acquired, there is no doubt that the most important factor in determining overall susceptibility to food allergy is genetic predisposition and inheritance of atopy. An atopic phenotype in one or both parents (and possibly particularly in the mother) substantially increases the likelihood of atopy in the infant [55, 56]. Taken together the available evidence suggests that against a generalized predisposition to develop IgE-mediated responses to food proteins (which may be largely inherited, but possibly impacted also by certain environmental factors), it is the conditions, timing, and route of exposure that will determine to which particular food proteins allergic responses are directed.

THE INFLUENCE OF ANTIGEN AND THE BASIS FOR HAZARD IDENTIFICATION

The question that is addressed here is the nature of characteristics that confer on proteins the ability to induce allergic sensitization (in an inherently susceptible subject, and under appropriate conditions of exposure). It can be argued that, if levels of exposure are sufficient, any food protein has some potential to induce allergic sensitization. Although this concept is, in practice, difficult to disprove, experience suggests that this is not the case. Only a fraction of food proteins experienced in the diet are implicated as food allergens. The arguments are: (1) that most or all food proteins are inherently immunogenic and potentially able to stimulate immune responses characterized by IgG

antibody; and (2) that a fraction (a minority) of these food proteins have properties that allow (in susceptible subjects, and under appropriate conditions of exposure) stimulation of the quality of response required for the sustained production of IgE antibody and allergic sensitization.

The challenge then for immunotoxicologists is to identify those proteins that have the ability to induce allergic sensitization and to distinguish these from non-allergenic, but nevertheless immunogenic, proteins that do not possess sensitizing activity. Strategies for hazard identification are reviewed in the next section, but in advance of examining practical approaches to safety assessment it is relevant to consider the characteristics of proteins that serve to confer sensitizing activity. There are available comprehensive reviews of the area [2, 57–63]. A similarly detailed account is beyond the scope of this chapter, but it is relevant nevertheless to list below the various properties that are considered to be of relevance for sensitizing activity. The characteristics summarized here (in no order of importance with respect to their perceived contribution to sensitizing potential) are not necessarily exhaustive. Nor in many instances has the mechanistic link between possession of a particular trait and the potential to induce sensitization been established.

The most important properties are thought to be:

- Size of the protein.
- General structural and functional properties.
- Glycosylation status (quality and quantity).
- Resistance to proteolytic digestion.
- Nature of interactions with, and processing by, dendritic cells and other antigen presenting cells.
- The expression of conformational and/or linear allergenic epitopes.

It is proposed that these characteristics, either singly or collectively, play decisive roles in determining whether or not a protein will have the inherent potential to cause allergic sensitization in a susceptible subject. It is important to appreciate, however, that these attributes do not necessarily act independently. Thus, for instance, there is reason to believe that carbohydrate residues will affect the recognition of proteins by antigen presenting cells, and the vigor and quality of resulting immune responses. Moreover, the resistance of proteins to proteolytic digestion will impact not only on the integrity of expression of allergenic epitopes, but is likely to influence also intracellular processing by antigen presenting cells.

Clearly the relationships between protein structure and function and inherent sensitizing activity are complicated and far from fully defined, and it is against this background of uncertainty that it is necessary to develop approaches suitable for hazard identification.

HAZARD IDENTIFICATION AND SAFETY ASSESSMENT

General surveys of strategies available for characterizing the allergenic properties of proteins, or the novel foods from which they derive, are available for reference [4, 11,

64–69]. As indicated above, approaches to identify proteins that have the inherent ability to induce allergic sensitization are based upon the assumptions that: (1) proteins vary with respect to their allergenic potential, and that, in practice at least, there are proteins that lack inherent sensitizing activity; and (2) that there are properties of allergenic proteins that can be used as the basis for *in silico, in vitro* or *in vivo* discrimination from those that are unable to cause sensitization. Predicated on these assumptions, the modern history of protein safety assessment paradigms in the context of food allergy can be reasonably be said to date from 1996 when the first conceptual framework was proposed [70]. The suggested strategy resulted from a collaboration between the International Food Biotechnology Council (IFBC) and the International Life Sciences Institute (Allergy and Immunology Institute), and took the form of a decision tree. This tiered structure comprised considerations of: (1) whether or not the protein of interest derived from a source established previously to be associated with allergy in humans, (2) amino acid sequence homology with confirmed protein allergens, and (3) the degree of resistance to digestion by simulated gastric fluid (SGF). In addition, the recommendation was that where the protein of interest derives from a source known to be allergenic (or where screening reveals amino acid sequence homology with a known allergen), then testing for serological identity with potentially cross-reactive allergens should be conducted using serum drawn from subjects known to be sensitized to the relevant protein(s) [70]. Since publication of the IFBC/ILSI recommendations in 1996 other decision trees have been proposed, most notably that deriving from a joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) consultation in 2001 [71]. Compared with the IFBC/ILSI proposals, the FAO/WHO decision tree had some unique features, one of which related to homology with known protein allergens. Specifically, the recommendation was that greater than an overall 35% amino acid sequence homology, and/or identity at the level of 6 or more contiguous amino acids, with an allergen provide alerts for potential allergenic activity [71]. This was in contrast to the basis for a structural alert in the IFBC/ILSI scheme in which identity at the level of 8 contiguous amino acids was identified as the threshold for concern [70]. In addition, two forms of serum screening were suggested: targeted serum screening and specific serum screening. It was intended that the first of these, targeted screening, should be deployed under circumstances where the novel gene derived from a source considered not be allergenic, and would comprise a general assessment of sera drawn from panels of allergic patients to react with the protein of interest. If, however, the gene derived from a known allergenic source then it was proposed that a more specific screen should be conducted using serum from patients with confirmed sensitization to the source material, or to closely-related allergen sources [71]. In practice, neither of these modifications to the first decision tree has been used in the way that was originally intended. Finally, unlike the IFBC/ILSI approach, the decision tree proposed by FAO/WHO made mention of the possibility of integrating into evaluations data derived from suitable animal models [70, 71], and this aspect of safety assessment will be discussed later.

It is appropriate here to consider briefly some of the experimental approaches that are contained within such decision trees, and that continue to provide the bases for protein allergenicity safety assessment.

Structural and Sequence Homology

As previously indicated, considerations of amino acid sequence homology have constituted a core element of safety assessment strategies for protein allergenicity [11, 72]. The purpose of this approach is to examine whether the protein of interest has areas of identity with known allergenic proteins that might represent T lymphocyte epitopes on the latter (and which might therefore indicate potential sensitizing activity), and to determine if there is sufficient similarity (cross-reactivity) with existing allergens for provocation of a reaction in a previously sensitized subject. For this purpose there are available a variety of allergen databases and bioinformatics tools [11, 72–78]. The original proposal that eight contiguous amino acids should represent a flag for potential identity was based on an understanding that optimal peptide length for T lymphocyte recognition is in the range of between 8 and 12 amino acids, with B lymphocyte epitopes being generally somewhat larger. The subsequent proposal resulting from the FAO/WHO consultations was even more conservative suggesting a match of 6 or more contiguous amino acids should be viewed as an alert. There is a general consensus that the latter threshold of six amino acids is of little practical value, being associated with an unacceptably high frequency of false positive alignments [11, 74]. However, there is growing evidence also that the predictive value of eight amino acid identity may also be low [75, 79]. In fact, on the basis of recent analyses, it was concluded that searches of eight amino acids or fewer to identify proteins as potential cross-reactive allergens yield results that are a product of chance and of little practical value with respect to assessment of the allergenic potential of novel proteins [79]. It has been observed that as more information becomes available about the structural bases and minimum requirements for immunologic cross-reactivity between proteins, there has been greater emphasis placed on the use of FASTA and BLAST algorithms to identify potentially cross-reactive proteins. Such algorithms are based on overall levels of identity (currently 35%) within larger fragments (80 amino acids or more) of polypeptides; this approach being better suited to identification of conformational epitopes [11].

Protein Resistance to Pepsin Digestion

It has been proposed that an important prerequisite for the acquisition of food allergy would be that the inducing protein should have the properties necessary to survive within the hostile environment of the stomach, at least for sufficient time to permit recognition by, and interaction with, the local immune system. Against this theoretical background Astwood and colleagues conducted a systematic evaluation of the ability of plant-derived food allergens, and of non-allergens, to resist digestion by pepsin in a low pH simulated gastric fluid (SGF) [80]. An apparent correlation was found between stability in SGF and allergenic activity, the conclusion drawn being that resistance to proteolytic digestion provides an important parameter for identification of potential food allergens, and for distinguishing these from non-sensitizing proteins [80]. Since that original description, measurement of the stability of proteins in SGF has become established as a cornerstone of the safety assessment process, and has been the subject

of extensive investigations and re-evaluations [81–84], including an initiative to provide a standard protocol to increase assay reproducibility [85]. Experiences are mixed and, although concerns have been raised about the sensitivity, selectivity and overall accuracy of the method, it is not without merit if the data are interpreted judiciously and in the context of other available information. Thus, there is no doubt that many important plant food allergens show high levels of resistance to proteolytic digestion at low pH. It must be acknowledged, however, that the relationship is by no means absolute and it is clear that not all stable proteins are allergenic, and that not all allergens are stable. This is not unexpected since there is sound evidence that sensitization to food proteins, or to proteins immunologically cross-reactive with food proteins, can be effected by routes of exposure other than oral ingestion, where stability to proteolytic digestion will not be required, at least not at the level necessary in the stomach, to permit successful engagement with the local immunological machinery.

It is relevant also to question whether the association between allergenic potential and stability in SGF is necessarily a reflection solely of the need for survival following oral ingestion. For instance, it has been found that even proteins that are digested very rapidly in SGF are able in mice to provoke strong IgG antibody responses when administered orally (by gavage) [86]. One interpretation is that relative resistance to pepsin digestion more importantly reflects the ways in which proteins will be degraded within antigen presenting cells for subsequent presentation to T lymphocytes. It is possible that differential intracellular processing driven by sensitivity to proteolytic digestion may in turn impact on immunogenicity and allergenicity.

In summary, it is reasonable to conclude that bioinformatic analysis of structural similarities to, or of sequence homology with, known protein allergens, and evaluation of stability with SGF, can both contribute, if interpreted cautiously, to an overall assessment of sensitizing hazard. However, these analyses, neither alone nor in combination, provide a definitive answer regarding allergenic potential, and for this reason there has been interest in exploring a more holistic approach (and more definitive assessment) that may be provided by suitable animal models.

Animal Models for Assessment of the Allergenic Potential of Proteins

The approach to safety assessment recommended by IFBC/ILSI [70] was somewhat guarded about the potential utility of animal models and noted at that time that there were available no suitable methods. However, a somewhat different view was expressed in 2001 in the recommendations deriving from the FAO/WHO joint consultation [71]. In this case one of the conclusions reached was that animal models might contribute valuable information on the likely allergenicity of foods derived from GM crops. Although progress in the development of animal models appropriate for safety assessment predated the publication of the FAO/WHO recommendations, further momentum was provided for research in this area [4, 9, 87–98].

With the aim of developing animal models appropriate for safety assessment of allergenic potential a number of species has been considered, including swine [94, 99] and dogs [94, 100–102]. Although these species have their advantages, discussion here

will focus exclusively on approaches based upon characterization of immune responses to proteins in rats and mice.

The strain of rat favored for studies of food allergy has traditionally been Brown Norway (BN). This strain has an atopic-like phenotype, insofar as these rats are recognized as being high responders with respect to IgE antibody production. Various approaches using BN rats have been described [103–106], but perhaps the best characterized is the model developed by Knippels, Penninks and colleagues [95, 107]. In this experimental system rats are administered test proteins (in the absence of adjuvant) daily by gavage and responses measured at various times (characteristically 42 days) after the initiation of exposure. Using ovalbumin (OVA) as a model food allergen it was shown that exposure resulted in both IgG and IgE antibody responses, together with various clinical signs and symptoms consistent with an allergic reaction [107–109]. In supplementary experiments it was found that in response to gavage administration of either hens' egg white or cows' milk proteins over a period of 42 days BN rats mounted a pattern of IgG and IgE antibody responses comparable in specificity with that found in humans sensitized to the same proteins [95, 110].

One attractive attribute of this approach is that exposure is via a route that is relevant for the induction of food allergy in humans. However, oral administration of test proteins is not without challenges with respect to criteria that need to be fulfilled by suitable animal models [111]. It is relevant to acknowledge that even when it has proven possible to induce in BN strain rats IgE antibody responses to OVA by repeated gavage (bolus) exposure, administration of the same protein *ad libitum* in the drinking water (gradual exposure via drinking) provoked no, or only very low frequency, IgE responses, presumably as under these circumstances immunological tolerance is favored [108]. In this context it is also of importance to recognize that unscheduled dietary pre-exposure of test animals or their parental generations to the protein under investigation is of relevance since this may result in tolerance [112]. This may also explain why even when gavage administration is used the induction of IgE responses in BN can be variable, and in some instances unsuccessful. Thus, in other independent laboratories, using a comparable experimental regimen, it either proved impossible to stimulate by gavage in BN strain rats IgE antibody responses to OVA, or to another allergen (peanut agglutinin), under conditions where these same proteins were able to elicit strong IgG antibody responses [113], or IgE antibody production to OVA was achieved [114, 115].

In parallel, there has been interest in exploring the utility of different models that employ other routes of exposure. The approach that has received most attention is based upon characterization of immune responses induced by proteins in BALB/c mice; this strain of mouse (like BN rats) having an atopic-like phenotype [93, 94, 96]. This model was built on preliminary investigations in which the nature of humoral immune responses was analyzed following intraperitonal (ip) exposure of mice to protein allergens [116, 117]. Since then more detailed investigations have been conducted incorporating a wider range of test proteins and a consideration of dose-response relationships [118, 119]. The evidence available to date indicates that it is possible using this approach to identify protein allergens as a function of their ability to elicit IgE antibody responses (measured by passive cutaneous anaphylaxis), and to distinguish allergens from other

proteins that are immunogenic, but apparently lack allergenic activity. Thus, both protein allergens and protein immunogens stimulate IgG antibody responses, but only the former are able to induce the production of IgE antibody. It has now been possible, within the context of an inter-laboratory investigation, to demonstrate that the elicitation of IgE antibody responses by protein allergens of apparently differing potency is a relatively robust phenomenon and transferable between laboratories [120]. Moreover, it has been found also that exposure of mice to protein allergens is associated with the development of a selective Th2-type immune response consistent with the elaboration of IgE antibodies [121]. The premise of this approach is, therefore, that it will be possible to identify potential protein allergens as a function of their ability to induce IgE antibody responses, and to distinguish these from non-allergens that, despite being administered under conditions where an IgG antibody response is elicited, fail to cause the production of IgE. Further, the rationale for using ip, rather than oral, administration is to avoid opportunity for the generation of immunological tolerance and maximize induced immune responses such that sensitive detection of potential allergens is facilitated, and confidence increased in the identification of non-allergens.

It will be apparent from this brief survey that both the models in which most investment has been made show promise, and each offer certain advantages. In both cases there is a need for further investment in research to develop a clearer and more confident understanding of how best these methods can be integrated effectively with other information relating to allergenic activity to provide a holistic assessment of likely sensitizing potential.

GENERAL CONCLUSIONS

Food allergy represents a relatively new area of interest and responsibility for immunotoxicologists. It is an important and increasing health problem that provides many intriguing challenges for physicians and immunologists. For toxicologists the primary goal is the development of robust and effective methods for identification and characterization of the inherent potential of proteins to cause allergic sensitization and food allergy. Progress in this area will require not only further refinement of existing approaches and their effective integration with new methods, but also a parallel and coordinate investment in developing an increased appreciation of the immunological mechanisms that impact on the acquisition of sensitization to food proteins, the ways in which the conditions of exposure influence the development of food allergy, and the factors responsible for inter-individual differences in susceptibility.

Some of the key needs in this area were discussed during a workshop "Assessment of the Allergenic Potential of Genetically Modified Foods" held in 2001 in North Carolina, [122], and many of the research requirements identified then are still current today.

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36 Drug Allergy

Kenneth L. Hastings

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INTRODUCTION

Drug allergy is essentially a colloquial term used to describe pathologies that seem to have an immunological basis. It is important to remember when discussing drug allergy that an uncertain fraction of this group of phenomena only resembles what would be considered immunologic reactions, and at this time there is great uncertainty concerning the size of the fraction. As will be discussed below, there are many pathologies that are grouped under the general heading of "immune-mediated drug hypersensitivity reactions" (IDHR). Consider, for example the classic drug allergy reaction anaphylaxis. A quick search on the term in many databases (such as the *Physicians Desk Reference*—the PDR) gives a clue to the problem. In the case of the PDR, the latest edition lists anaphylaxis as a drug-associated adverse reaction in 235 product labels. It is highly unlikely that all of these products have the ability to induce true anaphylaxis. Most likely, signs associated with anaphylaxis—basically shock-like reactions- were reported when the drug was administered. Why so many drugs have the ability to produce shock reactions, interpreted as anaphylaxis, is unknown. What is clear, however, is that in the absence of relatively specific biomarkers–such as anti-drug IgE antibodies–the diagnosis is likely incorrect. In order to understand why it is so difficult to correctly diagnose IDHR it

is necessary to understand the complex factors that seem to be involved in true drug allergy.

The Hapten Hypothesis

Long ago, Landsteiner [1] determined that in order for small molecular weight compounds to induce allergic reactions in the skin, these need to covalently bind to proteins. Coincidentally, some of the most potent drug allergens were being used for the first time in clinical medicine: penicillin and sulfa drugs among them. In fact, covalent binding appears to be the mechanism by which penicillin has the capacity to induce all types of immunopathies (not just anaphylaxis or allergic contact dermatitis) [2]. Thus, the chemistry of drug allergy was established long before the complexities of hapten immunology became apparent. That is, if covalent binding of a reactive compound to a protein was both *necessary* and *sufficient* to induce drug-specific adverse immune responses, drug allergy would likely not be such a great problem. However, drug companies have not adopted covalent binding assays as part of routine drug development, primarily because these do not appear to be sufficiently predictive. Much more is involved in drug allergy, and the hapten hypothesis, though useful, is not sufficient.

Genetic Predisposition

Even a potent allergen such as penicillin does not cause IDHR in all patients. The actual prevalence of penicillin hypersensitivity is not known precisely, but anaphylaxis rates appear to be in the range of 1 per 5,000 exposures [3]. Penicillin probably accounts for around 75% of fatal drug-associated anaphylactic reactions in the United States [4,5]. What is particularly striking is that penicillin does not require metabolism in order to react with endogenous proteins: that is, it is inherently reactive and virtually anyone exposed is likely to form potentially immunogenic haptens [6]. Why is it, then, that only a fraction of patients suffer from some form of allergic reaction to penicillin? It is quite common in the biologic sciences to attribute such puzzles to genetics. Unfortunately, this is the important factor in many otherwise difficult to explain phenomena—unfortunate because it is so hard to establish the exact predisposing genetic factor(s).

The good news is that with advances in technology, understanding genetic predisposition has become (or is becoming) a practical reality. Thus, it is likely in the future that, in understanding genetic predisposition, the mechanisms of IDHR will be elucidated to the degree needed for adequate predictive models. Until recently, the level of understanding genetic predisposition was limited to ethnicity, sex, certain relatively crude parameters such as body weight, atopy (patients with elevated baseline serum IgE levels), and genetic polymorphisms related to drug metabolism. The level of understanding has increased greatly, however. For example, very specific predisposing factors have been identified, such as haplotypes associated with abacavir and carbamazepine hypersensitivity [7,8]. Even the gold standard of drug allergy, penicillin, has been associated with a specific polymorphism (E237G variant of FcεRIβ) [9].

The Danger Hypothesis

As with virtually all biologic phenomena, IDHR has both a genetic and an environmental component. The hapten hypothesis and understanding genetic predisposition can only explain a portion of the IDHR conundrum. A major addition to our understanding of drug allergy is the "danger hypothesis," originally proposed by Matzinger [10]. This theory builds on the knowledge that in order for a molecule to induce an immune response, two separate signals are necessary: one involving appropriate presentation of the potential immunogen, and the second a signal that essentially stimulates mechanisms involved in recruitment of immune effector factors (such as T cells), resulting in immunologic memory. The important facts to consider are (1) in the absence of a second signal, immune cells that respond to the potential immunogen undergo apoptosis; and (2) the nature of the second signal is such that the immunogen is recognized as potentially harmful. Any number of molecular and cellular events can stimulate the second signal, but the most important seems to be related to necrosis [11]. That is, the effects may be described as irritation, inflammation, stress, release of cytokines, and/or chemokines in the absence of necrosis, or other phenomena, but the pivotal event is the immune system recognizing exposure to an immunogen as harmful. This may be a property of the drug-immunogen itself (such as irritation or liver cell necrosis), or exposure to the drug-immunogen in the context of an ongoing disease process (such as treatment of an infection). Thus, in the case of drug allergy, formation of a hapten (via metabolism or some other form of biotransformation) in and of itself is not likely to induce an immunopathy. But in the case of a drug such as penicillin, the hapten(s) it forms *in vivo* are likely to be seen in the context of an alerted immune system. In a patient with the predisposing haplotype, this would constitute the triad of events that may be necessary to induce an allergic reaction. Penicillin represents an excellent example of context determining effect: it is not a particularly toxic drug in and of itself, but under the appropriate conditions it can induce a life-threatening immune reaction. There are problems with the danger hypothesis, however, an important one being the hygiene hypothesis [12]. Briefly, the prevalence of allergy in a given population seems to be directly related to the "cleanliness" of the environment in which people live. An argument could be made that poor hygiene would increase the "danger signals" to which an individual would be exposed. Without belaboring the issue, the hygiene hypothesis includes the idea that exposure to many potential immunogens actually acts to "instruct" the immune system to recognize what is versus what is not a true danger signal. That is, however accurate the hygiene hypothesis, it does not necessarily represent a contradiction to the danger hypothesis.

A HOLISTIC APPROACH TO DRUG ALLERGY

There have been several attempts over the years to classify adverse drug reactions, primarily based on the perception of what can and what cannot be predicted given knowledge of the pharmacology and toxicology of the drug. For example, Edwards and Aronson [13] proposed what is probably the most extensive classification system:

- Type A: dose-related, relatively predictable, and likely related to pharmacological action of the drug.
- Type B: not related to dose, not generally predictable, likely to have an immunological basis or related to a metabolic idiosyncrasy.
- Type C: cumulative-dose related, likely associated with defects in clearance or other pharmacokinetic parameter.
- Type D: time-related, such as chronic exposure resulting in drug-induced tumors.
- Type E: withdrawal of drug resulting in adverse effects.
- Type F: failure of efficacy.

Although this classification system is admirably comprehensive, for the purposes of drug allergy, the simpler system proposed by Rawlins and Thompson [14] is more appropriate: Type A reactions are predictable and dose-related, Type B reactions are neither and probably have an immunologic basis. Several factors should be considered in determining that an adverse drug reaction is a form of drug allergy [15]:

- The reaction is not expected based on the pharmacology of the drug.
- Sensitization precedes the reaction.
- The adverse reaction is not associated with an efficacious dose.
- The clinical symptoms are consistent with known forms of allergic reactions.
- Symptoms resolve with discontinuation of therapy.
- Cross-reactivity can occur with structurally similar drugs.

None of these factors alone should be considered to indicate a Type B reaction, and any taken singly can be associated with a Type A reaction. This list should be taken as a starting point in determining if an adverse drug reaction is a type of drug allergy. The following are additional considerations.

Is the Drug Inherently Immunogenic?

This applies primarily to protein drugs and has been a continuous problem in developing biotherapeutics. Consistent with the hapten hypothesis, simply demonstrating that a protein drug can induce an immune response does not seem to be sufficient to predict drug allergy, but can be useful under certain circumstances [16]. For example, if drug-specific IgE responses are observed in nonclinical toxicology studies, this should be taken as a warning sign. Comparative immunogenicity might also be useful: if changes in manufacturing of the drug result in enhanced immune responses, or production of antibodies that neutralize the activity of physiologic proteins with non-redundant activity, this could be an especially serious indicator [17].

Is the Drug Inherently Reactive?

This applies primarily to such drugs as cancer chemotherapeutics that covalently bind to nucleic acids or other potential tumor targets and therefore have the inherent capability

to form haptens. Various types of drug allergies are commonly seen in cancer chemotherapy [18]. Other drug types can fall into this category as well. For example, Pichler has proposed that sulfamethoxazole can directly interact with T-cell MHC antigens and produce drug allergic reactions [19].

Is the Drug Biotransformed to Protein-Reactive Products?

This is a very complicated subject and is often the critical point missed in drug development. First, metabolism is not the only way in which a drug can be biotransformed into a protein-reactive product, as exemplified by the β-lactam antibiotics [20]. Drugs that can readily bind to proteins *in vitro* are likely to have the potential to induce some form of drug allergy. Structure-activity relationships predictable using various databases can be useful in screening for compounds that do not require metabolism to form haptens [21]. Another form of biotransformation—photoactivation—can result in hapten formation and photoallergy [22].

The more complicated situation is metabolism. There are several factors to consider: enzyme polymorphisms, xenobiotic-induced metabolism, and even body weight (primarily lean to fat tissue ratio) can be involved [23, 24]. One very interesting proposal is the *reactive metabolite syndrome*: this idea incorporates both the danger and the hapten hypotheses [11]. The drug is metabolized to a form that can bind to proteins, but the metabolites are themselves cytotoxic. Thus, although formation of haptens and induction of an immune response *could* result in drug allergy, it might be possible that metabolite cytotoxicity is the critical second signal. In this situation, pro-inflammatory cytokines and related factors could be pivotal in induction of an immune response, and it is even possible that drug-specific antibodies could be biomarkers rather than mediators of drug allergy. Although this paradigm does not address the issue of immune memory, an essential component of truly immune-mediated reactions, this could explain why in so many cases an adverse drug reaction appears to have an allergic basis, but no drug-specific antibodies or T cells can be demonstrated.

Although in the past biotransformation to produce protein-reactive products has not been considered important in discovery toxicology studies, this situation appears to be changing. The limiting factor has been availability of radiolabeled drug to conduct appropriate covalent binding studies, but strategies have been proposed to overcome this obstacle [25].

Have Signs Consistent with Drug Allergy Been Observed in Nonclinical Toxicology Studies?

At this time, it is generally accepted that the only form of drug allergy for which truly predictive assays exist is allergic contact dermatitis [26]. These assays have been discussed in other chapters, but it is important to consider the following situation: if a drug is identified as a contact allergen using a standard assay such as the guinea pig maximization test or the murine local lymph node assay, should it be considered a potential allergen if administered by a non-dermal route? There have been attempts to answer

this question, but it should be considered open for research and discussion [27]. In the case of drugs to be administered by the dermal route, a positive finding in a dermal sensitization assay can be considered a definitive answer and can be used to support the ultimate risk/benefit determination pivotal in drug marketing approval [26]. For drugs to be administered by the inhalation or intranasal route, adaptations of the local lymph node assay to determine cytokine patterns consistent with ability to induce respiratory sensitization seems to be a sensible approach [28]. In general, nonclinical studies have not proven to be useful in predicting other forms of drug allergy.

What Effects Have Been Observed in Clinical Trials Suggestive of Drug Allergy?

The spectrum of drug allergy is daunting. Some, such as contact dermatitis, are relatively straightforward. Some, such as maculopapular (morbilliform) skin reactions following oral administration of a drug, are almost impossible to predict and appear to involve complex mechanisms (although there are exceptions to this, such as the rat model for nevirapine-induced rash) [29, 30]. One of the issues that remains unresolved has been whether skin reactions such as maculopapular rash indicates that the drug has the potential to produce truly serious reactions such as toxic epidermal necrolysis/Stevens-Johnson syndrome [31–33].

Blood dyscrasias and vascular effects represent difficult diagnostic challenges. For example, anemia can have many causes, drug-induced intravascular hemolysis being one of them. In the case of immune-mediated anemia, demonstration of drug-specific antibodies (usually IgM) and a positive direct Coomb's test could be diagnostic of a hapten-mediated reaction [34]. However, there are other immune mechanisms that could be involved: immune complex absorption to red blood cells resulting in complement-mediated hemolysis, true drug-induced anti-erythrocyte autoimmune reactions, and even immune mediated aplastic anemia. The complexity of the issue can best be illustrated by the recent catastrophy in which recombinant erythropoietin was found to induce neutralizing antibodies resulting in fatal aplasia [35, 36]. Anti-drug immune responses have been associated with thrombocytopenia and neutropenia [37, 38]. Drug-induced immune-complex deposition can result in various forms of vasculitis as well as organ specific immune-complex diseases such as glomerulonephritis, pneumonitis, and cutaneous necrotizing vasculitis [39]. Finally, immune complexes can produce generalized serum sickness, which presents with a spectrum of signs (fever, arthralgias, etc.) [18].

Anaphylaxis is another complex clinical diagnosis. True drug-induced anaphylaxis in humans is mediated by drug-specific IgE. However, even with the case of penicillin, it is often difficult to demonstrate the presence of penicillin-specific (or, more accurately, penicillin-derived hapten-specific) IgE in a patient following an episode of penicillin-associated anaphylaxis [40]. Although there are methods to model true anaphylaxis in animals (e.g., passive cutaneous and active systemic anaphylaxis assays), with the exception of protein allergens, these have not proven to be reliably predictive [41]. To make the situation even more complex, signs of anaphylaxis vary with species, and even the mediators of anaphylaxis may vary with species [42, 43]. Drug-specific IgE

can also be associated with urticaria in the absence of anaphylaxis [39]. Finally, many drugs can produce a type of pseudoallergy usually referred to as an *anaphylactoid reaction*. There are three known mechanisms for anaphylactoid reactions: direct action of the drug on mast cells/basophils, activation of the alternate complement pathway, and alterations in arachidonic acid metabolism [43]. Any of these can result in release of anaphylaxis mediators. One interesting observation is that some drugs can induce both anaphylactoid reactions and true anaphylaxis [44]. It is at least possible that mediators of anaphylaxis act as danger signals, thus providing the second signal needed to induce a true immune reaction [10].

Organ-specific drug allergy is an especially interesting, if often devastating, phenomenon. A classic example is halothane hepatitis: about 20% of patients demonstrate a transient increase in liver serum enzyme levels following use of this anesthetic [45]. However, in about 1 in 35,000 uses, the patient will develop fulminant liver disease that is frequently fatal [46]. Halothane is metabolized to a simple hapten (trifluoroacetylated protein), which appears to induce a type of allergic hepatitis [47]. There are other examples of allergic hepatitis, but this appears to be the best studied. The important point here is that, for unknown reasons, trifluoroacetylation of hepatic proteins is a uniquely immunogenic event which can be reliably demonstrated in animals. Compounds structurally related to halothane, which can be metabolized to form the same (or closely related) haptens, have also been associated with liver disease [48].

A PHENOMENOLOGY OF DRUG ALLERGY

The Gell and Coombs' classification scheme for immune-mediated toxicity has been discussed elsewhere, but it is important to emphasize that, with perhaps a few exceptions, none of the drug allergies appears to be of a single type. Anaphylaxis and immune-mediated urticaria appear to be Type I, IgE mediated reactions, and allergic contact dermatitis a Type IV, cell-mediated reaction. The Gell and Coombs' system is very useful in understanding mechanisms of immunopathy, but in understanding drug allergy it is much less useful. Consider the syndrome referred to as *drug reaction with eosinophilia and system symptoms* (DRESS). This syndrome is associated with anticonvulsant therapy (primarily phenytoin, phenobarbital, and carbamazepine), as well as other drugs, and is characterized by fever, rash, lymphadenopathy, eosinophilia, interstitial pneumonitis, myocarditis, and, in most cases, hepatitis [49]. It is very difficult to describe DRESS as a single type of immunopathy using the Gell and Coombs' system.

Many drug allergies appear to be combinations of Gell and Coombs' types. For example, consider halothane hepatitis: the cumulative data seems to indicate that this disease has features of both Types II and III immunopathies, as well as an autoimmune component [50]. Procainamide induces a lupus-like syndrome, and autoimmune reactions due to drugs can be considered a form of drug allergy [51]. Finally, drugs such as sulfonamides and β-lactams can cause any of the Gell and Coombs' immunopathies, and occasionally signs of more than one form are observed concurrently (especially true for sulfa drugs) [39].

The Th1/Th2 paradigm and the danger hypothesis have been useful to some extent

in understanding drug allergy from a mechanistic perspective [52, 53]. As discussed in connection with the murine local lymph node assay, cytokine patterns may be predictive of certain adverse effects. However, even in well-studied immunological diseases such as asthma and Type I diabetes, the patterns of causation are still unclear. The same is true for drug allergy.

CONCLUSION

Drug allergy remains one of the most challenging problems in toxicology. Arguably, this represents one of the major public health issues in immunotoxicology. As has been pointed out by a task force of experts in the area, it is doubtful that any single approach can be used to determine the ability of a drug to cause IDHR [54]. But many tools may be available in the future to deal with this complex issue.

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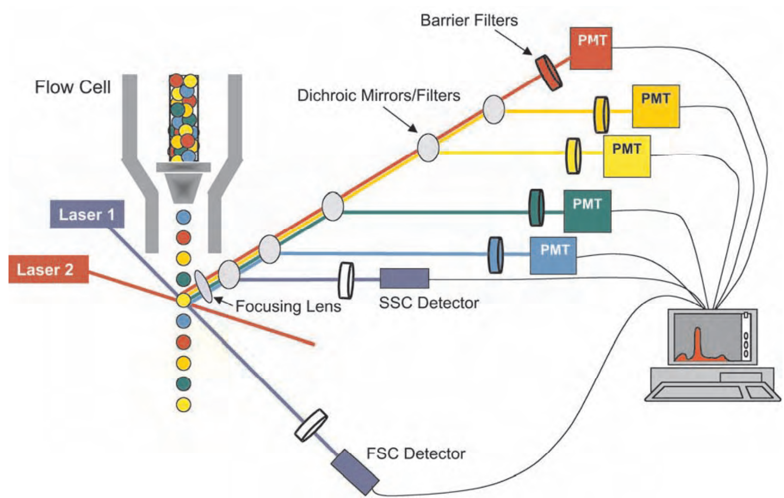

FIGURE 7.1 Representative diagram of the mechanics of a flow cytometer. For description, see page 98.

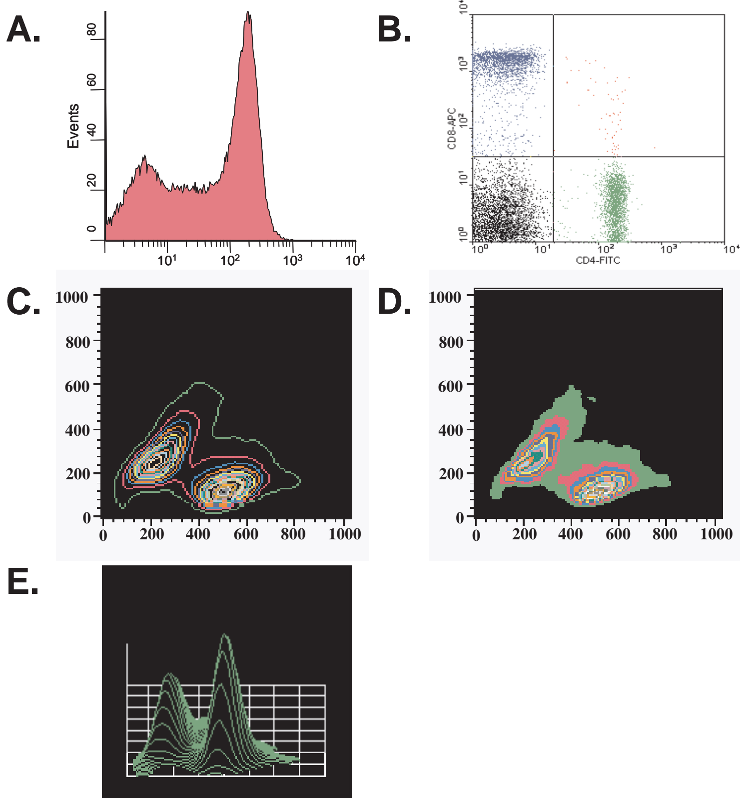

FIGURE 7.2 Representative graphical data displays. For description, see page 99.

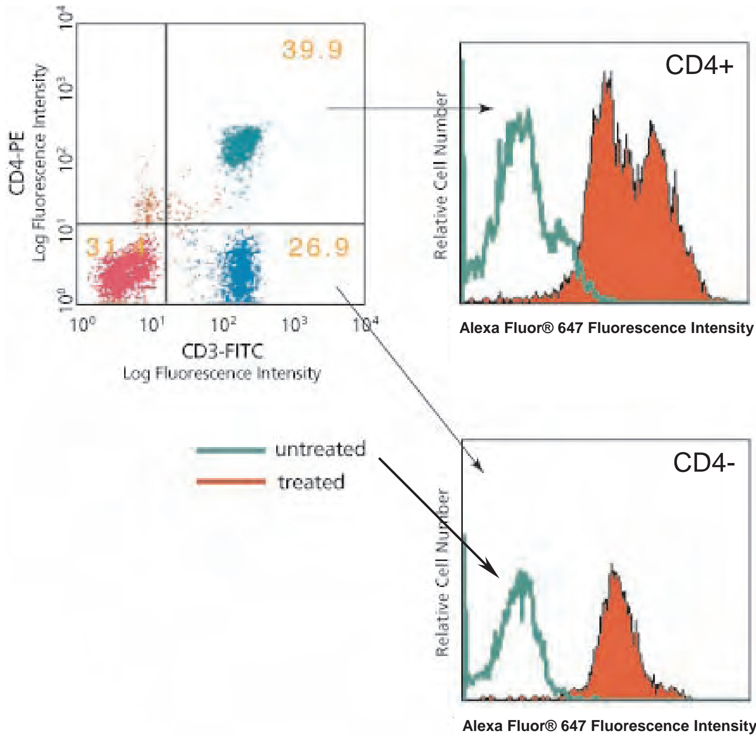

FIGURE 7.6 Detection of intracellular tyrosine phosphorylation (pY) of ZAP70 in CD4+ and CD4- human peripheral blood leukocytes (HPBL) following stimulation with H_2O_2. For description, see page. 110.

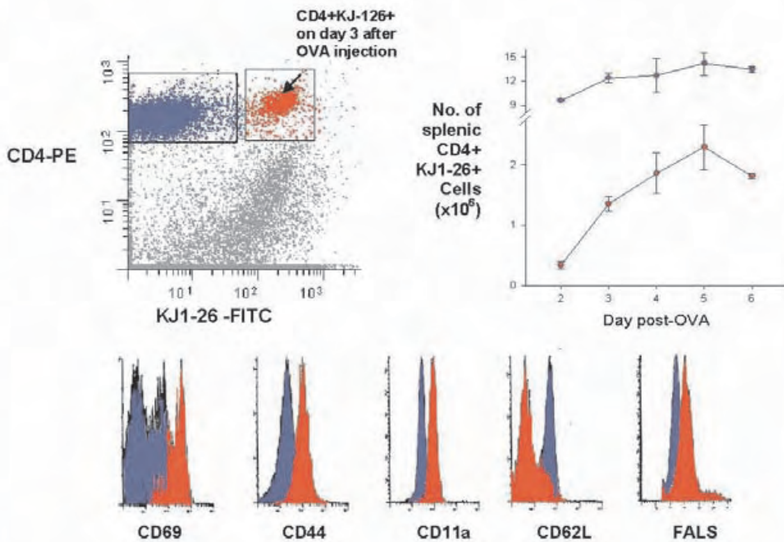

FIGURE 7.7 Adoptively transferred DO11.10 transgenic T cells can be identified by expression of CD4+ and KJ-126 in spleen cell suspension from Balb/c mice after ovalbumin (OVA) immunization. For description, see page 112.

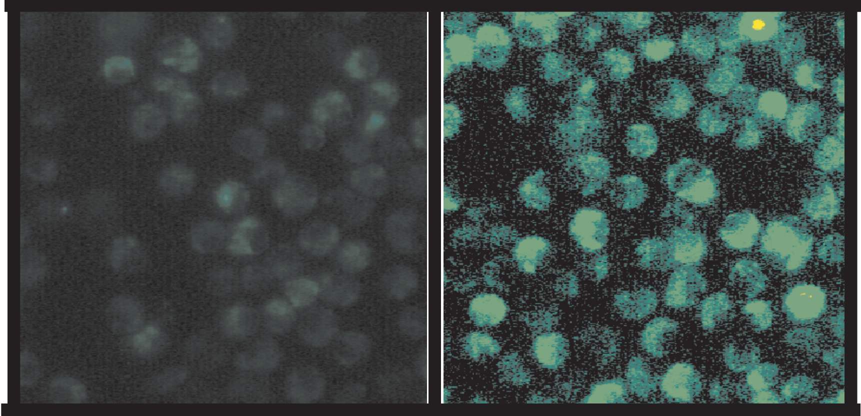

FIGURE 7.9 Peroxynitrite production by activated macrophages. For description, see page 115.

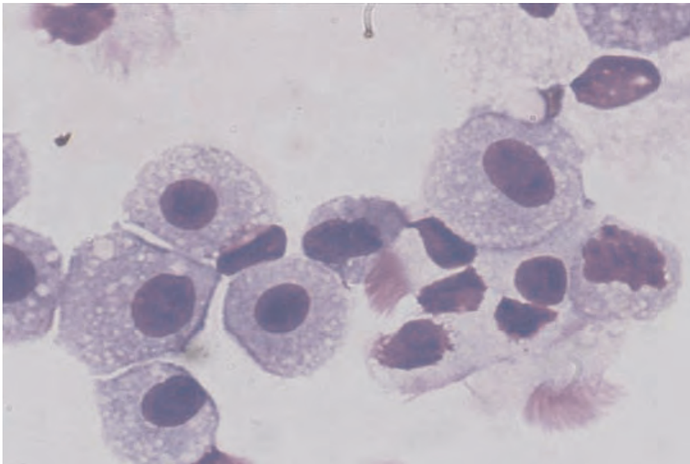

FIGURE 18.1 Pulmonary cells obtained from a rat lung wash 24 hours after infection with rat adapted influenza virus. For description, see page 312.

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