



## Sensitivity and predictivity in immunotoxicity testing: immune endpoints and disease resistance

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### Abstract

In spite of extensive laboratory data on the effects of chemicals and drugs on immunologic parameters in laboratory animals, and a well established correlation between suppression of immune function and increased incidence and/or severity of certain infectious and neoplastic diseases, interpreting data from experimental immunotoxicology studies for risk assessment purposes has proved challenging. This is particularly true when the immunological effects are minimal-to-moderate in nature, as might be expected from inadvertent chemical exposures. This review examines the methods used to evaluate immune responses in laboratory rodents and their utility to predict disease outcomes. The available data suggest that if a large enough population is exposed and that the challenge dose or virulence of pathogenic organisms or tumor cells is sufficient, small changes in immune surveillance could increase the background incidence and burden of disease in the human population.

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*Keywords:* Immunotoxicity; Disease resistance; Infection; Neoplasia; Rodents

### 1. Introduction

The potential for chemicals to cause injury to the immune system is of considerable public health significance, as alterations in immune function can lead to increased incidence of hypersensitivity disorders, autoimmune and infectious diseases or neoplasia. Experimental animal data has provided a broad range of information from which the sensitivity and predictability of tests commonly used for the screening of chemicals for immunotoxicity have been evaluated (Luster et al., 1988, 1992a, 1993; Vos and Van Loveren, 1987). Because it is relatively difficult to determine the contribution of chronic low-level immunosuppression or

the cumulative effects of modest changes in immune function to the background incidence of disease in the human population, efforts have been made to examine the predictive value of laboratory measures of immune response for disease resistance in rodent models.

### 2. Host resistance assays

The major function of the immune system is to protect the host from infectious or neoplastic disease; thus most immunotoxicologists consider “host resistance assays” as the most relevant for validating the usefulness of other detection methods and for extrapolating the potential of environmental agents to alter host susceptibility in the human population. In these

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Table 1  
Commonly employed disease resistance models

| Challenge agent                 | Endpoint measured                    |
|---------------------------------|--------------------------------------|
| <i>Listeria monocytogenes</i>   | Liver CFU, spleen CFU, morbidity     |
| <i>Streptococcus pneumoniae</i> | Morbidity                            |
| <i>Plasmodium yoelli</i>        | Parasitemia                          |
| Influenza virus                 | Morbidity, viral titer/tissue burden |
| Cytomegalovirus                 | Morbidity, viral titer/tissue burden |
| <i>Trichinella spiralis</i>     | Encysted larvae, adult parasites     |
| PYB6 sarcoma                    | Tumor incidence (subcutaneous)       |
| B16F10 melanoma                 | Tumor burden (lung nodules)          |

assays, groups of experimental animals are challenged with either an infectious agent or transplantable tumor at a challenge level sufficient to produce disease at a low level or in a small number of control animals (Table 1). As the endpoints in these tests have evolved from relatively non-specific (e.g., animal morbidity and mortality) to quantitative (e.g., tumor numbers, viral titers or bacterial cell counts), the sensitivity of these models has significantly increased. However, in contrast to functional tests, they are still somewhat limited by inter-animal variability and the number of animals that realistically can be devoted to any laboratory study. As a surrogate for measuring actual disease resistance, panels of assays, applied as tiers of tests, are used to evaluate the potential immunotoxicity of chemicals. These assays evaluate numerous endpoints, including the measurement of antibody responses following antigen stimulation, thus simultaneously assessing multiple, regulated components of the immune response. These tiered screening panels have been the basis for several risk assessment guidelines and a number of regulatory agencies in the United States, European Union, and Japan have established or are developing requirements or guidelines for the assessment of potential adverse effects on the immune system (FDA, 2002; JPMA, 1999; IPCS, 1996; OECD, 1995; USEPA, 1998). In general, host resistance assays represent the final level of the screening process and are usually only conducted when there are indications of alterations in immune function in the primary screen and if it is desirable to evaluate the severity of altered function. Although host resistance assays are considered to be the ultimate predictor of adverse effects, there are data to suggest that compounds which produce no evidence of changes in functional immune tests will not likely

affect disease resistance in the commonly used host resistance models (Luster et al., 1993). Selection of particular challenge models is based upon experimental considerations such as the route of chemical exposure and results obtained from initial immune evaluations, which provide an indication of the immune cells or processes targeted by the xenobiotic.

### 3. Immune endpoints and host resistance

Tests used to screen for immunotoxicity range from in vitro culture systems to simple quantitative measures (e.g., serum immunoglobulins or complete blood count) or to fairly complex experimental animal studies. For any screening test to be meaningful, however, it is important to identify both the limitations of the test as well as its concordance (i.e., how accurately does the test predict the interest of concern). In addition, before a test can be considered generally applicable, potential inter- and intra-laboratory variability needs to be considered. A number of studies have examined inter-laboratory variability and the robustness of tests to measure specific endpoints, such as antibody responses (Temple et al., 1993), histopathology (ICICIS Group Investigators, 1998; Kuper et al., 2000), quantitation of cell surface markers by flow cytometry (Zenger et al., 1998; Burchiel et al., 1997), and cytokine production (Langezaal et al., 2002; Herman et al., 2003).

Surprisingly, there have been relatively few studies that have evaluated the sensitivity and predictive value of individual measures of immune outcomes with endpoints that have relevance in the risk assessment process, such as resistance to infection or neoplasia. One of two early efforts of this type was begun in 1979, under the auspices of the US National Toxicology Program (NTP), when a panel of experts gathered to prioritize a list of immunological assays that would be suitable for use in rodent studies. Four laboratories participated in the ensuing validation effort to determine whether the selected tests by the panel had the required sensitivity and reproducibility to successfully detect subtle alterations in immune function and host resistance in mice (Luster et al., 1988). Subsequent studies examining the results of using this testing panel to evaluate 51 chemicals established correlations between specific immune function and host resistance

tests (Luster et al., 1992a,b). The rat has also been a focus in immunotoxicity testing, primarily because of its use in routine toxicity studies. In the late 1970s, a testing panel using the rat, and based on the OECD 407 guidelines was developed at the National Institute of Public Health and the Environment Vos, 1977, 1980; van Loveren and Vos, 1989). The most significant difference between the two testing paradigms was the inclusion of functional tests in Tier I of the NTP rodent screening panel and in the selection of the highest dose. Based on the OECD 407 guideline, the highest dose used in the RIVM panel was at or near the MTD, i.e., the dose at which some overt toxicity (i.e., body weight changes and gross pathological lesions) would be observed. In the NTP screen the highest dose was chosen so that no overt toxicity would be observed, to avoid potentially confounding stress-related effects on the immune system (Pruett et al., 1993, 1999). The utility of the rat model for immunotoxicity testing has been validated in a number of inter-laboratory studies with known immunosuppressive agents (White, 1992; Richter-Reichhelm et al., 1995; ICICIS Group Investigators, 1998). Both testing panels have been updated to include additional endpoints, such as “enhanced histopathology,” routine enumeration of lymphocyte subsets, etc. and new techniques (particularly in vitro methods) are being evaluated for their utility as predictors of potential toxicity to the immune system.

Several investigators have addressed the relationship between changes in immune function measurements and many of the host resistance tests used in rodent studies. While it is rare for a single component of the immune system to be solely responsible for resistance to specific infectious agents or tumor types, certain immune measures show increased correlation with the outcomes of individual host resistance assays. For example, reduction in natural killer (NK) cell activity has been shown to correlate with increased susceptibility to challenge with PYB6 sarcoma cells, B16F10 melanoma cells, and murine CMV (Luster et al., 1988, 1993; Selgrade et al., 1992). Suppression of cell-mediated immunity, complement deficiency, depressed macrophage, and neutrophil function has been associated with decreased resistance to *Listeria monocytogenes* (Petit, 1980; Luster et al., 1988; Bradley, 1995). Clearance of parasitic infections, such as *Plasmodium yoelii* and *Trichinella spiralis*, is mediated by both cellular and humoral components

and decreased resistance has been shown following depression of both arms of the immune system (van Loveren et al., 1995; Luebke, 1995). While the predictive values of individual immune tests for host resistance tests range from relatively good (plaque forming cell assay: 73%; NK cell activity: 73%; delayed type hypersensitivity response 82%) to poor (lymphoproliferative response to LPS <50%), combinations of multiple immune tests increased concordance rates to as high as 100% (Luster et al., 1993). Efforts are ongoing to correlate additional endpoints with their ability to predict changes in host resistance (Germolec et al., unpublished data).

Deletion or ‘functional blocking’ of specific immune components in experimental animals have been used to elucidate the relative contributions of specific molecules, signaling pathways and cells to disease resistance (Hickman-Davis, 2001). This can be achieved via targeted gene disruption, resulting in animals deficient in specific cell populations or soluble mediators which contribute 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. Previous studies have suggested that alterations in one or more lymphocyte subpopulations are predictive of changes in host resistance approximately 70% of the time, although no attempt was made to correlate changes in specific cell types with specific infection models (Luster et al., 1993). A study by Wilson et al. (2001) was specifically designed to determine the magnitude of NK cell suppression that would translate into altered resistance in three host resistance models. The studies utilized NK cell depletion with an antibody to the cell surface molecule, Asialo GM1, using a treatment regimen that did not alter the outcome of other standard immune function tests used in the assessment of immunotoxicity in rodents. These authors demonstrated that at low-levels of tumor challenge, an approximate reduction of 50% or more in NK cell activity was required before significant effects on resistance to an NK-sensitive tumor could be observed. These studies also demonstrated that the level of suppression needed to alter host resistance was related to the challenge level of the tumor. Conversely, studies that have used monoclonal antibodies to deplete CD4+ and CD8+ T lymphocytes

have found little evidence of altered resistance to challenge with PYB6 sarcoma cells, a model which was thought to be dependent on cell-mediated immunity (Weaver et al., 2002).

While these experiments contribute to our understanding of the process of disease resistance, the evaluation of a single immune parameter to characterize the relationship between immune function and host resistance tests has some limitations. In studies designed to specifically address these limitations, Keil et al. (2001) demonstrated that evaluating the suppression of several immunological parameters concurrently provides information that might not be evident from studies using single tests. Using the prototypical immunosuppressive agent dexamethasone, these authors demonstrated that contrary to what would be expected based on the compound's suppressive effects on cytokine production, T cell function and NK cell activity, relatively high levels of dexamethasone were needed to decrease resistance to *L. monocytogenes*. At doses that suppressed many immune parameters, an increase in neutrophil numbers and nitrite production by peritoneal macrophages was observed. It was suggested that at lower doses of dexamethasone, the significant increase in the concentration of neutrophils in the blood in conjunction with increased production of nitric oxide compensated for the decrements in other immune parameters so that overall resistance to the pathogen was not compromised (Keil et al., 2001). Herzyk et al. (1997) have developed a testing paradigm that evaluates immune function within the context of resistance to a specific infection. Following infection with *Candida albicans*, a four parameter model was used that includes survival, spleen colony forming units (CFU), muscle CFU, and antibody titers. The authors suggest that these four parameters allow an inclusive evaluation of both non-specific, cell- and humoral-mediated immune responses. This approach has proved successful in identifying both immunosuppressive and immunostimulatory compounds. The advantages of being able to evaluate multiple immune endpoints in an intact animal and directly relate them to a clinical endpoint are obvious. However, the utility of the method as a screening tool has yet to be evaluated.

Additional variables such as virulence and dose of the infectious agent may impact the ability to clear the challenge organism. In general, these remain a con-

stant in most individual laboratory investigations, but are likely to vary between laboratories and in human exposures. A study by Luster et al. (1993) demonstrated the relationship between test outcome and challenge level. These investigators found that control mice developed a high frequency of tumors provided the challenge dose of PYB6 sarcoma cells was sufficiently high, while severely immunosuppressed mice developed tumors at all of the challenge doses examined. Of significant interest was the finding that in animals treated with a minimally suppressive dose of cyclophosphamide (50 mg/kg), evidence of increased susceptibility appeared to be a function of the dose of the challenge agent. Similar findings were reported following cyclosporine treatment and challenge with group B streptococcus in the Keil et al. (2001) studies described above. However, their results with *L. monocytogenes* indicate that this may be somewhat dependent on the composite effects on all immune parameters.

#### 4. Conclusion

As disease resistance may be dependent on multiple immune processes, and as individuals are exposed to a variety of chemicals that may modulate one or more of these processes, the level of suppression for any one process required to increase the risk of disease may be difficult to predict. However, these studies suggest that assuming a large enough population is exposed and that the challenge dose or virulence of pathogenic organisms or tumor cells is sufficient, small changes in immune surveillance could increase the background incidence and burden of disease in the human population.

#### Acknowledgements

The author would like to thank Yvette Rebeloso and Drs. Kevin Trouba, Robert Luebke, and Daniel Morgan for their thoughtful comments on the manuscript. This review was prepared as part of the efforts of the Immunotoxicology Workgroup supported by the Environmental Protection Agency (EPAs), Office of Research and Development (National Center for Environmental Assessment), EPAs

Office of Childrens's Health Protection, National Institute of Environmental Health Sciences (National Toxicology Program), and National Institute for Occupational Safety and Health (Health Effects Laboratory Division). Members of the workgroup not included as authors are Drs. Laura Blanciforti (NIOSH), David Chen (EPA/OCPH), Michael Kashon (NIOSH), Marquee King (EPA/ORD/NCEA), Michael Luster (NIOSH), Robert Luebke (EPA, NHERL), Christine Parks (NIEHS), and Yung Yang (EPA, OPPTS). Special thanks to Dr. Bob Sonawane (EPA/ORD/NCEA) for helping to organize this effort.

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