

PART I

CURRENT REGULATORY EXPECTATIONS FOR IMMUNOTOXICITY EVALUATION OF PHARMACEUTICALS

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CURRENT REGULATORY EXPECTATIONS FOR IMMUNOTOXICOLOGY EVALUATION OF PHARMACEUTICALS

KENNETH L. HASTINGS

Immunotoxicology is generally perceived to be a relatively new area of concern in the development of human pharmaceuticals, but in fact adverse effects on immune function have been a significant problem for decades. For example, penicillin, arguably the most important pharmaceutical ever developed from a public health perspective, is also one of the most allergenic drugs. Penicillin is the most common cause of fatal drug-related anaphylaxis, which is a Type I immunopathy (Joint Task Force, 1998; Neugut et al., 2001). The problem is that anaphylaxis, as well as drug allergy in general, has not traditionally been considered a form of immunotoxicity. However, when all forms of drug-induced immune reactions are combined, immunotoxicity probably accounts for around 10% of total adverse drug reactions (Bala et al., 2005). These may manifest as increased susceptibility to infections and tumors, hemolytic anemias, systemic inflammatory reactions, and organ-specific autoimmune disease. It is the *spectrum* of immunotoxic drug reactions that has led to a misunderstanding of how important the subject really is. A related issue is the actual interpretation of the term *immunotoxicity*; this has traditionally been applied to drugs that impair immune function primarily by bone marrow and/or lymphatic system toxicity. It has become increasingly clear that drug allergy is likely to be more

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important than direct immunotoxicity. Thus, ICH S8 defines immunotoxicity as both unintended immunosuppression and immunoenhancement (ICH Harmonised Tripartite Guideline, 2006). However, the entire linear paradigm of immunotoxicity, in which a drug either suppresses or enhances immune function, appears to be misleading. A more accurate term is *adverse immunomodulation*, which captures the concept that drugs may disrupt immune function with often unpredictable results (House and Hastings, 2004). Nevertheless, the regulatory approach to evaluating the potential immunotoxicity of drugs still relies on this linear concept, and is reflected in current guidance documents.

IMMUNOSUPPRESSION

There are many methods for assessing the potential of drugs to produce immunosuppression. Most of these are adaptations of standard immunology methods, many of which are quite old. As a practical matter, the most important tools are those used to evaluate general toxicity, and rely on the skill of pathologists examining tissues from drug-treated animals for signs of immunotoxicity (Kuper et al., 2000). There is a commonly cited list of effects observable in standard nonclinical acute and repeat-dose toxicity studies which have proven to be sufficiently reliable in order to screen for “unintended immunosuppression” (USFDA, 2002). These include: (i) hematological changes; (ii) changes in immune system organ weights and/or histology; (iii) changes in serum immunoglobulin levels; (iv) increased incidence of infections; and (v) increased incidence of tumors. Of course, these signs are subject to many interpretations and should be considered carefully in evaluating potential relationship to immunosuppression.

Hematological Changes

In most nonclinical toxicology studies, the following clinical hematology parameters are assessed: packed cell volume (hematocrit), red blood cell counts, total hemoglobin levels, various red cell parameters (mean and variability in cell volume, mean hemoglobin, and mean hemoglobin concentration), white cell counts, absolute and relative white cell types (granulocytes, lymphocytes, monocytes, eosinophils, and basophils), and platelet counts (see Chapter 2.1). Each of these parameters could provide a signal that the drug being tested has an adverse effect on immune function. What should be kept in mind is that observed changes may not always have a simple relationship to effects such as bone marrow toxicity.

Anemia is an excellent example of how complex interpretation of findings can be. Often, the first assumption made when treatment-related anemia is observed is that the drug being tested is a bone marrow toxin. Evaluating this possibility can be relatively straightforward. Examination of the bone marrow, usually by simple microscopic examination of “smears,” can give a clue that

the basis of anemia is destruction of red cell precursors. Flow cytometry can also be used to obtain more accurate cell counts, although morphologic signs that could be useful are not obtained by this method. Other considerations would be whether reticulocyte counts have changed: a decrease (“right shift”) would be consistent with bone marrow toxicity, whereas an increase (“left shift”) would indicate some other reason for the observed anemia (such as a hemolytic process). Other clues would be white cell and platelet counts: these tend to decrease before observing anemia in bone marrow toxicity, and neutrophil counts are especially sensitive indicators. Of course, as is stated in ICH S8, it is important to consider the intended use of the drug. For example, traditional cytotoxic chemotherapeutic agents being developed to treat cancer often produce bone marrow toxicity, and anemia is a likely finding in nonclinical toxicity studies. However, even in this situation, it could be useful to determine the relative sensitivities of bone marrow progenitor cells. Erythrocyte and granulocyte colony-forming cell assays have been used to estimate safe doses for clinical trials in cancer patients, for instance.

When anemia is observed in the absence of bone marrow toxicity, determination of cause can be especially difficult. Direct drug-induced hemolysis appears to be a relatively rare phenomenon, but *in vitro* assessment can be informative. Also, red cell morphology can be a clue: poikilocytosis is a likely finding where intravascular hemolysis is occurring. However, a much more difficult problem is immune-mediated hemolysis. First, this represents the “other end” of the traditional immunotoxicology continuum: the immune system has been adversely stimulated in some way. Where anemia is observed in nonclinical toxicology studies and bone marrow toxicity has been excluded as the cause, it may be useful to perform a direct Coombs’ test for antibodies bound to red cells. A positive finding likely indicates drug (haptens)-bound red cells which have induced an immune reaction; that is, a form of drug hypersensitivity rather than unintended immunosuppression (USFDA, 2002).

An even more complex situation has been observed with recombinant proteins (biotherapeutic drugs, biopharmaceuticals). ICH S8 does not apply to these drugs, but increasingly adverse immune effects are being observed with biopharmaceuticals. Probably the best-known example involved recombinant erythropoietin (EPO), indicated for patients with anemia associated with cancer chemotherapy. For reasons that are not been completely understood, reformulated recombinant EPO, when administered to patients, was associated with pure red cell aplastic anemia. These patients developed neutralizing antibodies to EPO, resulting in ablation of both endogenous and recombinant molecule activity (Schellekens and Jiskoot, 2006).

With respect to hematology, examples similar to anemia have been observed with both leukocytes and thrombocytes: the range of causes can vary from bone marrow toxicity to drug-induced antibody formation. An important consideration is that bone marrow toxicity probably is more often associated with exaggerated pharmacodynamics, whereas antibody-mediated cytopenia often is not. Unfortunately, as is clear from ICH S8, test methods for the latter effect

are not readily available, and require imaginative problem solving. Another consideration is that leukocytosis can be an indicator of infection, and thus paradoxically associated with unintended immunosuppression.

Changes in Immune System Organ Weights and Histology

Immune organ weights are relatively insensitive indicators of immunotoxicity, but should not be ignored. Potent immunosuppressants can produce significant decreases in thymus, spleen, and lymph node weights. One consideration is that necropsy technique is an important cause of variability, resulting in statistically insignificant findings. Thus, individual animal findings may yield important signals. Another consideration in evaluating immune organ weights is that both increases and decreases may be signs of immunotoxicity. There are situations in which increased lymph node weights can be associated with immunosuppression, as this effect may be related to increased susceptibility to infections, especially viral. Thus, lymphadenopathy could represent a counterintuitive effect observed in nonclinical toxicology studies. It is even conceivable that lymph node weights could exhibit a dose-related bell-shaped curve, with mean decreases in the high-dose groups and increases, compared to controls, in lower-dose groups. Inflammation due to drug-induced autoimmunity should also be considered in evaluating immune organ weight increases. Often these changes are not given the consideration needed for proper evaluation of toxicology study findings.

Histologic examination of immune system tissues obtained from animals in nonclinical toxicology studies is the most important single method for detection of immunotoxicity (see Chapter 2.2). This issue has been the subject of much debate, but as a practical matter, it is an obvious conclusion. The immune system should not be considered so significantly different from other organ systems that histology could not be considered the benchmark determination. Obviously there are potential functional effects of drugs that may not be detected by morphological examination, but as a practical consideration, these are likely to represent a minority. ICH S8 contains a list of tissues that should be specifically evaluated by histological examination for signs of immunotoxicity: thymus, spleen, lymph nodes draining the anatomical site of maximum drug exposure (assumed to be the route of administration), at least one lymph node distal to the site of maximum drug exposure, bone marrow, Peyer's patch for drugs administered by the oral route, and bronchus- and nasal-associated lymphoid tissues for drugs administered by the inhalation or nasal route. For intravenously administered drugs, the spleen is considered to be the draining lymph node equivalent.

Much has been made of the "enhanced histopathology" concept. In reality, this is simply a reminder to pay close attention to the histology of immune system tissues. ICH S8 recommends that a semiquantitative description of observed changes be used. This reflects the fact that lymphoid tissues demonstrate cellular dynamics indicative of potential functional changes. Once again,

hypocellularity is not the only effect that can signal immunotoxicity. For example, drug-related increases in lymphoid germinal centers (usually taken to indicate “immune activation”) can indicate increased susceptibility to infections and/or hypersensitivity/autoimmunity. It is in this context that immunohistochemistry can be especially valuable: demonstrating increased expression of markers associated with inflammation could indicate potential adverse drug reactions not generally thought of as immunotoxicity.

Changes in Serum Immunoglobulin Levels

Basal immunoglobulin changes are not considered to be sensitive indicators of toxicity, but this conclusion is arguably an artifact of standard immunotoxicity studies conducted in rodents. These studies are usually 28-day repeat-dose toxicity studies, and basal immunoglobulin levels are unlikely to change significantly in this context. Experience suggests that chronic repeat-dose toxicity studies (of at least 3-month duration) are needed to detect the potential of xenobiotics to produce alterations in basal immunoglobulin concentrations in the blood. This is likely due, at least in part, to the circulating half-life of immunoglobulins. As discussed in Chapter 3.1, antigen challenge assays involving specific antibody response are much more sensitive indicators of immunoglobulin levels. However, elevations in serum immunoglobulin levels may be observed occasionally in repeat-dose toxicology studies, but this appears to be rare. When observed, under most circumstances it is likely associated with infection, and thus could constitute a pattern indicative of immunosuppression. This is especially important when accompanied by leukocytosis and signs of inflammation. Another potential consideration is unintended immune enhancement, such as induction of autoimmunity. Although not likely to be associated with drugs covered under ICH S8, this could be an important signal when evaluating the safety of biotherapeutics (Chapters see 6 and 7).

Increased Incidence of Infections

Increased infections in repeat-dose toxicology are an important, and all too often overlooked, indicator of unintended immunosuppression. There are several issues that should be considered. With respect to rodents used in GLP studies, these are assumed to be free of specific pathogens, so if treatment-related infections are observed, this should be taken as a presumptive sign of unintended immunosuppression. For non-rodents, it is less likely that these can be assumed to be completely free of potential pathogens, especially in the case of nonhuman primates. Thus, non-rodents may be more sensitive to immunosuppressive drug effects. If signs of infection are observed in nonclinical toxicology studies, an attempt should be made to isolate and identify the causative organism(s). In rodents, an important finding would be infections due to opportunistic saprophytes (especially given that these are assumed to be free of known pathogens). In nonhuman primates, a more important pos-

sibility is activation of silent infections due to potential pathogens. An especially interesting situation would be emergence of infections such as malaria or other parasitic diseases in wild-caught nonhuman primates. These findings could be considered “adventitious” host-resistance assays. Unfortunately, the most important examples of drug-associated infections have been related to biopharmaceuticals where arguably the adverse effects represented exaggerated pharmacodynamics. Two examples in particular are therapeutic monoclonal antibodies directed at TNF α and α 4-integrin, which in clinical use induced active tuberculosis and progressive multifocal leukoencephalopathy, respectively. Neither was found to have infection-inducing potential in nonclinical toxicology studies, and the causative organisms, *Mycobacterium tuberculosis* and JC virus, have not been used in host-resistance assays.

Increased Incidence of Tumors

One of the most difficult issues in drug development is evaluating potential carcinogenicity. As a general rule, drugs that produce compelling signs of genotoxicity are not likely to be developed (with obvious exceptions such as cancer chemotherapeutics). Therefore, when significant drug-related carcinogenicity is demonstrated in standard lifetime rodent bioassays, it is often important to determine the probable mechanism. Chronic immunosuppression is a known cause of tumors, especially types that appear to have viral etiology. When assessing the cause of positive carcinogenicity findings, especially where other non-genotoxicity mechanisms have been excluded (e.g., hormonal effects, liver enzyme induction), unintended immunosuppression should be investigated. Since carcinogenicity due to immunosuppression appears to be both dose- and duration-related, information useful in risk management could be obtained from proper evaluation of immune impairment parameters.

IMMUNE ENHANCEMENT

ICH S8 includes unintended immune enhancement as an adverse effect, which should be considered in evaluating the potential immunotoxicity of drugs. Specifically excluded are drug-specific allergenicity and autoimmunity. This is perhaps a confusing exemption and should be discussed.

First, it was recognized by the authors of ICH S8 that signs consistent with immune enhancement could, and often are, observed in nonclinical toxicology studies. These include, for example, leukocytosis, splenomegaly/lymphadenopathy, or other findings, characteristic of organ-specific and/or systemic inflammation. As is discussed above, these signs may in fact be associated with unintended immunosuppression. Also, changes in a hematologic parameter, which is red cell count, can be due to drug-induced immune stimulation.

A classic example is penicillin-induced hemolytic anemia. Associated with long-term use of the drug, it is caused by hapten-red blood cell complexes

inducing primarily antidrug antibodies. This usually results in a Gell & Coombs Type II immunopathy (antibody-mediated cytotoxicity), but can also present as immune-complex disease (Type III), and even result in autoimmune hemolytic anemia (where autoantibodies to red cell antigens persist after withdrawal of penicillin therapy). It may be possible to model such reactions in animals, but more likely this would be observed clinically.

Adverse immunostimulation is in fact a broad category of effects and can be associated with both chemical drugs and biopharmaceutical products. One example is anaphylactoid reaction, often referred to as a type of “pseudo-allergy.” This reaction appears to be IgE mediated (Type I), but in fact involves only the *effector* mechanisms of anaphylaxis. There are three known primary causes: (i) direct drug interaction with mast cells/basophils; (ii) activation of the alternate complement pathway; and (iii) dysregulation of arachidonic acid metabolism. The signs of anaphylactoid reaction resemble anaphylaxis (especially angioedema, urticaria, and cardiopulmonary crisis associated with release of histamine and other endogenous vasoactive compounds), but are not caused by IgE. To make the situation even more complex, some drugs known to cause anaphylactoid reactions (certain radioimaging agents, fluoroquinolone antibiotics) may also induce true IgE-mediated anaphylaxis. One interesting aspect of anaphylactoid reactions is that they can be detected in standard nonclinical toxicology studies and animals can be used to model reaction parameters, such as intravenous infusion rates, useful in risk management.

Finally, in very rare instances elevations in particular immunoglobulin classes, especially IgE or IgA may be detected upon evaluation of serum immunoglobulins. IgE elevations may indicate allergenic potential, but it is highly unlikely to be observed outside of special assays such as adaptations of the murine local lymph node assay. Elevated IgA levels are associated with certain human diseases, such as a form of glomerulonephritis, but this phenomenon appears to be rarely observed in animal studies and even when seen, would be of uncertain predictive value.

There are also examples of drugs that produce systemic effects consistent with adverse immunostimulation. The recent example of an anti-CD28 monoclonal antibody is an extreme case, and is in a class not covered by ICH S8.

SUMMARY

ICH S8 contains a list of issues to consider in interpreting findings of unintended immunomodulation in drug development. An overall consideration is the intended use of the drug. Therapeutics intended to have effects on immune function should be evaluated in a context different from those that are not. Many biotherapeutics are intended to alter immune function and safety evaluation is often integral to pharmacodynamic studies. This is the primary reason that ICH S8 does not apply to this class of pharmaceuticals.

For drugs covered by ICH S8, where signs of immunotoxicity are observed, additional considerations should be given to statistical and biological significance of effects, as well as severity of effects. Some immunotoxic effects may be statistically significant, but are of dubious biological relevance. This is a very complex and contentious subject and is not amenable to simplification. However, consider statistically significant, dose- and duration-related increases in albumin/globulin ratios. Although this could reflect decreases in basal serum immunoglobulin levels, the basis of the effect could be unrelated to immune impairment, and could in fact be toxicologically insignificant based on the actual degree of the change.

For drugs not covered by ICH S8—protein biotherapeutics—evaluation of potential immunotoxicity is likely to be conducted as an integral component of overall pharmacology as opposed to separate studies driven by observed “cause for concern.” There will be examples of protein therapeutics that may need to be assessed for unintended adverse effects on immune function independent of pharmacology studies, but these cases are unlikely to be common. The single general exception is likely to be immunostimulatory drugs, where such issues as “cytokine release syndrome” may need to be assessed. Even in this case, it is likely that adverse immune effects would be exaggerated pharmacodynamics. This is essentially the approach advocated by ICH S6, and even if the document is updated in the near future, it is unlikely that the basic approach would be changed.

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