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Immunogenicity/Hypersensitivity of Biologics

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ABSTRACT

This continuing education course was designed to provide an overview of the immunologic mechanisms involved in immunogenicity and hypersensitivity reactions following administration of biologics in nonclinical toxicity studies, the methods used to determine whether such reactions are occurring, and the associated clinical and anatomic pathology findings. Hypersensitivity reactions have classically been divided into type I, II, III, and IV reactions; type I and III reactions are those most often observed following administration of biologics. A variety of methods can be used to detect these reactions. Antemortem methods include hematology; detection of antidrug antibodies, circulating immune complexes and complement fragments, and immunoglobulin E in serum; tests for serum complement activity; and evaluation of complement receptor 1 on erythrocytes. Postmortem methods include routine light microscopy and electron microscopy, which can demonstrate typical findings associated with hypersensitivity reactions, and immunohistochemistry, which can detect the presence of immune complexes in tissues, including the detection of the test article. A final determination of whether findings are related to a hypersensitivity reaction in individual animals or across the entire study should rely on the overall weight of evidence, as findings indicative of these reactions are not necessarily consistent across all affected animals.

Keywords: antidrug antibodies; biologics; complement; drug development; electron microscopy; hypersensitivity; immunogenicity; immunohistochemistry.

The immune system in animals and humans is fundamentally designed to differentiate self from nonself, thus allowing detection of foreign (nonself) molecules (Jiang and Chess 2009). In many cases, the recognition of a structure as foreign results in an immune response. Such responses can be critical to survival, for example, when the foreign molecule is part of an infectious agent that, in the absence of an immune response, might otherwise injure or kill the host.

Drugs administered to animals or humans are usually not exactly the same structure as the native, endogenous molecules.

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Abbreviations: ADA, antidrug antibody; APC, antigen presenting cell; CIC, circulating immune complexes; CR1, complement receptor 1; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; FDA, Food and Drug Administration; ICH, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; Ig, immunoglobulin; MHC, major histocompatibility; PD, pharmacodynamic; PK, pharmacokinetic.

Thus, they can be perceived as foreign and elicit an immune response. The likelihood of a drug to be immunogenic is dependent in part on its basic structure. In this regard, drugs can generally be divided into the broad categories of small or large molecules; the latter are also typically known as biologics. Small molecules are created by chemical reactions and generate molecules that are very uniform and relatively simple from a structural standpoint. Molecules in this class have been synthesized and administered to animals and humans for over a century, for example, aspirin (Ugurlucan et al. 2012), and there is immense experience with this overall class. In general, small molecules are not often recognized by the immune system as foreign, and thus immune responses to small molecules are relatively uncommon (Gruchalla 2003; Riedl and Casillas 2003). That said, certain small molecules (such as penicillin and other β -lactam drugs) do have a propensity to induce hypersensitivity reactions, often by binding covalently to protein molecules *in vivo*, thus rendering the modified protein immunogenic in some individuals (Naisbitt et al. 2001).

Over the past several decades, biologics have become a growing part of the pipeline of pharmaceutical companies, based to a large extent on their ability to be more specific in

their effects than small molecules and their ability to target molecules that were previously “undruggable.” In contrast to small molecules, biologics are typically large proteins with complex structures that usually require the machinery of a living organism or cell (bacteria, animal, insect, or plant cell) for synthesis. This leads to greater structural variability and with it an increased risk of recognition as foreign by the immune system. Drugs in this category include monoclonal antibodies, cytokines, growth factors, enzymes, and peptides. Compared with small molecules, biologics are relative newcomers; the first genetically engineered biologic (insulin, Humulin[®]; Eli Lilly & Co, Indianapolis, IN) received approval in the United States in October 1982 (Altman 1982; Junod 2007). However, in the past decade, many more biologics have entered development; and as of March 2013, over 900 biologic drugs and vaccines are under development to treat patients with a wide variety of diseases ranging from genetic deficiencies of a protein to chronic inflammatory diseases to cancer (Taylor 2013). The larger number of biologics in drug development, coupled with their propensity for inducing immune reactions, has led to a need to understand the induction of an immune response and subsequent hypersensitivity reactions to biologics. In the context of nonclinical drug development, it is critical to understand how to identify such reactions and understand their relevance to humans.

Immunogenicity to biologics can result in a variety of effects (Leach 2013). The immune response can reduce or eliminate the activity of the test article (or rarely enhance or prolong the activity), neutralize the endogenous molecule, and/or result in hypersensitivity reactions, typically in the form of either immediate (type I) or immune complex-mediated (type III) reactions. While many reactions are related to the development of an adaptive immune response, the innate immune system may also be primarily involved in some reactions.

Immunogenicity in animals is not considered predictive of immunogenicity in humans (Bugelski and Treacy 2004). In this regard, it is critically important to determine whether findings seen in nonclinical toxicity studies are related to pharmacology (and are thus likely relevant to humans) or whether they are related to immunogenicity (and are thus less likely to be relevant to humans). Erroneous decisions can lead to inappropriate termination of programs or conversely may represent a failure to recognize potential negative effects in humans. At the present time, a variety of assays and methodologies can be used to determine whether immunogenicity is occurring in nonclinical toxicity studies, and these will be discussed subsequently. In the future, additional methods, such as predictive modeling, may prove useful in understanding and limiting immunogenicity of biologics (De Groot and Martin 2009; Koren et al. 2007).

James Rottman presented an overview of the mechanisms associated with hypersensitivity reactions. The Gell and Coombs classification of hypersensitivity divides the reactions into types I, II, III, and IV, based on the immune component involved (Table 1), and recently this classification has been modified (Pichler 2007). The most common types of hypersensitivity

reactions in nonclinical toxicity studies are related to type I and type III reactions.

Fundamental to the induction of an immune response to an administered biologic are the interactions of antigen presenting cells (APCs), T cells, and B cells. APCs process administered biologics into peptides that are then loaded onto major histocompatibility (MHC) complex class I and II molecules for presentation to T cells. Animals (or humans) that develop hypersensitivity reactions have a small subset of T cells that are capable of recognizing the peptide–MHC complex via specific binding to the unique T cell receptor expressed on the surface of this small population of cells in a lock and key interaction (Bulek et al. 2012). This initial binding event provides the first activation signal to the T cell, but to be fully activated, the T cell must receive signals through costimulatory molecules on the surface of the APC. These costimulatory molecules are induced when the APC is activated by stimulation of pattern recognition receptors (Toll-like receptors, mannose receptors, nucleotide-binding oligomerization domain receptors, etc.) following binding of their ligands (lipopolysaccharide, bacterial nucleic acid, etc.). The cytokine milieu at the site of activation then drives the phenotype of the antigen-bearing T cell. T cells that do not receive a costimulatory signal become anergic or undergo apoptosis. Native or unmodified proteins do not generally elicit upregulation of costimulatory molecules and thus should usually be nonimmunogenic. Likewise, fully human biologics that are identical or nearly identical to the endogenous proteins are expected to be relatively nonimmunogenic in humans but may be more “foreign” and thus more immunogenic in animals used in nonclinical toxicity studies.

B cells can be activated via T-dependent or T-independent mechanisms to produce antibodies, including antidrug antibodies (ADAs). For activation via T-dependent mechanisms, three signals are required, including engagement of the B-cell receptor, a pattern recognition receptor, and CD40 on the surface of the B cell. Activated B cells initially produce immunoglobulin (Ig) M, then following interaction with antigen-specific T helper cells, class switch to IgG or IgE, and undergo somatic hypermutation to produce higher affinity antibodies. A subpopulation of these B cells then matures into long-lived plasma cells, the majority of which reside in the bone marrow. Upon subsequent exposure to antigen (i.e., the biologic), these plasma cells then rapidly respond with secretion of large amounts of antibody, representing an anamnestic or memory response. Type I and III hypersensitivity reactions typically occur after antibody isotype class switching, which usually happens around 10 to 14 days after the initial exposure to the antigen (i.e., test article in the context of nonclinical toxicity studies). In contrast, T-independent B-cell activation can also occur, resulting in ADAs that are usually of IgM isotype, and the plasma cells are typically short lived.

A variety of factors appear to predispose an animal (or human) to ADA production. These can include factors related to the biologic and/or those that are specific to the individual animal. Factors related to the biologic include differences between the molecular structure and amino acid sequence of

TABLE 1.—Revised Gell and Coombs' classification of hypersensitivity reactions.

Hypersensitivity reaction type							
	Type I	Type II	Type III	Type IVa	Type IVb	Type IVc	Type IVd
Immune reactant	IgE	IgG1, IgG3, IgM	IgG	IFN- γ , TNF- α (TH1 cells)	IL-4, IL-5/IL-13 (TH2)	Perforin, Granzyme B, FasL (CTL)	CXCL8, GM-CSF (T cells)
Antigen	Soluble antigen	Cell- or matrix-associated antigen	Soluble antigen	Antigen presented by APCs	Antigen presented by APCs	Antigen presented by target cells	Antigen presented by APCs
Effector cells	Mast cells	Complement, FcR+ cells	Complement, FcR+ cells	Macrophages	Eosinophils	CD8+ T cells	Neutrophils
Examples	Anaphylaxis	Hemolytic anemia, thrombocytopenia	Vasculitis, serum sickness, Arthus reaction	Tuberculin reaction	Asthma, allergic rhinitis	Contact dermatitis, Stevens Johnson syndrome, toxic epidermal necrolysis	Acute generalized exanthematous pustulosis, Behcet's disease

Note: ADAs = antidrug antibodies; APC = antigen presenting cell; FcR+ = Fc receptor positive; Ig = immunoglobulin; IFN- γ = interferon γ ; IL = interleukin; TNF = tumor necrosis factor. Reactions are classified into types I, II, III, and IV based on the immune reactant, antigen, and effector cells. Type IV reactions are further subdivided into IVa, b, c, and d. Examples of each type of reaction are shown. When hypersensitivity reactions occur against administered biologics in the context of nonclinical toxicity studies, type I and III reactions (shaded) are the most common. Both involve generation of antibodies against the administered biologic, that is, ADAs.

Source: Adapted from Pichler (2007).

the native protein and the test article, the amount of protein aggregation, the use of surfactants, the presence of impurities/cofactors/adjuvants in the test article formulation, and the glycosylation/pegylation status. Factors related to the individual animals include the dose level, frequency of dosing, route of administration, genetic predisposition (human leukocyte antigen class and presence of genetic defects), age, immune status and competence, and presence of other diseases. The nature of the target protein, in the case of antibody or antibody-like therapeutics, can also play a role, as it appears that cell surface targets may have a greater likelihood of inducing immunogenicity than other targets. Although dogma suggests that intravenous dosing is less immunogenic than subcutaneous or intradermal dosing, this has not been the experience of the authors. The aforementioned factors may combine to play a role in the variability of immunogenicity and hypersensitivity reactions that are observed between individual animals in nonclinical toxicity studies.

As noted previously, when hypersensitivity reactions occur in nonclinical toxicity studies, type I and type III are the most common which occur in animals, and both involve ADAs in their pathogenesis. In type I reactions, ADAs of the IgE isotype are formed during an initial exposure to the biologic and bind to mast cells and basophils via their Fc receptors. When the test article is administered again, it binds to these antibodies, causing rapid release of histamine and other mediators, resulting in typical anaphylaxis that can range from relatively mild to fatal. An additional mechanism for inducing type I hypersensitivity has been described in mice and may also occur in other species. This mechanism involves the binding of ADAs of the IgG isotype to neutrophils via their Fc receptors. Upon reexposure, the biologic cross-links the surface IgG molecules causing neutrophil activation and release of platelet-activating factor, which is up to 10,000 times more potent than histamine and can initiate an atypical anaphylactic response (Jönsson et al. 2011, 2012).

Type III hypersensitivity reactions involve the formation of biologic/ADA immune complexes in the circulation which, when present in the correct stoichiometric ratio, become deposited in tissues. Once immune complexes are deposited, they can elicit complement activation and inflammation, leading to tissue damage. The need for the correct ratio to have tissue deposition may explain some of the individual animal variability in hypersensitivity reactions, as each individual will likely have differing amounts of ADAs and test article and different stoichiometric ratios. When immune complexes are deposited in tissues, they tend to localize in small postcapillary venules where there is loss of laminar blood flow, in sites of ultrafiltration where there is high pressure and fenestrated endothelium (e.g., choroid plexus, ciliary body, synovium, and glomeruli), in sites of turbulent blood flow (e.g., coronary artery branches off aorta, aortic bifurcations, and cardiac valve leaflets), and in renal glomerular endothelium. Although these are predilection sites, any vessel of any size in any organ can be affected, and changes are usually multifocal and often sporadic and scattered throughout the tissues of affected animals.

Ben Hock presented an overview of regulatory guidance, immunogenicity testing strategies, and methods for detection of ADAs including neutralizing ADAs. ADAs are related to the development of an adaptive immune response to an administered biologic. Because the biologic is usually a foreign (i.e., human) protein in nonclinical test species, the development of an adaptive immune response and ADAs in these animal species is certainly not unexpected (see appendix E within FDA 2013).

Understanding ADAs and their potential impact is critical to accurately interpreting responses to biologics in nonclinical toxicity studies, in particular because findings that can be attributed to immunogenicity in nonclinical studies are not considered predictive of what will occur in humans (Bugelski and Treacy 2004).

While an assessment of ADAs is not necessary in every study (ICH 2011), it is recommended that appropriate samples be collected from all studies in case they are needed. The ICH S6(R1) guidance document describes scenarios where measurement of ADA should be done, including when there is evidence of altered pharmacodynamic (PD) activity, unexpected changes in test article exposure (i.e., altered pharmacokinetics [PKs]) in the absence of a PD marker, or evidence of immune-mediated reactions (immune complex disease, vasculitis, anaphylaxis, etc.; ICH 2011). An understanding of these first two scenarios requires a good understanding of PD markers and assays and PK assays. A good PD marker should be sensitive to concentrations of the test article in a timely manner, should have well-understood variability in the test species, and the assay detecting the PD marker should be qualified or validated to determine assay performance and robustness. Regarding PK assays, the ability of the assay to detect active drug in the presence of ADA should be determined. If the PK assay is sensitive to the presence of neutralizing ADAs, the combination of an appropriate PD marker and PK data may eliminate the need to perform ADA analysis. For further details on this topic, please see Ponce et al. (2009) and Swanson and Bussiere (2012).

In those cases where ADA analysis is warranted, a two-tier immunoassay approach (a binding assay followed by an orthogonal confirmatory assay) is suggested, which allows for sensitive detection of binding ADAs and a reduction in the possibility of false-positive results (Wakshull 2011). Under particular circumstances (the absence of a PD marker or the presence of findings consistent with immune-mediated reactions), neutralizing ADA and characterization assays may also be conducted. Characterization assays may include analyses of circulating immune complexes (CICs; Stubenrauch et al. 2010) or drug-specific IgE (Clark et al. 2013).

ADAs can be detected using several types of assays. The fundamental challenge in detecting ADAs is the development of a robust and sensitive assay capable of detecting ADAs in the presence of circulating drug, which is often present at high concentrations (i.e., a drug-tolerant assay). The choice of assay platform is informed by the modality of the therapeutic, likely PK parameters, and past experience with nonclinical dosing (if

possible). The surface plasmon resonance (Biacore®; GE Healthcare, Piscataway, NJ) ADA assay requires no labeling of substrates, is sensitive to ADAs of all isotypes of varying affinities (Barger et al. 2012), and allows for “on-the-spot” characterization but generally has poor drug tolerance. The electrochemiluminescence ADA assay (conducted on the Meso Scale Discovery platform) can be conducted in several ways. It can use an acid-dissociation bridging format that has a low background, good sensitivity, and drug tolerance, but it may require efforts to demonstrate that soluble ligands or other non-ADA factors are not contributing to the response. It can also use an indirect format that relies on species-specific ADA detection reagents (Bautista, Salimi-Moosavi, and Jawa 2012), which has the advantage of a standardized platform for monoclonal antibody therapeutics and good drug tolerance but requires reagents of exquisite specificity. Finally, the colorimetric enzyme-linked immunosorbent assay (ELISA) ADA assay has high throughput and is cost effective but often has greater background and reduced sensitivity. Recommendations of design and validation parameters are covered by several articles (Devanarayan and Tovey 2011; Shankar et al. 2008) and guidance documents (EMA 2007; FDA 2009).

Several neutralizing ADA assay formats are commonly used. The gold standard has been a cell-based neutralizing ADA assay. Possible outputs include functional consequences (e.g., proliferation or cytokine production), cell-signaling events (e.g., p-ERK), and transcriptional responses (e.g., endogenous targets or reporter genes). In the absence of an appropriate cell line, receptor- or target-binding assays also have good sensitivity and may be less sensitive to interference from matrix components (Wu et al. 2011). Excellent reviews on neutralizing ADA assay development (Civoli et al. 2012) and validation requirements (Finco-Kent and Grenham 2011) are available.

Deborah Finco presented an overview of additional methods beyond determination of ADAs that can be used to detect evidence of an immune response using blood or serum. Some possible assays for investigating immune-related toxicity study findings may include measurement of CICs, complement receptor 1 (CR1), complement split products, CH50, IgE levels, autoantibodies, as well as use of mast and basophil activation assays.

CICs can readily be measured in serum, and a commercially available ELISA kit (catalogue #A002; Quidel, San Diego, CA) to measure CIC has been qualified for use in cynomolgus monkeys at Pfizer. In addition, serum samples collected for other reasons (clinical chemistry, PK) can be used for this assay, in cases where samples were not specifically collected for this purpose. Increases in CICs in the serum postdrug exposure provide supportive evidence of immunogenicity against an administered biologic. However, there are other causes of increased CICs, including infection or drug aggregation, and data must be considered in light of other findings in the study.

CR1 on erythrocytes is important as a normal physiologic mechanism for removing immune complexes from circulation in humans and many nonhuman primate species (Birmingham

and Hebert 2001; Hebert 1991), while in other species, platelets play a larger role (Edberg, Tosic, and Taylor 1989; Quigg and Holers 1995). Decreased CR1 expression, which can be measured by flow cytometry, may be associated with chronic increased clearance of immune complexes (Cosio et al. 1990) and may be suggestive of ongoing immune complex formation that might occur when a sensitized animal is dosed repeatedly with the test article. The ensuing lower CR1 expression may then reduce clearance of CICs, leading to higher CIC levels, and potentially increasing the risk of immune complex disease. In addition, low baseline CR1 expression has been observed in approximately 10% of healthy cynomolgus monkeys (Sokolowski, unpublished data), and it is hypothesized these animals might have reduced ability to clear CICs, leading to higher CICs in these animals and possibly increasing the risk of immune complex-mediated tissue damage. If correct, this may contribute to the individual animal variability that is observed in immunogenicity-related findings in nonclinical toxicity studies.

Assays to assess complement are available. Commercially available ELISA (Quidel MicroVue C3a and SC5b-9 Plus EIA, San Diego, CA) can measure C3a and C5b-9 and have been qualified for use with cynomolgus monkeys. The presence of increased C3a and C5b-9 is suggestive of complement activation. For optimal plasma results, K2 EDTA collection tubes are recommended for measurement of C3a and C5b-9. Serum complement activity can also be assessed using the CH50 assay, which determines the ability to lyse sheep erythrocytes coated with anti-sheep erythrocyte antibody. The CH50 assay has some inherent variability, and results are reported as the reciprocal of the serum dilution lysing 50% of the sheep erythrocytes reported in CH50 units/mL of serum. Thus, lower values indicate reduced complement activity in the serum and suggest activation of complement has occurred, leading to complement consumption. Historically, assays to measure complement activity and function have been hampered by a lack of standardization between laboratories worldwide. The recently adopted international standards for use in complement assays may help bring greater consistency to assays and allow better interpretation of results (Bergseth et al. 2013).

The evaluation of histamine or total IgE and the use of mast cell and basophil assays may be helpful to determine whether a test article is inducing an allergic or pseudoallergic immune response. Total IgE can be monitored in cynomolgus monkeys with a commercial ELISA kit specific for monkeys (catalogue #7070; Alpha Diagnostics, Harrisburg, PA). Healthy monkeys have fairly constant levels of IgE when tested longitudinally over 6 months (Finco, unpublished data). Thus, an increase in IgE could be detected with this assay. Furthermore, mast cell and basophil activation assays (using donor cells with serum and drug combinations) can be used to evaluate a functional effect of a drug either directly or in concert with serum from an animal observed with a possible hypersensitivity-type reaction. Histamine release can be detected using commercial ELISA kits (catalogue #RE95000; IBL, Toronto, Canada), or one can also look at activation markers such as CD63 or CD203 on basophils or mast cells by flow cytometry.

In addition to inducing hypersensitivity reactions, drugs can induce autoimmune disorders (Verdier, Patriarca, and Descotes 1997). Thus, evaluation of autoimmune antibodies may provide useful data. A wide spectrum of autoantibodies can be evaluated, including antinuclear, antideoxyribonucleic acid, antierythrocyte antigens, and anti-Ig (rheumatoid factor). The assays used are commercially available ELISAs developed for humans and qualified for use with cynomolgus monkey samples; a Coombs' assay can be used to assess antibodies to erythrocyte antigens.

Although not an all inclusive list of assays, incorporating these types of assays may provide information to help determine whether an immune response is occurring in animals. These assays should not be considered as routine end points but rather used to investigate potential mechanisms to explain findings in toxicity studies.

Jennifer Rojko presented an overview of immunohistochemical and electron microscopic methods that can be used to detect evidence of an immune response in tissues. Immunohistochemistry has a number of advantageous characteristics in detecting immune responses. It can often be done on sections from existing fixed tissue paraffin blocks, thus eliminating the need to conduct an additional study. In addition, it can allow localization of immune complexes in regions that show damage on routine light microscopic examination of hematoxylin and eosin-stained tissue sections, thus providing a link between the immune complexes and the changes observed. The detection of immune complexes typically involves immunohistochemical staining for Ig (IgG, IgM, and/or IgA) from the animal as well as colocalization of C3 and the terminal complement complex sC5-9. A comparison of staining patterns in test article-dosed animals with controls is critical for interpretation of findings. Immune complexes highlighted by immunohistochemistry typically appear as granular deposits in tissues. Staining for albumin may also be included to demonstrate patterns of leakage of serum components along tissue planes, which must be differentiated from immune complex-related granular deposits.

An additional and very powerful immunohistochemical technique involves the detection of the test article in the tissue sections. Demonstration of the test article in immune complexes may support the interpretation that a finding is related to immune complex formation secondary to an immune response to the test article. When attempting to detect the test article, it is critical to use the correct reagents. In particular, the antibody used to detect the test article must be specific for the test article and must not bind to endogenous Ig from the animal. As most biologic test articles currently in development are humanized or fully human forms of a human protein, this is usually not a challenge when the test species is a rodent or a dog. However, when the test species is a monkey, many commercially available anti-human protein Igs also bind to the monkey protein. As an example, if the test article is a humanized IgG, the detecting antibody would be an anti-human IgG. That anti-human IgG would need to be specific for human IgG and not bind to monkey IgG to ensure accurate detection and localization of the test article. Reagents that can differentiate

human and monkey IgG can be prepared by extensive cross adsorption of the detection reagents with Igs, serum, and tissue. Ideally, the following cross-adsorbed reagents would be prepared: monkey-adsorbed, anti-human IgG (to detect the test article) and human-adsorbed, anti-monkey IgG (to provide context for the normal biodistribution staining patterns of IgG in the test species as well as to detect antidrug antibody components of any immune complexes).

Blood vessel walls and renal glomeruli are two of the most common sites for immune complex deposition secondary to antidrug antibody formation, with subsequent development of inflammation and tissue damage. When compared to controls, animals with immune complex disease demonstrate increased staining of Ig, complement products, and test article, and importantly the staining appears as granular material at the site of immune complex deposition. Because Igs and complement are normally present in animals, it is critical to compare changes in test article-dosed animals with controls to reach accurate interpretations. An understanding of how endogenous IgG and IgM are transported in the kidney is also essential.

Electron microscopy (EM) can be used to provide morphologic evidence of immune complex deposition and can provide very specific localization of the immune complexes. However, EM has the limitation that only a very small amount of tissue is usually examined in detail, raising the possibility that the area evaluated may not be representative. Because of the limitations in the amount of tissue that can be evaluated, EM is often best used in conjunction with immunohistochemistry, which allows evaluation of much larger sections of tissue, to support an overall interpretation. In the context of detecting evidence of hypersensitivity reactions, EM is most useful in demonstration of immune complexes in kidney glomeruli, where granular electron-dense deposits consistent with immune complexes can be seen in subendothelial or subepithelial locations in glomeruli. While technically challenging, immunoelectron microscopy can be performed to detect the test article and co-localize it with immune complexes, providing strong evidence that any observed immune complexes are related to ADAs.

Joseph Beyer and Michael Leach each presented multiple case studies of immunogenicity and hypersensitivity reactions in the context of nonclinical toxicity studies in animals. Clinical findings in animals with immunogenicity to administered biologics can be quite variable and may differ between species and between animals in the same study. Clinical findings may include no signs, local to generalized pruritis, somnolence, loss of consciousness, death without preceding signs, and anaphylaxis. At necropsy, affected animals often show no changes unless they have developed full-blown anaphylaxis. When anaphylaxis occurs in monkeys, there may be edema and hemorrhage in and around the respiratory tract and hemorrhage in the digestive tract. Microscopically, findings may include localized infiltrates of cells and perivascular cuffing in the dermis and/or subcutis around injection sites, lymphoid hyperplasia, and in the case of anaphylaxis, edema and hemorrhage in and around the respiratory tract. Immune

complex disease may also be present, which often manifests as vasculitis and/or glomerulonephritis secondary to tissue deposition of complexes of antigen (i.e., test article) and antibody (i.e., ADA). Signs may be apparent clinically or only microscopically.

Several key messages were evident from the case studies:

- Effects related to immunogenicity often do not appear until after multiple doses and usually not until after approximately 10 to 14 days. In some cases, the first observed effects may not occur until months after initiation of dosing, despite weekly administration of the test article. However, if animals have preexisting ADAs, or the test article activates the innate immune system, effects can be seen after the first dose.
- Thrombocytopenia, decreased red cell mass, and neutrophilia can be seen in the peripheral blood.
- There can be significant variability of responses between individual animals in the same study, even at the same dose level.
- The total percentage of animals affected can vary from one animal to all animals in a given dose group or study.
- Effects can vary in the same animal if dosing is continued and may get worse, improve, or not change.
- There is not always a good association of all the findings that one would associate with immunogenicity in the same animal. For example, ADA titers sometimes correlate with clinical reactions, microscopic findings, loss of PD effect, evidence of complement activation, and so on, but not always. In some animals, all laboratory parameters behave as expected, while in other animals only some or no laboratory parameters seem to correlate well with clinical and/or microscopic observations.
- Dose responses can be bell shaped or inverse, with greater evidence of immunogenicity and hypersensitivity in mid- or low-dose groups, compared with high-dose groups.
- ADA and associated hypersensitivity reactions can occur in animals even when the test articles are immunosuppressive, including those that deplete B cells.
- Administration of antihistamines such as diphenhydramine may not ameliorate responses in animals.
- In rats, immunogenicity may manifest as hepatic necrosis and/or fibrosis, and each administration of the test article does not appear to result in the same severity of effects.
- Use of genetically-modified rodents can be helpful in determining whether the immune system is involved in any reactions observed in animals. For example, in one case study in which hepatic necrosis was observed in rats, administration of the test article to nude rats prevented the development of ADAs and any evidence of hepatic damage.

- Less frequent dosing may increase the risk of immunogenicity.

In conclusion, immunogenicity resulting in hypersensitivity reactions may occur following administration of biologics to animals. These reactions can result in anaphylaxis, vasculitis, and/or glomerulonephritis. It is critical to determine whether effects observed in animals are related to test article pharmacology or are related to immunogenicity, because the former may be relevant to humans while the latter is less likely to be relevant to humans. A number of assays can be used to determine whether effects observed in animals are related to an immune response. These include assays that can be done in blood or serum (drug concentrations, detection of ADAs, complement activity, complement split products, hematology, etc.) or analyses that can be done in tissues (routine light microscopic examination, immunohistochemistry, and EM). Experience has shown that evidence of an immune response to biologics can be variable between individual animals within a study or between different studies. When effects occur in only one or a few animals, and the data are not consistent in clearly indicating the presence of immunogenicity and hypersensitivity, making accurate conclusions can be challenging. However, experience suggests that a weight of evidence approach is usually effective in deciding whether findings in animals are likely related to an immune response, thus allowing scientists to reach appropriate mechanistic conclusions and determine the relevance of the findings to humans.

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