Guidance for Industry

Immunogenicity Assessment for Therapeutic Protein Products

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
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Guidance for Industry[1]
Immunogenicity Assessment for Therapeutic Protein Products

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I. INTRODUCTION

This guidance is intended to assist manufacturers and clinical investigators involved in the development of therapeutic protein products for human use.[2] In this document, FDA outlines and recommends adoption of a risk-based approach to evaluating and mitigating immune responses to or adverse immunologically related responses associated with therapeutic protein products that affect their safety and efficacy. Any given approach to assessing and mitigating immunogenicity is determined on a case-by-case basis and should take into consideration the risk assessment we describe. For the purposes of this guidance, immunogenicity is defined as the propensity of the therapeutic protein product to generate immune responses to itself and to related proteins or to induce immunologically related adverse clinical events.

This guidance describes major clinical consequences of immune responses to therapeutic protein products and offers recommendations for risk mitigation in the clinical phase of development. It also describes product- and patient-specific factors that can affect the immunogenicity of therapeutic protein products. For each factor, recommendations are made for sponsors and investigators that may help them reduce the likelihood that these products will generate an immune response. Appendix A provides supplemental information on the diagnosis and pathophysiology of particular adverse consequences of immune responses to therapeutic protein products and brief discussions of the uses of animal studies and the conduct of comparative

[1] This guidance has been prepared by the Division of Medical Policy Development, Office of Medical Policy, in the Center for Drug Evaluation and Research (CDER) in coordination with the Center for Biologics Evaluation and Research (CBER) at the Food and Drug Administration.

[2] See the draft guidance for industry *Biosimilars: Questions and Answers Regarding Implementation of the Biologics Price Competition and Innovation Act of 2009* for FDA's interpretation of the category of "protein (except any chemically synthesized polypeptide)" in the amended definition of "biological product" in section 351(i)(1) of the Public Health Service Act (PHS Act). When final, this guidance will represent the FDA's current thinking on this topic. We update guidances periodically. To make sure you have the most recent version of a guidance, check the FDA Drugs guidance Web page at http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm.

immunogenicity studies. Although this guidance focuses on therapeutic protein products, the scientific principles may also apply to related products and biological entities, for example, peptides. Although this guidance encompasses products used to modulate or modify the immune system, including those that are antigen specific, it does not cover products that are intended to induce a specific immune response to prevent or treat a disease or condition (such as vaccines to prevent infectious diseases) or to enhance the activity of other therapeutic interventions. This guidance does not address assay development, which is covered in a separate guidance.[3]

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

Immune responses to therapeutic protein products may pose problems for both patient safety and product efficacy. Immunologically based adverse events, such as anaphylaxis, cytokine release syndrome, and cross-reactive neutralization of endogenous proteins mediating critical functions (see Appendix A.3), have caused sponsors to terminate the development of what otherwise may have been efficacious therapeutic protein products. Unwanted immune responses to therapeutic protein products may also neutralize their biological activities and result in adverse events not only by inhibiting the efficacy of the therapeutic protein product, but also by cross-reacting to an endogenous protein counterpart, leading to loss of its physiological function (e.g., neutralizing antibodies to therapeutic erythropoietin cause pure red cell aplasia by also neutralizing the endogenous protein) (Hermeling et al. 2004; Rosenberg and Worobec 2004; Rosenberg and Worobec 2005; Koren et al. 2008; Murphy 2011). Because most of the adverse effects resulting from elicitation of an immune response to a therapeutic protein product appear to be mediated by humoral mechanisms, circulating antibody to the therapeutic protein product has been the chief criterion for defining an immune response to this class of products.[4]

Both patient-related and product-related factors may affect immunogenicity of therapeutic protein products. These factors are critical elements in the immunogenicity risk assessment. Ideally, these factors should be taken into consideration in the early stages of therapeutic protein product development. Section III contains a detailed discussion of the nature of and the risk factors for immune responses to therapeutic protein products as well as possible mitigation strategies that may be employed.

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[3] See the draft guidance for industry *Assay Development for Immunogenicity Testing of Therapeutic Proteins*. When final, this guidance will reflect the Agency's current thinking on this topic.

[4] IgG and IgE antibody responses are those most often associated with clinical adverse events, and their generation generally requires collaboration between antigen-specific T-helper cells and B-cells. Murphy, K. (2011). The Humoral Immune Response. Janeway's Immunobiology. New York, Garland Science Publishing. 8th: 367-408.

III. CLINICAL CONSEQUENCES

Treatment of patients with therapeutic protein products may result in immune responses of varying clinical relevance, ranging from antibody responses with no apparent clinical manifestations to life-threatening and catastrophic reactions. During therapeutic protein product development, elucidation of a specific underlying immunologic mechanism for immunologically related adverse events is encouraged, because this information can facilitate the development of strategies to help mitigate their risk (see section III.B.1–5). The extent of information required to perform a risk-benefit assessment will vary among individual products, depending on product origin and features, the immune responses of concern, the target disease indication, and the proposed patient population.

A. Consequences for Efficacy

Development of antibodies can limit product efficacy in patients treated with therapeutic protein products. Neutralizing antibodies can block the efficacy of therapeutic protein products by specifically targeting domains critical for efficacy. For example, antibodies binding to either the uptake or catalytic domain of a therapeutic enzyme may lead to loss of product efficacy. Loss of efficacy is problematic for all products, but is of utmost concern if the product is a lifesaving therapeutic. Neutralizing antibodies that cross-react with a nonredundant endogenous counterpart of the therapeutic protein product can also impact safety, as discussed in section III.B. Both neutralizing and non-neutralizing antibodies may alter the pharmacokinetics of the product by enhancing clearance (and thereby shortening serum half-life) or, conversely, by prolonging serum half-life and product activity. If present at high enough titer, a non-neutralizing antibody may mistarget the therapeutic protein product into Fc receptor (FcR) bearing cells, thereby reducing or eliminating product efficacy (Brooks et al. 1998; Wang et al. 2008). Furthermore, although some antibody responses to therapeutic protein products may have no apparent effect on clinical safety or efficacy, they may promote the generation of neutralizing antibodies via the mechanism of epitope spreading of antibody responses (Disis et al. 2004; Hintermann et al. 2011). Pharmacodynamic biomarkers may be useful in the assessment of antibody-mediated interference with product activity, although correlation with clinical response is usually necessary to determine clinical relevance.

B. Consequences for Safety

The safety consequences of immunogenicity may vary widely and are often unpredictable in patients administered therapeutic protein products. Therefore, a high index of suspicion should be maintained for clinical events that may originate from such responses, even if the initial risk assessment suggests a lower risk of immunogenicity. The applicant should provide a rationale for the proposed immunogenicity testing paradigm, based on product- and patient-specific concerns. The following sections describe a few of the major safety concerns associated with immunogenicity:

1. Anaphylaxis

Anaphylaxis is a serious, acute allergic reaction characterized by certain clinical features. The definition currently accepted by the Agency relies on clinical diagnostic criteria and does not specify a particular immunologic mechanism (Sampson et al. 2006) (also see Appendix A.1). Historically, the definition of anaphylaxis has invoked the involvement of specific IgE antibodies. However, such a mechanistic definition may be problematic in the context of therapeutic protein product development and other clinical settings where it may not always be possible to identify a specific immunologic mechanism as the basis of an adverse event. To capture all potential adverse events of interest, the Agency recommends identifying all cases meeting the clinical diagnostic criteria of anaphylaxis, regardless of the presumed pathophysiology. Additional information, such as the assessment of serum histamine, serum tryptase, and complement components, following a reaction or the detection of product-specific IgE antibodies may help elucidate the pathophysiology of the anaphylactic response and thus guide control and mitigation strategies.

Furthermore, the presence of anti-drug antibody (ADA) alone is not necessarily predictive of anaphylaxis or other hypersensitivity reactions. Correlation with clinical response is typically required to determine the clinical relevance of these antibodies. Determination of the underlying mechanism remains of interest, however, because anaphylaxis with confirmation of IgE involvement has certain prognostic implications for repeat exposure as well as for potential therapeutic options for mitigation.

2. Cytokine Release Syndrome

Cytokine release syndrome is a symptom complex caused by the rapid release of proinflammatory cytokines from target immune cells (Stebbings et al. 2007; Stebbings et al. 2013). Although cytokine release syndrome is not directly related to immunogenicity, the clinical presentation of cytokine release syndrome overlaps with anaphylaxis and other immunologically related adverse reactions. Distinguishing this symptom complex from these other types of adverse reactions is potentially useful for the purpose of risk mitigation. Although the underlying mechanisms may not be fully understood, in some cases the mechanism appears to relate to the cross-linking of activating cell surface expressed receptors, which are the targets of the therapeutic protein product (e.g., CD28 expressed on T-cells). A risk-based evaluation, focused on the mechanism of action of the therapeutic protein product as well as results of animal and in vitro evaluations should be performed to determine the need for collection of pre- and post-dose cytokine levels in the early phase of clinical development. In case of a clinical adverse event, such an evaluation may provide evidence to support the clinical diagnosis of cytokine release syndrome and help distinguish this entity from other acute drug reactions (e.g., anaphylaxis, see Appendix A.2).

3. "Infusion Reactions"

Therapeutic protein products may elicit a range of acute effects, from symptomatic discomfort to sudden, fatal reactions that have often been grouped as "*infusion reactions"* in the past. Although the term implies a certain temporal relationship, infusion reactions are otherwise not

well defined and may encompass a wide range of clinical events, including anaphylaxis and other events that may not be directly related to antibody responses, such as cytokine release syndrome. In the absence of an agreed-upon definition for infusion reaction, the categorization of certain adverse events as infusion reactions without further detail is problematic and is not recommended. Sponsors are encouraged to use more-descriptive terminology when possible, noting the timing, duration, and specific signs and symptoms observed upon administration of a therapeutic protein product and to provide data from mechanistic studies which may facilitate a mitigation strategy.

4. Non-Acute Reactions

Anaphylaxis, cytokine release syndrome, and other acute reactions are temporally linked to administration of a therapeutic protein product. Delayed hypersensitivity (i.e., serum sickness) and immune responses secondary to immune complex formation typically have a subacute presentation. As a result, the association between administration of a therapeutic protein product and non-acute reactions may be more difficult to establish, and assessment of the underlying mechanism will likely require evaluation of circulating immune complexes and complement activation. Clinical signs may include delayed onset of fever, rash, arthralgia, myalgia, hematuria, proteinuria, serositis, central nervous system complications, and hemolytic anemia in the face of an ongoing antibody response to the therapeutic protein product (Hunley et al. 2004; Goto et al. 2009). When such a reaction is suspected, laboratory assessment for circulating immune complexes may help confirm the diagnosis. The necessity and details of a laboratory assessment will depend on the individual situation and should be discussed with the respective review division for the therapeutic protein product.

5. Cross-Reactivity to Endogenous Proteins

ADA can have severe consequences if it cross-reacts to and inhibits a nonredundant endogenous counterpart of the therapeutic protein product or related proteins (Macdougall et al. 2012; Seidl et al. 2012). If the endogenous protein is redundant in biological function, inhibition of the therapeutic and endogenous proteins may not produce an obvious clinical syndrome until the system is stressed, because not all biological functions of an endogenous protein may be known or fully characterized (Stanley et al. 1994; Bukhari et al. 2011). Moreover, the long-term consequences of such antibodies may not be known. An additional potential consequence of cross-reactivity to an endogenous protein results from antibody responses to a therapeutic protein product that is a counterpart of an endogenous cell surface receptor or a counterpart of an endogenous cytokine that is membrane-expressed. Such antibodies may cross-reactively bind to the respective cell surface receptors or proteins, causing cytokine release or other manifestations of cellular activation.

For therapeutic protein product counterparts of endogenous proteins that are critical to normal fetal or neonatal development, neutralization of such endogenous proteins, resulting from antibodies to the therapeutic protein product that cross react to the endogenous counterpart, has the potential to negatively impact fetal or neonatal development when these immune responses are generated or boosted during pregnancy or breast feeding. As part of the risk evaluation, sponsors should consider the potential transmission of antibodies to the fetus by the placenta or

to the developing neonate by human milk. Therefore, the risk of neutralizing antibody development following administration of such therapeutic protein products to women of childbearing potential should be strongly considered in light of the potential benefit. Moreover, the risk of neutralizing antibody development to endogenous proteins critical in growth and development beyond the neonatal period should be evaluated in studies in pediatric populations.

Although animal studies may provide useful information regarding the possible consequences of inhibition of an endogenous protein, particularly for endogenous proteins that are highly evolutionarily conserved, such studies are not considered to be predictive of the *likelihood* of an immune response to a therapeutic protein product in humans. Moreover, differences in the timing and extent of transplacental transfer of maternal antibodies may limit the utility of animal studies to assess in utero effects of cross-reactive antibodies to the endogenous counterpart of the therapeutic protein product.

IV. RECOMMENDATIONS FOR IMMUNOGENICITY RISK MITIGATION IN THE CLINICAL PHASE OF DEVELOPMENT OF THERAPEUTIC PROTEIN PRODUCTS

Given the variety of factors that can affect immunogenicity, the risk assessment and the control and mitigation strategies will depend on the individual development program and should be considered at the earliest stage and at each subsequent stage of product development. The extent of immunogenicity safety information required premarketing and postmarketing will vary, depending on the potential severity of the consequences of such immune responses and the likelihood of their occurrence.

In terms of evaluating the clinical relevance of immune responses, the Agency has the following recommendations:

Development of assays for anti-drug antibody (ADA)

- Sponsors should develop and implement sensitive immunoassays commensurate with the overall product development program.[5] Concomitant assessment of levels of therapeutic protein product in the sample is recommended to assess the potential for the presence of the product to interfere with detection of antibody in the assay.

Product-specific antibody sampling considerations

- Baseline samples for ADA testing should be collected, and the post-baseline sampling frequency and duration should reflect anticipated use of the product. More frequent

[5] See the draft guidance for industry *Assay Development for Immunogenicity Testing of Therapeutic Proteins*, where assay development is covered in detail. When final, this guidance will reflect the Agency's current thinking on this topic. Guidance on appropriate assay development for immunogenicity testing is also available in the ICH guidances for industry *Q2A Text on Validation of Analytical Procedures* and *Q2B Validation of Analytical Procedures: Methodology*.

sampling is appropriate during the initiation and early use of a new, chronically administered product; less frequent sampling may be appropriate after prolonged use. Repeat sampling should generally occur over periods of sufficient duration to determine whether these responses are persistent, neutralizing, and associated with clinical sequelae. Samples for antibody assessment should be drawn prior to administration of the therapeutic protein product.

- In addition to a prespecified sampling schedule, unscheduled sampling, triggered by suspected immunologically related adverse events, is necessary for establishing the clinical relevance of ADAs. Future sampling considerations for patients whose samples test positive for antibody at the end of a study should be discussed with the respective review division for the therapeutic protein product. Informed consent should address the possibility for sampling beyond study termination.

- Banking of serum samples from clinical trials under appropriate storage conditions for future testing is always advisable.

Dosing

- For first-in-human trials, a conservative approach in an appropriate medical setting with access to immediate supportive care in the event of a serious adverse event, such as anaphylaxis, should be taken. Staggered dosing among individual patients and dosing cohorts is appropriate. The trial design should include prespecified dose escalation criteria and adequate time intervals between dosing cohorts and, as appropriate for the pharmacokinetics and pharmacodynamics of the product, between individuals within a dosing cohort to assess toxicities prior to administration of subsequent doses or treatment of additional individuals. The need for such an approach will depend on individual circumstances.[6] Aside from first-in-human trials, there may be other situations where a similarly conservative approach is indicated, e.g., change in the route of administration, change in formulation, change in container closure system. As development progresses, dosing strategies and safety parameters can be modified based on clinical experience with the product and other products of the same class.

- Because it may be difficult to predict the incidence of product-specific antibodies in different clinical trial scenarios, dosing regimens in subsequent studies should be risk based and take into account the following: data from initial trials; the potential for and predicted effects of cross-reactivity to endogenous proteins; the severity of effects of neutralization of the therapeutic protein product (e.g., a lifesaving versus adjunctive treatment product); clinical parameters that impact immunogenicity in different

[6] See the guidance for industry *Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers*.

patient populations; and the adequacy of proposed safety monitoring (Koren et al. 2008).

- Higher doses of therapeutic protein products do not uniformly overcome high titer and/or sustained or neutralizing antibody responses and may impact safety, e.g., may precipitate immune complex mediated disease or cause other toxicities. The appropriateness of such a dose escalation strategy will depend on the specific product, the magnitude of the antibody response, and the disease indication. A protocol defining specific safety monitoring evaluations and stopping rules should be developed prior to implementation of dose escalation to overcome an antibody response.

Adverse events

- The development of neutralizing antibody activity or the presence of sustained antibody titers may lead to loss of efficacy or an increased risk of an adverse reaction. In certain high-risk situations (e.g., assessment of a product with a nonredundant endogenous counterpart), real-time assessments for antibodies during a clinical trial may be recommended for safety reasons. Real-time assessments entail analyses of the samples as soon as possible after sampling, before banking of the samples, and prior to additional dosing. The need for such intensive monitoring will depend on individual circumstances.

- If clinically relevant immune responses are observed, sponsors are encouraged to study the underlying mechanism and identify any critical contributing factors. These investigations can facilitate development and adoption of potential control and mitigation strategies, including modification of product formulation and screening of higher-risk patients (see section V).

- In some cases, sponsors may choose to explore premedication, desensitization, or immune tolerance induction procedures as potential mitigation strategies. Given the risks associated with desensitization/immune tolerance induction procedures and the potential for premedication to mask early signs and symptoms of adverse events, the appropriateness of such procedures will depend on the nature of the specific indication, the target patient population, and the stage of development.

Comparative immunogenicity studies

- For all comparative immunogenicity studies (e.g., those comparing immunologically related adverse events, antibody incidence, titer, or neutralizing activity to product pre- and post-manufacturing changes), a strong rationale and, when possible, prespecified criteria should be provided to justify what differences in incidence or severity of immune responses would constitute an unacceptable difference in product

safety.[7] The same antibody assay should be used to enable valid comparisons (see Appendix A.6).

Postmarketing safety monitoring

- Robust postmarketing safety monitoring is an important component in ensuring the safety and effectiveness of therapeutic protein products. Because some aspects of postmarketing safety monitoring are product-specific, FDA encourages sponsors to consult with the appropriate FDA review division to discuss the sponsor's proposed approach to postmarketing safety monitoring. Rare, but potentially serious, safety risks (e.g., immunologically related adverse events) may not be detected during preapproval clinical testing, because the size of the population exposed may not be large enough to assess rare events. In some cases, such risks may need to be evaluated through postmarketing surveillance or required studies or clinical trials.

V. PATIENT- AND PRODUCT-SPECIFIC FACTORS THAT AFFECT IMMUNOGENICITY

A. Patient-Specific Factors That Affect Immunogenicity

Factors related to the target patient population may increase or decrease the potential for and the risk associated with an immune response. Therefore, caution is recommended when moving from one patient population to another, and a new risk assessment should be performed for each new patient population considered for treatment.

1. Immunologic Status and Competence of the Patient

Patients who are immune suppressed may be at lower risk of mounting immune responses to therapeutic protein products compared to healthy volunteers with intact immune responses. For example, 95 percent of immune-competent cancer patients generated neutralizing antibody to a granulocyte-macrophage colony-stimulating factor (GM-CSF) product; but only 10 percent of immune-compromised cancer patients did so in response to a GM-CSF product (Ragnhammar et al. 1994). Immune suppressive agents may diminish the immune response to therapeutic protein products. Thus, agents that kill antigen-activated lymphocytes and/or elicit activity of regulatory T-cells, such as methotrexate, have been shown to have a substantial effect on immunogenicity of co-administered monoclonal or other antibody products (Baert et al. 2003). In contrast to immune-deficient patients, patients with an activated immune system (e.g., patients with certain infections or autoimmune disease) may have augmented responses. Immune response generation may also be affected by patient age, particularly at the extremes of the age range (LeMaoult et al. 1997; PrabhuDas et al. 2011; Cuenca et al. 2013; Goronzy and Weyand 2013). Particular caution with regard to immunogenicity and immune responses should be used in studies

[7] For information on proposed biosimilar products, see the draft guidance for industry *Scientific Considerations in Demonstrating Biosimilarity to a Reference Product*. When final, this guidance will represent the FDA's current thinking on this topic.

evaluating novel therapeutics in healthy volunteers (Li et al. 2001; Stebbings et al. 2007; Colombel et al. 2010; Garces et al. 2013).

Recommendation

In the development of therapeutic protein products, a rationale should be provided to support the selection of an appropriate study population, especially for first-in-human studies. The potential influence of concomitant medications on ADAs should be taken into consideration during all stages of clinical development.

2. Prior Sensitization/History of Allergy

Prior exposure to a therapeutic protein product or to a structurally similar protein may lead to pre-existing antibodies at baseline. This is a particular concern for patients receiving a replacement product, such as clotting factors or an enzyme replacement therapy, who may have antibodies to a previous product that could cross-react to an analogous product.

Sensitization to the excipients or process/product-related impurities of a therapeutic protein product may also predispose a patient to an adverse clinical consequence. For example, products produced from transgenic sources may contain allergenic foreign proteins, such as milk protein or protein from chicken eggs.

Because patient history may not capture all prior exposures that could generate a pre-existing antibody response or predict anaphylaxis, screening for pre-existing antibodies, e.g., inhibitors or neutralizing antibodies in factor replacement therapy, should be considered when appropriate.

Recommendation

Screening for a history of relevant allergies pertaining to the source material of the therapeutic protein product (e.g., produced in transgenic hen eggs versus mammalian cells) is recommended, and the appropriateness of additional clinical or laboratory tests prior to administration should be considered in light of the overall risk-benefit assessment.

3. Route of Administration, Dose, and Frequency of Administration

Route of administration can affect the risk of sensitization. In general, intradermal, subcutaneous, and inhalational routes of administration are associated with increased immunogenicity compared to intramuscular and intravenous (IV) routes. The IV route is generally considered to be the least likely to elicit an immune response. In conjunction with the route of administration, dose and frequency can also affect immunogenicity (Rosenberg and Worobec 2004). For example, in certain circumstances, a lower dose administered intermittently may be more immunogenic than a larger dose administered without interruption. It should be noted that the effects of dose and frequency on ADA development may be affected by other factors, such as route of administration, product origin, and product-related factors that influence immunogenicity.

Recommendations

Immunogenicity should be considered when selecting an appropriate route of administration, especially for high-risk therapeutic protein products (e.g., therapeutic counterparts of nonredundant endogenous proteins) in first-in-human dosing.

Changes in the route of administration or dosing during product development may be associated with changes in the immunogenicity profile, and clinical safety data should be obtained to support such changes.

4. Genetic Status

Genetic factors may modulate the immune response to a therapeutic protein product. In particular, some human leukocyte antigen (HLA) haplotypes may predispose patients to development of undesirable antibody responses to specific products (Hoffmann et al. 2008). If both appropriate and feasible, HLA mapping studies may help define a subset of the patient population at increased risk. Moreover, genetic polymorphisms in cytokine genes may upregulate or downregulate immune responses (Donnelly et al. 2011).

Recommendation

Evaluation of genetic factors that may modulate the immune response to a therapeutic protein product is recommended in circumstances in which a subset of treated patients lose the clinical benefit of treatment or experience severe adverse events. For example, the subset of patients that generate neutralizing antibodies to IFN-beta products are more likely to possess distinct HLA haplotypes (Hoffmann et al. 2008). Thus, knowledge of the heightened susceptibility of patients with such HLA haplotypes may allow for measures to prevent such responses or for pursuit of other treatment options.

5. Status of Immune Tolerance to Endogenous Protein

Humans are not equally immunologically tolerant to all endogenous proteins. Thus, the robustness of immune tolerance to an endogenous protein affects the ease with which a therapeutic protein product counterpart of that endogenous protein can break such tolerance. Immunological tolerance in both protein-specific T- and B-cells depends on many factors, prominent among which is the abundance of the endogenous protein: immune tolerance is weaker for low-abundance and stronger for high-abundance proteins (Weigle 1980; Goodnow 1992; Haribhai et al. 2003).

The human immune system is not fully tolerant to low-abundance endogenous proteins, such as cytokines and growth factors, for which serum levels may be in the nanogram (ng)/milliter (mL) to picogram (pg)/mL range. This point is underscored by the presence of autoantibodies to cytokines and growth factors in healthy individuals, the development of antibodies to inflammatory cytokines, and the breaking of tolerance to endogenous proteins by administration of exogenous recombinant therapeutic protein products (Hermeling et al. 2004; Rosenberg and Worobec 2004; Rosenberg and Worobec 2005; Koren et al. 2008).

When a human therapeutic protein product is intended as a replacement for an absent or deficient endogenous protein, patients with genetic mutations conferring a protein *knock out* phenotype may respond to the therapeutic protein product as to a foreign protein or neoantigen or may already be sensitized as a result of previous exposure to a similar therapeutic protein or related proteins from other sources. Such responses may abrogate the efficacy of the replacement therapy.

Recommendations

For a therapeutic protein product that is a counterpart of an endogenous protein, the robustness of immune tolerance to the endogenous protein should be investigated before initiating a clinical trial and such evaluation should consider the following as preeminent risks: if the clinical study is a first-in-human use, if the endogenous protein has a nonredundant physiological function, and if immune responses to the endogenous protein have been detected in the context of autoimmune diseases. Suggested evaluations include:

- Quantitating or gathering information on the level of the endogenous protein in serum in the steady state, as well as in conditions that may specifically elicit its production (Weigle 1980).
- Assessing for or gathering information on the presence of pre-existing antibodies in healthy individuals and patient populations and on the frequency and role of such antibodies in autoimmune diseases (Bonfield et al. 2002; Hellmich et al. 2002).
- Evaluating immunogenicity, immune cell activation, inflammatory responses, and cytokine release in relevant animal studies to obtain insight and provide guidance for clinical safety assessments (Koren 2002) (also see Appendix A.5).
- In patients requiring factor/enzyme replacement therapies, evaluation of patient tissue samples for detection of endogenous protein or peptides (e.g., cross-reactive immunologic material (CRIM)), as well as for genetic mutations and HLA alleles (as appropriate), should be strongly considered to better predict the development of immune responses to the replacement therapy and to evaluate the need for tolerance induction mitigation strategies (Pandey et al. 2013).
- Evaluating the extent of polymorphisms, including single nucleotide polymorphisms, when appropriate, in relevant patient populations to identify potential mismatches with the therapeutic protein product (Jefferis and Lefranc 2009; Viel et al. 2009; Pandey et al. 2013).

B. Product-Specific Factors That Affect Immunogenicity

Product-specific factors may increase or decrease the potential for and the risk associated with an immune response. Immunogenicity testing should be considered when changes are made to product-specific factors.

1. Product Origin (foreign or human)

Immune responses to nonhuman (i.e., foreign) proteins are expected and, as previously explained, may be anticipated for endogenous human proteins. Moreover, mismatches between the sequence of the endogenous protein of the patient and that of the therapeutic protein product caused by naturally occurring polymorphisms are one risk factor for the development of immune responses to the therapeutic protein product (Viel et al. 2009). However, the rapidity of development, the strength (titer), and the persistence of the response may depend on a number of factors, including the following: previous and ongoing environmental exposure and the mode of such exposure; the presence in the product of immunity-provoking factors, such as product aggregates and materials with adjuvant activity; and the product's inherent immunomodulatory activity (see section V.B.6). For example, environmental exposure to bacterial proteins from either commensal or pathogenic bacteria on skin or in the gut may predispose to generation of immune responses when such bacterial proteins (either recombinantly or naturally derived) are used as therapeutics.

For proteins derived from natural sources, antibodies can develop not only to the desired therapeutic protein product, but also to other foreign protein components potentially present in the product. Furthermore, such foreign proteins may contain regions of homology to endogenous human proteins. The capacity of the foreign protein to break tolerance and induce antibody responses to the homologous human factor should be evaluated in the clinical trial. For example, during treatment with a bovine thrombin product, immune responses to bovine coagulation factor V, incidentally present in the product, led to development of antibodies that cross-reacted to human factor V and resulted in life-threatening bleeding in some patients (Kessler and Ortel 2009).

For monoclonal antibodies, product origin is an important factor that can influence immunogenicity. Although mouse antibodies have been shown to robustly elicit immune responses in humans as compared to chimeric, humanized and human monoclonal antibodies, it should be noted that chimeric, humanized and human monoclonal antibodies can also elicit a high rate of immunogenicity depending on the dosing regimen and patient population (Singh 2011). In fact, some human antibodies developed using phage display may have significant ADA responses.

Moreover, novel structural formats, including fusion proteins, bispecific or multispecific antibodies (bivalent or tetravalent), single chain fragments, single domain antibodies, and specifically engineered antibodies with mutations in the constant or variable regions, may elicit immune responses, as such novel structures may create neoantigens or expose cryptic epitopes. In addition, site-specific mutations in constant regions may create novel *allotypes*, and the use of an in vitro affinity maturation process may result in novel *idiotypes*. An understanding of the increased immunogenicity associated with certain antibody products will require more complete characterization of the ADA response, such as identification of the target epitope(s) (Singh 2011).

Recommendations

All therapeutic protein products should be evaluated for their content of and immune responses directed to incidental product components, including proteins and nonprotein components. A risk-based evaluation of potential immune responses to such process- and product-related impurities should be performed, and a testing program should be designed based on this evaluation. Foreign proteins intended for therapeutic use should be evaluated for molecular regions that bear strong homology to endogenous human proteins. When such homologies exist, assessment of antibodies to the homologous human protein should be made in addition to assessment of antibodies to the foreign therapeutic protein.

When developing assays to assess the immunogenicity of novel antibody-related products, appropriate controls should be incorporated into the assays to determine if the ADA response is directed against novel epitopes.

2. Primary Molecular Structure/Posttranslational Modifications

Primary sequence, higher-order structure, species origin, and molecular weight of therapeutic protein products are all important factors that may contribute to immunogenicity. Primary sequence analysis can reveal potentially immunogenic sequence differences in proteins that are otherwise relatively conserved between humans and animals. In some cases, nonhuman epitopes may elicit T-cell help or facilitate epitope spreading to generate an antibody response to the conserved human sequences (Dalum et al. 1997). Per section V.A.4, it is important to note that therapeutic protein products of human origin may elicit immune responses in subsets of patients with distinct HLA haplotypes as well as in patients whose endogenous protein amino acid sequence differs from that of the therapeutic protein product, even by single nucleotide polymorphisms.

Additional advanced analyses of primary sequence are also likely to detect HLA class II binding epitopes in nonpolymorphic human proteins. Such epitopes may elicit and activate regulatory T-cells, which enforce self-tolerance, or, opposingly, could activate T-helper (Th) cells when immune tolerance to the endogenous protein is not robust (Barbosa and Celis 2007; Tatarewicz et al. 2007; De Groot et al. 2008; Weber et al. 2009). However, if considered appropriate, engineering of changes to the primary sequence to eliminate immunogenic Th cell epitopes or addition of tolerogenic T-cell epitopes should be done cautiously, because these modifications may alter critical product quality attributes such as aggregation, deamidation, and oxidation and thus alter product stability and immunogenicity. Therefore, extensive evaluation and testing of critical product attributes should be performed following such changes. Primary sequence considerations are especially important in evaluation of the immunogenicity of fusion proteins, because immune responses to neoantigens formed in the joining region may be elicited (Miller et al. 1999) and may then spread to conserved segments of the molecule. Fusion proteins consisting of a foreign protein and an endogenous protein are of particular concern because of the capacity of the foreign protein to elicit T-cell help for generation of an antibody response to the endogenous protein partner. Similarly, bioengineered proteins involve the introduction of sequences not normally found in nature and may thus contain neo-epitopes. These epitopes have

the potential to broadly elicit immune responses or may instead interact with HLA alleles found only in a subset of patients to induce immune responses (Kimchi-Sarfaty et al. 2013).

Chemical modifications of therapeutic protein products, such as oxidation, deamidation, aldehyde modification, and deimination, may elicit immune responses by, for example, modifying primary sequence, causing aggregate formation, or altering antigen processing and presentation. Importantly, such changes may be well controlled during manufacture and storage, but may occur in vivo in the context of the relatively high pH of the in vivo environment or in inflammatory environments and cause loss of activity as well as elicitation of immune responses. Evaluation of therapeutic protein products in the context of the in vivo environments to which they are targeted can reveal susceptibility to such chemical modifications. (Huang et al. 2005; Demeule et al. 2006; Makrygiannakis et al. 2006). Susceptibility to chemical modifications of therapeutic protein products, and thus the possibility of loss of activity or induction of immune responses in vivo, should prompt consideration of careful protein engineering.

Recommendations

Careful consideration should be given to the primary sequences chosen for development of therapeutic protein products in general, and especially of therapeutic protein product counterparts of endogenous proteins in view of potential polymorphisms in endogenous proteins across human populations.

The ADA response to fusion molecules or engineered versions of therapeutic protein products should utilize assays that are able to assess reactivity to the whole molecule as well as to its distinct components. Immune responses directed to the intact protein product, but not reactive with either of the separate partner proteins, may be targeting novel epitopes in the fusion region.

Evaluation of therapeutic protein products in the in vivo milieu in which they function (e.g., in inflammatory environments or at physiologic pH) may reveal susceptibilities to modifications (e.g., aggregation and deamidation) that result in loss of efficacy or induction of immune responses. Such information may facilitate product engineering to enhance the stability of the product under such stress conditions. Sponsors should consider obtaining this information early in product design and development.

3. Quaternary Structure: Product Aggregates and Measurement of Aggregates

Protein aggregates are defined as any self-associated protein species, with monomer defined as the smallest naturally occurring and/or functional subunit. Aggregates are further classified based on five characteristics: size, reversibility/dissociation, conformation, chemical modification, and morphology (Narhi et al. 2012). Aggregates ranging from dimer to visible particles that are hundreds of micrometers in size (Narhi et al. 2012) have been recognized for their potential to elicit immune responses to therapeutic protein products for over a half-century (Gamble 1966). Mechanisms by which protein aggregates may elicit or enhance immune responses include the following: extensive cross-linking of B-cell receptors, causing efficient B-cell activation (Dintzis et al. 1989; Bachmann et al. 1993); and enhancing antigen uptake, processing, and presentation; and triggering immunostimulatory danger signals (Seong and

Matzinger 2004). Such mechanisms may enhance recruitment of the T-cell help needed for generation of high-affinity, isotype-switched IgG antibody, the antibody response most often associated with neutralization of product efficacy (Bachmann and Zinkernagel 1997).

The potential clinical consequences of immune responses induced by protein aggregates may in large measure depend on the loss or preservation of native epitopes in the aggregate. Some antibodies generated by aggregates containing native protein can bind to monomeric protein as well as to the aggregate, with the potential to inhibit or neutralize product activity. In contrast, some antibodies to denatured/degraded protein bind uniquely to the aggregated material, but not to native protein monomers, such as was the case with early preparations of human intravenous immune globulin (IVIG) (Barandun et al. 1962; Ellis and Henney 1969). Responses to aggregates containing degraded epitopes have been shown to cause anaphylaxis, but do not inhibit or neutralize activity of the native protein (Ellis and Henney 1969).

Critical information is lacking regarding the types and quantities of aggregates needed to generate immune responses for any given therapeutic protein product (Marszal and Fowler 2012), although there is evidence that higher-molecular-weight aggregates and particles are more potent in eliciting such responses than lower-molecular-weight aggregates (Dintzis et al. 1989; Bachmann et al. 1993; Joubert et al. 2012). The aggregates formed and the quantities that efficiently elicit immune responses also may differ for different products and in different clinical scenarios. Furthermore, the use of any single method for assessment of aggregates is not sufficient to provide a robust measure of protein aggregation. For example, sole use of size exclusion chromatography may preclude detection of higher-molecular-weight aggregates that fail to traverse the column prefilter, yet may be the most crucial species in generating immune responses. Moreover, it has been recognized that subvisible particulates in the size range of 0.1–10 microns have a strong potential to be immunogenic, but are not precisely monitored by currently employed technologies (Berkowitz 2006; Roda et al. 2009; Gross and Zeppezauer 2010; Mahler and Jiskoot 2012). These very large aggregates may contain thousands to millions of protein molecules and may be homogeneous or heterogeneous (e.g., protein molecules adhered to glass or metal particles).

Recommendations

It is critical for manufacturers of therapeutic protein products to minimize protein aggregation to the extent possible. Strategies to minimize aggregate formation should be developed as early as feasible in product development. This can be done by using an appropriate cell substrate, selecting manufacturing conditions that minimize aggregate formation, employing a robust purification scheme that removes aggregates to the greatest extent possible, and choosing a formulation (see section V.B.7) and container closure system (see section V.B.8) that minimize aggregation during storage. It is particularly important that product expiration dating take into account any increase in protein aggregates associated with protein denaturation or degradation during storage.

Methods that individually or in combination enhance detection of protein aggregates should be employed to characterize distinct species of aggregates in a product. Methods for measuring aggregation are constantly evolving and improving. Constant improvement and development of

these methods should be considered in choosing one or more appropriate assays. Assays should be validated for use in routine lot release and stability evaluations, and several of them should be employed for comparability assessments. Animal studies may be useful in identifying aggregate species that have the potential to be immunogenic, although additional considerations (amount and types of aggregates, route of administration, etc.) may determine the extent to which such aggregate species pose clinical risk.

Assessment should be made of the range and levels of subvisible particles (2–10 microns) present in therapeutic protein products initially and over the course of the shelf life. Several methods are currently qualified to evaluate the content of subvisible particulates in this size range (Mahler and Jiskoot 2012). As more methods become available, sponsors should strive to characterize particles in smaller (0.1–2 microns) size ranges. Sponsors should conduct a risk assessment of the impact of these particles on the clinical performance of the therapeutic protein product and develop control and mitigation strategies based on that assessment, when appropriate.

4. Glycosylation/Pegylation

Glycosylation may strongly modulate immunogenicity of therapeutic protein products. Although foreign glycoforms such as mammalian xenogeneic sugars (Chung et al. 2008; Ghaderi et al. 2010), yeast mannans (Bretthauer and Castellino 1999), or plant sugars (Gomord and Faye 2004) may trigger vigorous innate and acquired immune responses, glycosylation of proteins with conserved mammalian sugars generally enhances product solubility and diminishes product aggregation and immunogenicity. Glycosylation indirectly alters protein immunogenicity by minimizing protein aggregation, as well as by shielding immunogenic protein epitopes from the immune system (Wei et al. 2003; Cole et al. 2004). Pegylation of therapeutic protein products has been found to diminish their immunogenicity via similar mechanisms (Inada et al. 1995; Harris et al. 2001), although immune responses to the polyethylene glycol (PEG) itself have been recognized and have caused loss of product efficacy and adverse safety consequences (Liu et al. 2011). Anti-PEG antibodies have also been found to be cross-reactive between pegylated products (Garay et al. 2012; Schellekens et al. 2013).

Recommendations

For proteins that are normally glycosylated, use of a cell substrate production system and appropriate manufacturing methods that glycosylate the therapeutic protein product in a nonimmunogenic manner is recommended.

For pegylated therapeutic protein products, the ADA assay should be able to detect both the anti-protein antibodies and antibodies against the PEG moiety. The same principle may apply to modifications where the therapeutic protein products that are not pegylated but are modified with other high molecular weight entities, e.g., hydroxyethyl starch.

5. Impurities with Adjuvant Activity

Adjuvant activity can arise through multiple mechanisms, including the presence of microbial or host-cell-related impurities in therapeutic protein products (Verthelyi and Wang 2010; Rhee et al. 2011; Eon-Duval et al. 2012; Kwissa et al. 2012). These innate immune response modulating impurities (IIRMIs), including lipopolysaccharide**,** β−glucan and flagellin, high-mobility group protein B1 (HMGB1), and nucleic acids, exert immune-enhancing activity by binding to and signaling through toll-like receptors or other pattern-recognition receptors present on B-cells, dendritic cells, and other antigen-presenting cell populations (Iwasaki and Medzhitov 2010; Verthelyi and Wang 2010). This signaling prompts maturation of antigen-presenting cells and/or serves to directly stimulate B-cell antibody production.

Recommendations

It is very important for manufacturers to minimize the types and amounts of such microbial or host-cell-related impurities in therapeutic protein products.

Assays to evaluate the types of IIRMIs present should be tailored to the relevant cell substrate. Because even trace levels of IIRMIs can modify the immunogenicity of a therapeutic protein product, the assays used to detect them should have sensitivities to assess levels that may lead to clinically relevant immune responses.

If biomarkers are used to detect and compare the presence of IIRMIs, they should be tailored to the IIRMIs that could be present in the product. Examples of biomarkers could include cytokine release and transcription factor activation from defined cell populations.

6. Immunomodulatory Properties of the Therapeutic Protein Product

The immunomodulatory activity of any given therapeutic protein product critically influences not only the immune response directed to itself but also immune responses directed to other co-administered therapeutic protein products, endogenous proteins, or even small drug molecules and may not be predictable. For example, interferon-alpha (Gogas et al. 2006; Tovey and Lallemand), interleukin-2 (Franzke et al. 1999), and GM-CSF (Hamilton 2008) are not only relatively immunogenic of themselves but also are known to upregulate immune responses to endogenous proteins and to induce clinical autoimmunity. Immunosuppressive therapeutic proteins may globally downregulate immune responses, raising the possibility of serious infections. However, not all immunosuppressive therapeutic protein products suppress responses to themselves. For example, integrin and TNF monoclonal antibodies tend to be immunogenic. Thus, the immunogenicity of such therapeutic protein products should be evaluated empirically.

Recommendations

The immunomodulatory properties of therapeutic protein products, their effects on immune responses to themselves, and their capacity to induce autoimmunity should be monitored from the earliest stages of product development (Franzke et al. 1999; Gogas et al. 2006; Hamilton 2008).

Vaccination using live attenuated organisms should be avoided when the therapeutic protein product is immunosuppressive. Updated vaccination status, compliant with local health care standards, is recommended for patients before administration of the therapeutic protein product.

7. Formulation

Formulation components are principally chosen for their ability to preserve the native conformation of the therapeutic protein in storage by preventing denaturation due to hydrophobic interactions, as well as by preventing chemical degradation, including truncation, oxidation, and deamidation (Cleland et al. 1993; Shire et al. 2004; Wakankar and Borchardt 2006). Large protein excipients in the formulation, such as human serum albumin (HSA), may affect immunogenicity positively or negatively. Excipients such as HSA, although added for their ability to inhibit hydrophobic interactions, may coaggregate with the therapeutic protein or form protein adducts under suboptimal storage conditions (Braun and Alsenz 1997). Polysorbate, a nonionic detergent, is the most commonly used alternative to HSA. The stability of both types of excipients (i.e., HSA and polysorbate) should be kept in mind for formulation purposes because they too are subject to modifications (e.g., oxidation), which may then pose a threat to the integrity of the therapeutic protein product.

Formulation may also affect immunogenicity of the product by altering the spectrum of leachables from the container closure system. Leachables from rubber stoppers have been shown to possess immune adjuvant activity, as shown in an animal experiment (Mueller et al. 2009). Organic compounds with immunologic activity as well as metals have been eluted from container closure materials by polysorbate-containing formulations, leading to increased oxidation and aggregation (Seidl et al. 2012).

Recommendations

Excipients should be evaluated for their potential to prevent denaturation and degradation of therapeutic protein products during storage. Interactions between excipients and therapeutic proteins should be carefully evaluated, especially in terms of co-aggregation or formation of protein-excipient adducts.

Excipient stability should be carefully considered when establishing product shelf life. Thorough analyses of leachables and extractables should be performed to evaluate the capacity of container closure materials to interact with and modify the therapeutic protein product. A risk assessment should be conducted, and control and risk mitigation strategies should be developed as appropriate.

8. Container Closure Considerations

Interactions between therapeutic protein products and the container closure may negatively affect product quality and immunogenicity. These interactions are more likely with prefilled syringes of therapeutic protein products. These syringes are composed of multiple surfaces and materials that interact with the therapeutic protein product over a prolonged time period and thus have the

potential to alter product quality and immunogenicity. The following are other container closure considerations pertinent to immunogenicity:

- Glass and air interfaces can denature proteins and cause aggregation in glass syringes and vials.

- Glass vials have been known to delaminate at higher pH and with citrate formulations, potentially creating protein-coated glass particles, which may enhance immunogenicity of the therapeutic protein product (Fradkin et al. 2011).

- Silicone oil-coated syringe components provide a chemical and structural environment on which proteins can denature and aggregate.

- Appropriate in-use stability studies should be performed to confirm that conditions needed to maintain product quality and prevent degradation are adequately defined.

- Leached materials from the container closure system may be a source of materials that enhance immunogenicity, either by chemically modifying the therapeutic protein product or by having direct immune adjuvant activity, including the following:

    - Organic compounds with immunomodulatory activity may be eluted from container closure materials by polysorbate-containing formulations: a leachable organic compound involved in vulcanization was found in a polysorbate formulated product when the stopper surfaces were not Teflon coated (Boven et al. 2005).

    - Metals that oxidize and aggregate therapeutic protein products or activate metalloproteinases have been found in various products contained in prefilled syringes or in vials. For example, tungsten oxide that leached from the syringe barrel was reported to cause protein aggregation (Bee et al. 2009); and leached metals from vial stoppers caused increased proteolysis of a therapeutic protein because of activation of a metalloproteinase that co-purified with the product.

Recommendations

Whenever possible, sponsors should obtain detailed information regarding a description of all raw materials used in the manufacture of the container closure systems for their products. Sponsors should conduct a comprehensive extractables and leachables laboratory assessment using multiple analytical techniques to assess the attributes of the container-closure system that could interact with and degrade protein therapeutic products.

Because the United States Pharmacopeia *elastomeric closures for injections* tests do not adequately characterize the impact of leachables in storage containers on therapeutic protein

products under real-time storage conditions, leachables must be evaluated for each therapeutic protein product in the context of its storage container under real-time storage conditions[8].

Testing for leachables should be performed on the product under stress conditions,[9] as well as under real-time storage conditions, because in some cases the amount of leachables increases dramatically over time and at elevated temperatures. Product compatibility testing should be performed to assess the effects of container closure system materials and all leachables on product quality.

9. Product Custody

Products in their intended primary packaging container closure system should be tested for stability in protocols that include appropriate in-use conditions (e.g., light, temperature, and agitation) to identify conditions and practices that may cause product denaturation and degradation.

Given that most therapeutic protein products degrade on exposure to heat and light or with mechanical agitation, to ensure product quality, health care practitioners and patients should be educated regarding product storage, handling, and administration.

A secure supply chain is critical. Appropriate temperature-controlled transport and storage is of utmost importance in preserving product quality. For example, the storage of epoetin-α under inappropriate conditions by unauthorized vendors was associated with high levels of aggregates and antibody-mediated pure red cell aplasia (Fotiou et al. 2009).

Recommendations

Patient educational materials (e.g., FDA-approved patient labeling providing instructions for use as required under 21 CFR 201.57 and 201.80) should explicitly identify appropriate storage and handling conditions of the product. Appropriate patient instruction by caregivers is vital to ensuring product quality and helping to minimize adverse impacts on product quality during product storage and handling. Appropriate temperature-controlled transport and storage should be ensured.

VI. CONCLUSION

The consequences of immune responses to therapeutic protein products can range from no apparent effect to serious adverse events, including life-threatening complications such as anaphylaxis, neutralization of the effectiveness of lifesaving or highly effective therapies, or neutralization of endogenous proteins with nonredundant functions. Although immunogenicity risk factors pertaining to product quality attributes and patient/protocol factors are understood, immune responses to therapeutic protein products cannot be predicted based solely on

[8] 21 CFR 600.11(b) and (h).
[9] See the ICH guidance for industry *Q1A(R2) Stability Testing Of New Drug Substances And Products*.

characterization of these factors, but should be evaluated in the clinic. A risk-based approach, as delineated in this guidance, provides investigators with the tools to develop novel protein therapeutics, evaluate the effect of manufacturing changes, and evaluate the potential need for tolerance-inducing protocols when severe consequences result from immunogenicity.

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APPENDIX A

1. Diagnosis of Anaphylaxis

The diagnosis of anaphylaxis is based on the following three clinical criteria, with anaphylaxis considered as highly likely when one of these criteria is fulfilled (Sampson et al. 2006):

a. Acute onset of an illness (minutes to several hours) with involvement of the skin, mucosal tissue, or both (e.g., generalized hives, pruritus or flushing, swollen lips-tongue-uvula), **and at least one of the following:**

- Respiratory compromise (e.g., dyspnea, wheeze-bronchospasm, stridor, reduced peak expiratory flow, hypoxemia)
- Reduced blood pressure or associated symptoms of end-organ dysfunction (e.g., hypotonia [collapse], syncope, incontinence)

b. Two or more of the following that occur rapidly after exposure *to a likely allergen for that patient* (minutes to several hours):

- Involvement of the skin-mucosal tissue (e.g., generalized hives, itch-flush, swollen lips-tongue-uvula)
- Respiratory compromise (e.g., dyspnea, wheeze-bronchospasm, stridor, reduced peak expiratory flow, hypoxemia)
- Reduced blood pressure or associated symptoms (e.g., hypotonia [collapse], syncope, incontinence)
- Persistent gastrointestinal symptoms (e.g., crampy abdominal pain, vomiting)

c. Reduced blood pressure after exposure to *known allergen for that patient* (minutes to several hours):

- Infants and children: low systolic blood pressure (age specific) or greater than 30-percent decrease in systolic blood pressure
- Adults: systolic blood pressure of less than 90 mm Hg or greater than 30-percent decrease from that person's baseline

Although none of the clinical criteria provide 100-percent sensitivity and specificity, it is believed that these criteria are likely to capture more than 95 percent of cases of anaphylaxis (Sampson et al. 2006).

Laboratory tests for evaluating anaphylaxis:

At present, there are no sensitive and specific laboratory tests to confirm the clinical diagnosis of anaphylaxis. Skin testing and in vitro diagnostic tests to determine the level of specific IgE antibodies directed against the therapeutic protein product, mediator release, or basophil activation may be useful for characterizing the underlying pathophysiology and may provide insight into potential mitigation strategies (Simons 2010; Lee and Vadas 2011). However, the results of unvalidated tests should be interpreted with caution; and the clinical relevance of positive results from unvalidated tests may be uncertain during product development.

2. Cytokine Release Syndrome

Monoclonal antibodies specific for cell surface receptors or for cell membrane expressed cytokines, as well as antibodies that develop in patients to therapeutic protein products that bind to cell surface receptors, have the potential to augment a product's intrinsic agonist activity and exacerbate infusion-related toxicities. In vitro assessments of the capacity of such therapeutic protein products to mediate cellular activation, including proliferation and cytokine release in human whole blood or peripheral blood mononuclear cells, are recommended. For products with the potential to incur a cytokine release syndrome, an initial starting dose below that obtained by traditional calculations and slower infusion rates, where applicable, may also be recommended (Duff 2006). Pre- and post-administration levels of C-reactive protein and cytokines, such as TNF-α; IL-2; IL-6; IL-10; and IFN-γ, and certain clinical signs and symptoms, such as an acute elevation of body temperature, erythema, and hypotension, may serve as markers of a proinflammatory response pertaining to cytokine release.

Data from both animal studies and in vitro assessments may provide information to guide development of therapeutic protein products with the potential to induce cytokine release. Although data from both animal studies and in vitro assessments may supplement each other, they generally are not fully predictive of the clinical occurrence or outcome. Therefore it is imperative that great caution is always exercised in the clinical development of products with the potential to mediate receptor cross-linking (see sections III.B.1 and III.B.2). Although the traditional animal models used for toxicology testing (i.e., rat, mouse, dog, and cynomolgus monkey) rarely demonstrate overt toxicities related to lymphocyte activation and cytokine release, specific markers related to T-cell activation and cytokine release can be measured in routine toxicology studies, provided that the drug is pharmacologically active in the test species. These data may then be useful for predicting the potential for these agents to induce a cytokine release syndrome in the clinic or for evaluating the activity of second-generation agents that have been modified to reduce their level of T-cell activation. For example, cytokine production can be measured in blood samples obtained from treated animals during pharmacokinetic or general toxicology studies, provided that the amount of samples obtained does not compromise the health of the animals or the ability to evaluate the toxicology endpoints at study termination. When evaluation of cytokine release is included in animal testing, measurement of a cytokine panel that is as broad as possible and includes IL-2, IL-6, IFN-γ, and TNF-α, as well as other relevant cytokines indicative of cytokine release syndrome, is recommended. Such proposed animal studies should be discussed with FDA prior to initiation (Hsu et al. 1999; Norman et al. 2000). In vitro assessments of cellular activation, including proliferation and cytokine release in human whole blood or peripheral blood mononuclear cells, are important assessment tools that can help in overcoming the known limitations of animals in modeling activating stimuli in some T-cell subsets (Stebbings et al. 2007; Hellwig et al. 2008; Findlay et al. 2011; Romer et al. 2011; Stebbings et al. 2013). The impact of product cross-linking of cellular receptors should be considered in such studies. Signs of cellular activation in vitro should also be taken as an indication that the product has the potential to induce toxicities in the clinic, regardless of negative findings from animal studies.

3. Non-Acute Immune Responses

Type III hypersensitivity responses, including those mediated by immune complexes and T-cells (delayed hypersensitivity responses in the older literature), are relatively rare with respect to therapeutic protein products; and a high degree of clinical suspicion is necessary for the diagnosis (Dharnidharka et al. 1998; Hunley et al. 2004; Gamarra et al. 2006; Goto et al. 2009). Signs and symptoms of immune complex deposition may include fever, rash, arthralgia, myalgia, hematuria, proteinuria, serositis, central nervous system complications, and hemolytic anemia. Immune complexes, composed of antibody and a therapeutic protein product, have been responsible for the development of glomerulonephritis and nephrotic syndrome in patients undergoing tolerance induction treatment (with factor IX and α-glucosidase) in the face of a high titer and sustained antibody response (Dharnidharka et al. 1998; Hunley et al. 2004). There have been case reports of immune complex disease with immune responses to monoclonal antibodies (Gamarra et al. 2006; Goto et al. 2009) and situations in which large doses of a monoclonal antibody targeting high levels of a circulating multivalent antigen may increase the likelihood of immune complex deposition (Gonzalez and Waxman 2000).

If patients develop signs or symptoms suggestive of immune complex disease, appropriate laboratory assessments for circulating immune complexes and complement activation should be undertaken; and the administration of the therapeutic protein product should be suspended. In certain situations, development of tolerance induction therapies that eliminate the antibody response may be appropriate prior to further attempts at treatment.

4. Antibody Responses to Therapeutic Protein Products

Antibodies to therapeutic protein products are classified as either neutralizing or non-neutralizing. Neutralizing antibodies bind to distinct functional domains of the therapeutic protein product and preclude their activity. For example, antibodies to therapeutic enzymes may bind to either the catalytic site, blocking catalysis of substrate, or to the uptake domain, preventing uptake of the enzyme into the cell. In rare circumstances, neutralizing antibody may act as a *carrier* and enhance the half-life of the product and prolong its therapeutic effect. As discussed in section III of this guidance, non-neutralizing antibodies bind to areas of the therapeutic protein product other than specific functional domains and may exhibit a range of effects on safety and efficacy—enhanced or delayed clearance of the therapeutic protein product, which may prompt consideration of dosing changes, induction of anaphylaxis, diminished efficacy of the product by causing uptake of the therapeutic protein product into FcR-expressing cells rather than the target cells, and facilitation of epitope spreading, allowing the emergence of neutralizing antibodies. However, they may have no apparent effect on either safety or efficacy.

The development of neutralizing antibody is expected with administration of nonhuman proteins and in patients receiving factor/enzyme replacement therapies to whom such therapeutic protein products appear as foreign. However, neutralizing antibody to a therapeutic protein product that cross reacts to an endogenous protein does not always arise in situations in which the endogenous factor is defective or absent by genetic mutation, as in the case of hemophilia A or lysosomal storage diseases. Neutralizing antibodies can develop in healthy individuals to some

normal endogenous proteins because immune tolerance to some endogenous proteins is not robust and can be broken by a therapeutic protein homolog with sufficient provocation. For example, healthy volunteers treated with a thrombopoietin (TPO)-type protein mounted a neutralizing antibody response to the therapeutic, which cross-reactively neutralized endogenous TPO, inducing a prolonged state of thrombocytopenia in those formerly healthy individuals (Li et al. 2001). Thus, treatment with therapeutic counterparts of endogenous proteins serving a unique function or endogenous proteins present at low abundance should be undertaken with utmost caution. Neutralizing antibody to a therapeutic protein product can also be catastrophic when it neutralizes the efficacy of a lifesaving therapeutic such as therapeutic enzymes for lysosomal storage disorders, and immune tolerance induction should be considered in such circumstances (Wang et al. 2008).

Loss of efficacy of mAbs in patients caused by immune responses to the mAb can be highly problematic, and the clinical consequences should not be minimized. Sponsors may consider development of immune tolerance induction regimens in such patients.

As discussed in section III.B.5 of the guidance, if the endogenous protein is redundant in biological function (e.g., Type I interferons), neutralization of the therapeutic and endogenous protein may not appear to produce an obvious clinical syndrome. However, the more subtle effects of blocking endogenous factors, even though redundant in some functions, may not be apparent until the system is stressed, as not all biological functions of a factor may be known or fully characterized. Moreover, the effects of long-term persistence of neutralizing antibody, as have been observed, for example, in a small percentage of patients with antibodies to IFN-β (Bellomi et al. 2003), would not be known from short-term follow-up and should be studied longer term. Generally, for products given chronically, one year or more of immunogenicity data should be collected and evaluated unless a shorter duration can be scientifically justified. However, longer-term evaluation may be warranted depending on the frequency and severity of the consequences. In some cases, these studies may be done in the postmarket setting. Agreement with the Agency should be sought regarding the extent of data required before and after marketing.

In some circumstances, antibody responses, regardless of apparent clinical effect, should be serially followed until the levels return to baseline or an alternative approach is discussed with the Agency. Moreover, for patients in whom a therapeutic protein product appears to lose efficacy, regardless of the duration of the treatment course, it is important that an assessment be undertaken to determine whether the loss of efficacy is antibody mediated.

For patients who develop neutralizing antibodies or are considered at very high risk of developing neutralizing antibodies to a lifesaving therapeutic protein product (e.g., CRIM-negative patients with a deletion mutation for a critical enzyme who are given enzyme replacement therapy), consideration should be given to tolerance induction regimens in a prophylactic setting, before or concomitant with the onset of treatment (Wang et al. 2008; Mendelsohn et al. 2009; Messinger et al. 2012). Given the degree of immune suppression of such regimens, although far less than that of a therapeutic regimen to reverse an ongoing antibody response, careful safety monitoring should be undertaken throughout the duration of the protocol.

5. Utility of Animal Studies

Immunogenicity assessments in animals are conducted to assist in the interpretation of animal study results (e.g., toxicology studies) and in the design of subsequent clinical and nonclinical studies (for additional information, see the ICH guidance for industry *S6(R1) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals*).[10] They are generally limited in their ability to predict the *incidence* of human immune responses to a therapeutic protein product, but they may be useful in describing the *consequences* of antibody responses, particularly when an evolutionarily conserved, nonredundant endogenous protein is inhibited by cross-reactive antibodies generated to its therapeutic protein product counterpart. When available, animal models including hyperimmunized mice or gene knock out mice can be used to address potential consequences of inhibition of endogenous proteins. A special case is that of endogenous proteins that are vital to embryonic or fetal development whose elimination is embryonically lethal. In such situations, the use of conditional knock out mice may be useful for assessing potential consequences of neutralizing antibodies. As in human studies, consideration should be given to the potential transmission of antibodies to developing neonates by breast milk.

In contrast to proteins that mediate biologically unique functions, animal models are generally not useful for predicting consequences of immune responses to therapeutic protein products that are counterparts to endogenous proteins with redundant biological functions. Mice that are transgenic for genes encoding human proteins, humanized mice (i.e., immune-deficient mice with human immune systems), and mouse models of human diseases are increasingly being developed and may be considered for use in addressing multiple clinical issues, including immunogenicity.

In addition to appropriate animal studies, consideration should be given to in vitro and in silico analyses that may supplement animal studies to better or further elucidate risk for immunogenicity.

6. Comparative Immunogenicity Studies

The need for and the extent and timing of clinical immunogenicity studies in the context of evaluating the effects of a manufacturing change will depend on such factors as the degree of analytical comparability between the product before and after the manufacturing change, findings from informative comparative animal studies, and the incidence and clinical consequences of immune responses to the product prior to the manufacturing change. For example, if the clinical consequence of an immune response is severe (e.g., when the product is a therapeutic counterpart of an endogenous protein with a critical, nonredundant biological

[10] ICH guidance for industry *S6(R1) Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals* is available at http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm))

function or is known to provoke anaphylaxis), more extensive immunogenicity assessments will likely be needed.[11]

[11] Guidance on development programs for biosimilar products is available in the draft guidance for industry *Scientific Considerations in Demonstrating Biosimilarity to a Reference Product*. When final, this guidance will reflect the Agency's current thinking on this topic.