Guidance for Industry

Immunogenicity Assessment for Therapeutic Protein Products

DRAFT GUIDANCE

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Immunogenicity Assessment for Therapeutic Protein Products

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I. INTRODUCTION

This draft guidance is intended to assist manufacturers and clinical investigators involved in the development of therapeutic protein products for human use. In this document, FDA outlines and recommends adoption of a risk-based approach to evaluating and mitigating immune responses to therapeutic proteins that may adversely affect their safety and efficacy. We begin with a description of major clinical consequences of immune responses to therapeutic protein products and offer recommendations for risk mitigation in the clinical phase of development. Then, we describe product- and patient-specific factors that can affect the immunogenicity of therapeutic protein products, and for each factor, we make recommendations for sponsors and investigators that may help them reduce the likelihood that these products will generate an immune response. An appendix 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 immunogenicity studies.

Any given approach to assessing immunogenicity is determined on a case-by-case basis and should take into consideration the risk assessment we describe. The development of vaccines, such as cancer vaccines, is not addressed here, nor is assay development, which is covered in a separate guidance.[2]

[1] This guidance has been prepared by 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[2] See draft guidance *Assay Development for Immunogenicity Testing of Therapeutic Proteins* (December 2009). When finalized, this guidance will reflect the Agency's current thinking on assay development for immunogenicity testing of therapeutic proteins.

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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, so-called "infusion reactions," and nonacute immune reactions such as immune complex disease (see Appendix C), have caused sponsors to terminate the development of therapeutic protein products or limited the use of otherwise effective therapies. Unwanted immune responses to therapeutic proteins may also neutralize the biological activity of therapeutic proteins and may result in adverse events not only by inhibiting the efficacy of the therapeutic protein product, but by cross-reacting to an endogenous protein counterpart, if present (e.g., neutralizing antibodies to therapeutic erythropoietin may cause pure red cell aplasia by also neutralizing the endogenous protein) (Murphy 2011; Worobec and Rosenberg 2004; Rosenberg and Worobec 2004; Rosenberg and Worobec 2005; Koren, et al. 2008; Hermeling, et al. 2004). 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.[3]

Both patient-related and product-related factors may affect immunogenicity of therapeutic protein products. These factors provide the starting point for an immunogenicity risk assessment. Ideally, these factors should be taken into consideration in the early stages of therapeutic protein product development. Below is a more detailed discussion of the nature of, and risk factors for, the more common immune responses to therapeutic protein products as well as possible mitigation strategies that may be employed.

III. CLINICAL CONSEQUENCES

Treatment of patients with therapeutic protein products frequently results in immune responses of varying clinical relevance, ranging from transient 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 related adverse events is encouraged, because this information can facilitate the development of strategies to help mitigate the risk of clinically significant immune responses. The extent of information required to perform a risk-benefit assessment will vary among individual products,

[3] 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 2011).

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 both neutralizing and non-neutralizing antibodies can limit product efficacy in patients treated with therapeutic protein products. Neutralizing antibody can block the efficacy of the product, which is of utmost concern if the product is a life-saving therapeutic. Even if not in the context of a life-saving therapeutic, loss of efficacy can be problematic. Neutralizing antibody that cross-reacts with a nonredundant endogenous counterpart can also impact safety, as discussed in the next section. Non-neutralizing (binding) antibody may alter the pharmacokinetics of the product, by either diminishing or enhancing product pharmacokinetic parameters, and therefore may require dosing modifications (Wang, et al. 2008). However, if present at high enough titer, non-neutralizing antibody may also mistarget the therapeutic protein into Fc Receptor (FcR) bearing cells, thereby reducing product efficacy (Wang, et al. 2008). Furthermore, although some binding antibodies may have no apparent effect on clinical safety or efficacy, they may promote the generation of neutralizing antibodies via the mechanism of epitope spreading (Disis, et al. 2004). Correlation with clinical responses is usually necessary to determine the clinical relevance of both binding and neutralizing antibody responses.

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 for clinical events that may originate from such responses should be maintained, even if the initial risk assessment suggests a lower risk of immunogenicity. The following section describes 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 and see Appendix). Historically, the definition of anaphylaxis has invoked the involvement of specific IgE antibodies. However, such a mechanistic definition is problematic in the context of therapeutic protein product development and other clinical settings where it may not be possible to identify a specific immunologic mechanism as the basis of an adverse event. In the interest of capturing 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 detection of elevated serum histamine or tryptase levels following a reaction or product-specific IgE antibodies may help elucidate the pathophysiology of the anaphylactic response.

Furthermore, the presence of anti-product antibody alone is not necessarily predictive of anaphylaxis or other hypersensitivity reactions. Correlation with clinical responses is typically required to determine the clinical relevance of these antibodies. Determination of the underlying mechanism remains of interest, however, since 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). The underlying mechanism is not fully understood, and multiple mechanisms, such as binding of activating Fc Receptors and clustering of the antigen on target cells, may be involved for different products. Pre- and post-dose cytokine levels may provide evidence to support the clinical diagnosis and distinguish the symptom complex from other acute drug reactions (see Appendix).

3. "Infusion Reactions"

Therapeutic proteins 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 (see Appendices A and B). 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 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. Data from mechanistic studies may be able to discriminate specific antibody-mediated anaphylaxis from episodes pertaining to cytokine release phenomena.

4. Non-acute Reactions

Anaphylaxis, cytokine release syndrome, and other acute reactions are temporally linked to administration of a therapeutic protein product. Delayed hypersensitivity and immune responses secondary to immune complex formation typically have a subacute presentation. As a result, the association between a therapeutic protein product and these reactions may be more difficult to establish, and confirmation of the underlying mechanism may not be easily achieved. Clinical signs may include delayed onset of fever, rash, arthralgia, myalgia, hematuria, proteinuria, serositis, central nervous system complications, and hemolytic anemia (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.

5. Cross-reactivity to Endogenous Proteins

Anti-drug antibody can have severe consequences if it cross-reacts with and inhibits a non-redundant endogenous counterpart of the therapeutic protein product or related proteins. 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. Moreover, the long-term consequences of such antibodies may not be known.

For therapeutic protein 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 counterpart may potentially negatively impact fetal or neonatal development when such responses are generated during pregnancy or breast feeding. Indeed, the potential transmission of antibodies to developing neonates by breast milk must be considered. Therefore, the risk of neutralizing antibody development following administration of such therapeutic proteins to women of childbearing potential should be strongly considered in light of their potential benefit.

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 appropriate mitigation strategies will depend on the individual development program and should be considered at an early stage and at each stage of product development. The extent of immunogenicity safety information required premarketing and postmarketing will vary, depending on the potential severity of consequences of these immune responses and the likelihood of their occurrence.

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

Assay development

Assay development is covered in detail in draft guidance (see Draft Guidance for Industry entitled "*Assay Development for Immunogenicity Testing of Therapeutic Proteins*"). Sponsors should develop and implement sensitive, qualified immunoassays commensurate with the overall product development program. Concomitant sampling of therapeutic product levels is recommended to assess potential interference with the assay.

Product-specific antibody sampling considerations

- Baseline serum samples for anti-product antibody testing should be collected, and sampling frequency and duration should reflect anticipated use of the product. More

frequent 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 antibody responses are transient, whether a neutralizing antibody response has developed, and whether these responses are associated with long-term clinical sequelae.

- In addition to a prespecified sampling schedule, unscheduled sampling triggered by suspected immune-related adverse events is useful for establishing the clinical relevance of antiproduct antibodies.

- 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 staggered dosing among individual patients, dosing cohorts, and different routes of administration is generally appropriate. The trial design should include prespecified dose escalation criteria and adequate time intervals between dosage cohorts and, as appropriate for the pharmacokinetics and pharmacodynamics of the product, between individuals within a dose cohort to assess toxicities prior to administration of subsequent doses or treatment of additional individuals. The need for such an approach will depend on the individual circumstances. As development progresses, dosing strategies and safety parameters can be modified based on clinical experience with the product and related products.

- Because predicting the effects of product-specific antibodies may be difficult during therapeutic protein product development, dosing regimens in subsequent studies should be risk based, taking into account the data from initial trials, the potential for cross-reactivity to endogenous proteins or neutralization of the therapeutic protein product, clinical parameters that impact immunogenicity in different patient populations, and the adequacy of the proposed safety monitoring.

Adverse events

- The development of neutralizing antibody activity or the presence of sustained, high antibody titers may lead to loss of efficacy or an increased risk of an adverse reaction. In certain 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. The need for such intensive monitoring will depend on the 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 of potential mitigation strategies,

including modification of product formulation, screening of higher-risk patients, or adoption of risk mitigation strategies (see below).

- In some cases, sponsors may choose to explore desensitization or immune tolerance induction procedures as potential mitigation strategies. Given the risks associated with desensitization/immune tolerance induction procedures, the appropriateness of such investigations 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 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.[4]

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 appropriate FDA divisions to discuss the sponsors' proposed approach to postmarketing safety monitoring. Rare, but potentially serious, safety risks (e.g., immunogenicity) 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 particular cases, such risks may need to be evaluated through postmarketing surveillance or studies.

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 risk of an immune response. Therefore, caution is recommended when moving from one patient population to another.

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

[4] For information on proposed biosimilar products, see draft guidance titled *Scientific Considerations in Demonstrating Biosimilarity to a Reference Product* (February 2012).

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) product, but only 10 percent of immune-compromised cancer patients did so (Ragnhammar, et al. 1994). Immune suppression with agents that kill antigen-activated lymphocytes and/or elicit activity of regulatory T cells, such as methotrexate, can have a substantial effect on immunogenicity of co-administered therapeutic protein 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. Particular caution should be used in studies evaluating novel therapeutics in healthy volunteers with regard to immunogenicity and immune responses (Stebbings, et al. 2007; Li, et al. 2001).

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.

2. Prior Sensitization/History of Allergy

Prior exposure to a therapeutic protein or to a structurally similar protein may result in a sensitized patient at baseline. This is a particular concern for patients receiving factor or enzyme replacement therapy, who may have antibodies to a previous replacement product that could cross react on an analogous product.

Sensitization to the excipients or process/product related impurities of a therapeutic 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.

Recommendation

Screening for a history of relevant allergies is recommended, and the appropriateness of administration will depend on the individual 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 the intramuscular and the 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, the dose, frequency, and duration of treatment can also affect immunogenicity (Rosenberg and Worobec 2004). For example, a lower dose administered intermittently is typically more immunogenic than a larger dose administered without interruption. It should be noted that the effects of dose and frequency on immune responses to therapeutic protein products are not independent of other factors, such as route of administration, product origin, and product-related factors that influence immunogenicity (see below).

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 to support such changes are recommended.

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 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, Dickensheets, et al. 2011).

Recommendation

Evaluation of genetic factors that may modulate the immune response to a therapeutic protein product is recommended. 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 to pursue 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 (Worobec and Rosenberg 2004; Rosenberg and Worobec 2004; Rosenberg and Worobec 2005; Koren, et al. 2008; Hermeling,

et al. 2004). When a therapeutic protein is intended as a replacement for an absent or deficient endogenous protein, patients with genetic mutations conferring a "knock out" phenotype may respond to the therapeutic 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.

Recommendations

For a therapeutic protein product that is a counterpart of an endogenous protein, particularly if for first-in-human use, and for high-risk therapeutic proteins (e.g., those with endogenous protein counterparts with nonredundant functions), some understanding of the robustness of immune tolerance to that endogenous protein should be gained by the following:

- Quantitating or gathering information on the level of the endogenous protein in serum in the steady state, as well as in conditions that specifically elicit its production

- Assessing for, or gathering information on, the presence of pre-existing antibodies in healthy individuals and patient populations

- Incorporating evaluations of immunogenicity, immune cell activation, inflammatory responses, or cytokine release into relevant animal studies to obtain insight and provide guidance for clinical safety assessments (see Appendix, part E) (Koren 2002)

Consideration should also be given to the following:

- Evaluation of the genetic status (e.g., cross-reactive immunologic material or CRIM status) of patients requiring factor/enzyme replacement therapies for risk evaluation and mitigation.

- Evaluation of the extent of polymorphisms, including single nucleotide polymorphisms, in patient populations to identify potential mismatches with the therapeutic protein product.

B. Product-Specific Factors That Affect Immunogenicity

1. Product Origin (foreign or endogenous)

Immune responses to nonhuman (i.e., foreign) proteins are expected, and, as explained above, may be anticipated for some endogenous proteins. Moreover, mismatches between the sequence of the endogenous protein of the patient and that of the therapeutic protein product due to naturally occurring polymorphisms are a 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 6 below). 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. For example, during treatment with a bovine thrombin product, immune responses to bovine coagulation factor V, present in the product, led to development of antibodies that cross-reacted against human-Factor V and resulted in life-threatening bleeding in some patients (Kessler and Ortel 2009). Sponsors investigating such products should thus evaluate the risk posed by immune responses not only to the therapeutic moiety, but also to any known protein or other impurities that may be present.

Recommendation

Naturally sourced products should be evaluated for other components, protein and non-protein. A risk-based evaluation of immunogenicity of process and product related impurities should be performed and a testing program designed based on such an evaluation.

2. Primary Molecular Structure/Post Translational Modifications

Both the primary sequence and the higher-order structure of therapeutic protein products are important factors that contribute to immunogenicity. Primary sequence analysis can reveal potentially immunogenic sequence differences in proteins that are otherwise relatively conserved between humans and animals. In such cases, the 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). Primary sequence analyses may also reveal polymorphisms in relatively conserved human proteins that could lead to immune responses in patients whose endogenous protein amino acid sequence differs from that of the therapeutic protein product.

More 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 (Weber, et al. 2009; Barbosa and Celis 2007; Tatarewicz, et al. 2007; De Groot, et al. 2008). However, engineering of changes to the primary sequence to eliminate immunogenic Th cell epitopes or addition of toleragenic T cell epitopes should be done cautiously, because these modifications may alter critical product quality attributes such as propensity to aggregate, and susceptibility to deamidation and oxidation, and thus alter product stability. 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 from 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 (Dalum, et al. 1997).

Chemical modifications of therapeutic protein products such as oxidation, deamidation, aldehyde modification, and deimination may elicit immune responses by modification of primary sequence, by causing aggregate formation, or by 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 chemical degradation that may contribute to loss of activity and increased immunogenicity (Demeule, Gurny, et al. 2006; Makrygiannakis, et al. 2006; Huang, et al. 2005). 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 proteins in general and especially of therapeutic protein counterparts of endogenous proteins in view of potential polymorphisms in endogenous proteins across human populations.

For assessment of immune responses to fusion molecules, or to engineered versions of therapeutic protein products, antibody assays should be developed that enable assessment of responses to the intact protein product, as well as to each of the partner proteins separately or to novel regions. 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 withstand undesirable effects. Sponsors should consider this information in early product design and in development of improved products.

3. Quaternary Structure: Product Aggregates and Measurement of Aggregates

Protein aggregates have been recognized as potent elicitors of immune responses to therapeutic protein products for over a half-century (Gamble 1966). Mechanisms by which protein aggregates facilitate 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); enhancing antigen uptake, processing, and presentation; and triggering immunostimulatory danger signals (Seong and Matzinger 2004), thus recruiting 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).

Protein aggregates are composed either of intact native protein or of degraded or denatured protein which has lost epitopes of the normal protein. Antibodies generated by aggregates containing native protein can bind to monomeric protein as well, with the potential to inhibit or neutralize product activity. In contrast, 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). Such responses have been shown to cause anaphylaxis, but do not inhibit or neutralize activity of the native protein.

Critical information is lacking regarding the types and quantities of aggregates needed to generate immune responses for any given therapeutic protein product, although it is generally recognized that higher-molecular-weight aggregates (i.e., >100 kD) and particles are more potent in eliciting such responses than lower-molecular-weight aggregates (Bachmann, et al. 1993). 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; Wyatt Technology n.d.; Gross and Zeppezauer 2010; Roda, et al. 2009; 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. This can be done by using an appropriate cell substrate, selecting manufacturing conditions that minimize aggregate formation, employing a robust purification scheme that eliminates aggregates, and choosing a formulation and container closure that minimizes 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 these distinct species of aggregates in a product. One or more such assays should be validated for use in routine lot release, and several of them should be employed for comparability assessments. Methods include, but are not limited to the following: size exclusion chromatography, analytical ultracentrifugation (Berkowitz 2006), light scattering techniques (Wyatt Technology n.d.), Fourier transformed infrared spectroscopy (Gross and Zeppezauer 2010), and field flow fractionation (Roda, et al. 2009).

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 qualified to evaluate the content of subvisible particulates in this size range (Mahler and Jiskoot 2012). Sponsors should conduct a risk assessment of the impact of these particles on the clinical performance of the therapeutic protein product and develop a mitigation strategy 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, Martin, 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 (Lui, et al. 2011). Anti-PEG antibodies have also been found to be cross-reactive between pegylated products.

Recommendations

For proteins that are normally glycosylated, use of a cell substrate production system that glycosylates the protein in a nonimmunogenic manner and close to the normal human pattern is recommended.

For pegylated therapeutic proteins, assays for antibodies to PEG itself should be developed and implemented concomitantly with antibody assays to the therapeutic protein.

5. Impurities with Adjuvant Activity

Adjuvant activity can arise through multiple mechanisms, including the presence of microbial impurities in therapeutic protein products. These innate immune response modulating impurities (IIRMIs), including lipopolysaccharide**,** β–glucan, and flagellin, exert immune enhancing activity by binding to, and signaling through, Toll-like receptors or other pathogen recognition receptors present on B cells, dendritic cells, and other antigen presenting cell populations (Verthelyi and Wang 2010; Iwasaki and Medzhitov 2010). This signaling prompts maturation of antigen presenting cells and/or serves to directly stimulate B cell antibody production. It is very important to minimize the types and amounts of such microbial impurities in therapeutic protein products.

Recommendations

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 that are clinically relevant.

Biomarkers used to detect and compare the presence of IIRMIs should be tailored to the IIRMIs that could be present in the product.

6. Immunomodulatory Properties of the Therapeutic Protein Product

The immunologic activity of any given therapeutic protein product critically influences not only the immune response directed to it, 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 2010), 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 proteins suppress responses to themselves. For example, integrin and TNF monoclonal antibodies tend to be immunogenic. Thus, the immunogenicity of such protein therapeutics 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.

Vaccination using live attenuated organisms should be avoided when the therapeutic protein product is immunosuppressive. Updated vaccination status, compliant with local healthcare 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 protein in storage by preventing denaturation due to hydrophobic interactions, as well as chemical degradation, including truncation, oxidation, and deamidation (Cleland, Powell, et al. 1993; Shire, Shahrokh, 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 product or form protein adducts under suboptimal storage conditions (Braun and Alsenz 1997). Polysorbate, a nonionic detergent, is the most commonly used alternative to HSA because its association with proteins minimizes hydrophobic interactions. 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 causing leaching of materials with immune adjuvant activity from the container closure system. 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.

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 product-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. An appropriate risk mitigation strategy should be developed, as appropriate, following such an assessment.

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 product over a prolonged time period and thus have the potential to alter product quality. Other container closure considerations that are pertinent to immunogenicity include the following:

- Glass and air interfaces are hydrophobic surfaces that 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 (Frandkin, Carpenter, et al. 2011).

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

- 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 due to activation of a metalloprotease that co-purified with the product.

Recommendations

Sponsors should obtain a detailed description of all raw materials used in manufacture of the container closure systems for their products. Assays based on such techniques as reverse-phase high-performance liquid chromatography should be developed and used to assess the presence of leachables in therapeutic protein 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.

Testing for leachables should be performed on the product under stress conditions, 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 formulated in prefilled syringes should be tested for stability in protocols that include appropriate in-use conditions (e.g., light and temperature) to identify conditions and practices that cause product degradation.

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

A secure supply chain is critical. Cold chain security is of utmost importance in preserving product quality. For example, the custody of epoetin-α 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 ensure product quality and help minimize adverse events. Cold chain security should be ensured.

VI. CONCLUSION

Consequences of immune responses to therapeutic proteins can range from no apparent effect to serious adverse events, including life-threatening complications, such as anaphylaxis, neutralization of the effectiveness of life-saving or greatly needed 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 proteins cannot be predicted based solely on 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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VIII. APPENDIX

A. 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):

1. 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, wheezing/bronchospasm, stridor, reduced peak expiratory flow on pulmonary function testing, hypoxemia)
   - Reduced blood pressure or associated symptoms of end-organ dysfunction (e.g., hypotonia (collapse), syncope, incontinence)

2. 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, itching-flushing, swollen lips-tongue-uvula)
   - Respiratory compromise (e.g., dyspnea, wheezing/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)

3. 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% decrease in systolic blood pressure
   - Adults: systolic blood pressure of less than 90 mm Hg or greater than 30% decrease from that person's baseline

Although none of the clinical criteria provide 100% sensitivity and specificity, it is believed that these criteria are likely to capture more than 95% of cases of anaphylaxis.

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 may be useful for determining whether anaphylaxis is IgE-mediated. However, the results of unvalidated tests should be interpreted with caution and the clinical relevance of positive results may be uncertain during product development. Skin test methods should include positive and negative controls and delineate criteria for positive vs. negative skin reactions. The input of resources to develop and validate a prick and/or intradermal skin test for a respective therapeutic protein product (i.e., the

demonstration of high sensitivity and specificity) should be balanced by the utility of these tests in the confirmation of the diagnosis of anaphylaxis.

In vitro diagnostic tests that may be employed to determine the level of specific IgE antibodies directed against the therapeutic protein are the solid-phase radioallergosorbent test (RAST) and enzymatic assays (Sampson, et al. 2006). As with skin testing, application of such assays for evaluation of small molecule drugs or peptide therapeutics may be limited due to insufficient information about relevant metabolites or haptenated forms. RAST is of particular use in a number of situations: extensive skin disease, drug inhibition, and patient fear of skin testing. The presence of very high levels of nonspecific IgE can yield false positive results, whereas presence of IgG with the same specificity can yield false negative results via a 'blocking antibody' effect.

Plasma or urine histamine concentrations and total tryptase concentrations in serum or plasma may help support a clinical diagnosis of anaphylaxis and the pathophysiologic role of mast cell degranulation. However, these tests have intrinsic limitations (Simons 2009; Simons, et al. 2007; Sanz, et al. 2010). Accurate measurement of these mediators warrants careful timing of sampling and proper storage of the serum. Appropriate and meaningful interpretation of these results depends on the clinical context.

In humans, plasma histamine levels are reported to peak 5 to 15 minutes after an IgE-mediated anaphylactic episode and to return to baseline by 30 to 60 minutes. However, the accuracy of plasma histamine levels is limited by the requirement for immediate processing to prevent spontaneous basophil histamine release and the resulting artifactually elevated histamine levels that occur in unseparated blood. Urinary histamine and its metabolites are elevated for a longer period following an anaphylactic episode and, therefore, measurements of these levels may prove useful (Simons, et al. 2007; Lieberman, et al. 2010).

Similarly, tryptase levels may support the role of mast cell degranulation in an anaphylactic reaction. The majority of constitutively secreted tryptase is β-pro tryptase, an immature β tryptase, with α-tryptase contributing only a small amount. The marked increase in total tryptase observed during anaphylaxis is due to the rise in the mature β tryptase on degranulation (Lieberman, et al. 2010). Currently available tryptase assays detect both α- and β-tryptase, with a normal level below 11 ng/mL. During anaphylaxis, serum levels of β-tryptase have been reported to peak 30 to 60 minutes after the onset of symptoms and then decline, with a half-life of approximately 2 hours. The sensitivity and specificity of the assay may be enhanced if a 2-fold or greater increase in total tryptase over baseline levels is observed during the acute event. Baseline serum tryptase levels may be obtained either before the anaphylaxis event in question or 24 or more hours after resolution of clinical signs and symptoms (Shanmugam, et al. 2006). It should be noted that although an elevated total tryptase level supports the diagnosis of anaphylaxis, failure to document an elevation in total tryptase does not exclude the diagnosis even if the blood sample has been obtained within a few hours of the onset of symptoms (Simons, et al. 2007). Moreover, tryptase levels are elevated in patients with systemic mastocytosis. Therefore, mastocytosis should be excluded in the context of elevated tryptase levels during anaphylaxis (Brockow and Metcalfe 2010). Lack of correlation between

histamine and tryptase levels in anaphylaxis has been reported, with some patients exhibiting elevations of only one of these mediators (Sampson, et al. 2006).

Although only 42% of patients given the clinical diagnosis of anaphylaxis were found to have increased plasma histamine levels, and only 21% had increased plasma tryptase levels (Lin, et al. 2000), elevated mast cell mediators in the clinical setting of an anaphylactic episode strongly support the clinical diagnosis, especially if serial sampling demonstrates a significant change at the time of the inciting event when compared to baseline or post-recovery serum (Simons 2008). Other tests of immune responsiveness, such as T cell proliferation assays, are insufficiently specific to serve as indicators or predictors of anaphylaxis.

B. Cytokine Release Syndrome

Antibodies to therapeutic protein products have the potential to cross-link membrane-bound therapeutic proteins such as monoclonal antibodies (mAbs), possibly resulting in augmentation of a product's intrinsic agonist activity and exacerbation of infusion-related toxicities. In vitro assessments of 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 (e.g., receptors and products that either stimulate or demonstrate the ability to induce in vitro or in vivo cytokine release), 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, may serve as markers of a proinflammatory response.

Data from animal studies may provide information to guide development of therapeutic protein products with the potential to induce cytokine release. 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-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). Data from animal studies should be supplemented by in vitro assessments of cellular activation, including proliferation and cytokine release in human whole blood or peripheral blood mononuclear cells (Stebbings, et al. 2007; Hellwig, et al. 2008; Romer, et al. 2011). The impact of product cross-linking 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 preclinical animal studies.

C. 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 (Hunley, et al. 2004; Dharnidharka, et al. 1998; Goto, et al. 2009; Gamarra, et al. 2006). 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 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 (Hunley, et al. 2004; Dharnidharka, et al. 1998). There have been case reports of immune complex disease with immune responses to monoclonal antibodies (Goto, et al. 2009; Gamarra, et al. 2006) 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.

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

D. Antibody Responses to Therapeutic Proteins

Antibodies to therapeutic proteins are classified as either neutralizing or binding (non-neutralizing). Neutralizing antibodies bind to distinct functional domains of the therapeutic protein 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 other than specific functional domains and may exhibit a range of effects on safety and efficacy: enhanced or delayed clearance of the therapeutic protein, which may necessitate dosing changes; induction of anaphylaxis; diminished efficacy of the product by causing uptake of the therapeutic protein 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 proteins appear as foreign. However, neutralizing antibody 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 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, must be undertaken very cautiously. Neutralizing antibody to a therapeutic protein can also be catastrophic when it neutralizes the efficacy of a life-saving 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 due to 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 evaluated. 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 appears to lose efficacy, 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 life saving therapeutic protein (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 (Messinger, et al. 2012; Wang, et al. 2008; Mendelsohn, et al. 2009). Given the degree of immune suppression of such regimens,

although far less than that of a therapeutic regimen to reverse an ongoing response, careful safety monitoring should be undertaken throughout the duration of the protocol.

E. Utility Of Animal Studies

Immunogenicity assessments in animals are conducted to assist in the interpretation of animal study results and in the design of subsequent clinical and non-clinical studies (for additional information, see the Guidance to Industry ICH S6(R1): *Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals*, http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S6_R1/Step4/S6_R1_Guideline.pdf). They are generally limited in their ability to predict the *incidence* of human immune responses to a therapeutic protein, 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 (KO) 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 redundant therapeutic protein products. 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 to address multiple clinical issues, including immunogenicity.

F. Comparative Immunogenicity Studies

The need for, 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 extent 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 function or is known to provoke anaphylaxis), more extensive immunogenicity assessments will likely be needed.

Guidance on development programs for biosimilar products is available in a separate draft guidance (*Guidance for Industry on Scientific Considerations in Demonstrating Biosimilarity to a Reference Product*, February 2012).

Guidance on appropriate assay development for immunogenicity testing is available in a separate draft guidance *(Assay Development for Immunogenicity Testing of Therapeutic Proteins)*, in ICH guidance (ICH Q2A,B), and in recent publications (Koren, et al. 2008).