Guidance for Industry

Assay Development for Immunogenicity Testing of Therapeutic Proteins

DRAFT GUIDANCE

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Center for Drug Evaluation and Research (CDER)
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This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION

This guidance provides recommendations to facilitate industry's development of immune assays for assessment of the immunogenicity of therapeutic proteins during clinical trials.[2] This document includes guidance for binding assays, neutralizing assays, and confirmatory assays. While the document does not specifically discuss the development of immune assays for animal studies, the concepts discussed are relevant to the qualification and validation of immune studies for preclinical evaluation of data.

This document does not discuss the product and patient risk factors that may contribute to immune response rates (immunogenicity).

In addition, this document does not specifically discuss how results obtained from immunoassays relate to follow-on biologic therapeutic proteins. However, elements of assay validation may affect comparability determinations of immune responses. FDA 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. DISCUSSION

A. General

[1] This guidance has been prepared by the Office Biotechnology Products in the Office of Pharmaceutical Science, Center for Drug Evaluation and Research (CDER) and the Center for Biologics Evaluation and Research (CBER) at the Food and Drug Administration.

[2] This guidance does not pertain to immunogenicity assays for assessment of immune response to preventative and therapeutic vaccines for infectious disease indications.

The clinical effect of patient immune responses to therapeutic proteins has ranged from no effect at all to extreme harmful effects to patient health. The potential for such varied immune responses affect product safety and efficacy. Because this range exists, clinicians rely on the immunogenicity section of the package labeling that contains immunogenicity rates observed during clinical trials. This makes the development of valid, sensitive immune assays a key aspect of product development.

For new products, the design of such assays poses many challenges to applicants and FDA supports an evolving approach to assay development and validation during product development. Because these assays are critical when immunogenicity poses a high-risk and real time data concerning patient responses are needed, the applicant should implement preliminary validated assays early (preclinical and phase 1). Therapeutic proteins are frequently immunogenic in animals. Immunogenicity in animal models is not predictive of immunogenicity in humans. However, assessment of immunogenicity in animals may be useful to interpret nonclinical toxicology and pharmacology data. In addition, immunogenicity in animal models may reveal potential antibody related toxicities that could be monitored in clinical trials.

In other situations, FDA recommends the applicant bank patient samples so samples can be tested when suitable assays are available. FDA expects that the assays will be refined during product development and the suitability of the assays will be reassessed according to their use. For example, FDA does not require an applicant to establish interoperator precision early in clinical development if only a single operator is performing an assay. Nevertheless, at the time of license application, the applicant should provide data supporting full validation of the assays.

B. Immunogenicity Testing During Product Development

Even though different companies developing similar products employ fully validated assays to assess immunogenicity, such assays will differ in a number of parameters. These differences can make immunogenicity comparisons across products in the same class invalid. A true comparison of immunogenicity across different products in the same class can best be obtained by conducting head-to-head patient trials using a standardized assay that has equivalent sensitivity and specificity for both products. When such trials are not feasible, FDA recommends the applicant develop assays that are highly optimized for sensitivity, specificity, precision, and robustness.

FDA believes that such assays enable a true understanding of the immunogenicity, safety, and efficacy of important therapeutic protein products. The detection of antibodies is dependent on key operating parameters of the assays (e.g., sensitivity, specificity, methodology, sample handling) which vary between assays. Therefore, in the product labeling, FDA does not recommend comparing the incidence of antibody formation between products when different assays are used.

C. Principles of Immunogenicity Testing During Product Development

Multiple approaches may be appropriate for immunogenicity testing during clinical trials. However, when designing immunogenicity assays and their application to patient testing, the applicant should address the following:

- Sensitivity. The assays should have sufficient sensitivity to detect clinically relevant levels of antibodies.

- Interference. Assays results may be affected by interference from the matrix or from on-board product and this potential effect should be evaluated.

- Functional or physiological consequences. Immune responses may have multiple effects including neutralizing activity and ability to induce hypersensitivity responses, among others. Immunogenicity tests should be designed to detect such functional consequences.

- Risk based application. The risk to patients of mounting an immune response to product will vary with the product.

The applicant should provide a rationale for the immunogenicity testing paradigm. Further recommendations on assay development and validation are provided below. These recommendations are based on common issues encountered by the Agency upon review of immunogenicity submissions. In addition, other publications may also be consulted for additional insight (see section VIII, 1, 2).

III. APPROACH TO ASSAY DEVELOPMENT

A. Overview of Design Elements

1. Multi-tiered Approach

Because of the size of some clinical trials and the necessity of testing patient samples at several time-points, FDA recommends a multi-tiered approach to the testing of patient samples. In this approach, a rapid, sensitive screening assay should initially be used to assess samples. Samples testing positive in the screening assay should then be subjected to a confirmatory assay. For example, a competition assay could confirm that antibody is specifically binding to product and that the positive finding is not a result of non-specific interactions of the test serum or detection reagent with other materials in the assay milieu such as plastic or other proteins.

This approach should lead to a culling of samples that should then be tested in other assays, such as neutralizing assays, that are generally more laborious and time-consuming. Neutralizing antibodies (NAB) are generally of more concern than binding antibodies (BAB) that are not neutralizing, but both may have clinical consequences. Further, tests to assess the isotype of the antibodies and their epitope specificity may also be recommended once samples containing antibodies are identified by the screening assay.

Although results of patient sample testing are often reported as positive vs. negative, an assessment of antibody levels is informative. FDA, therefore, recommends that positive antibody responses be reported as a titer (e.g., the reciprocal of the highest dilution that gives a value equivalent to the cut point of the assay). Values may also be reported as amount of drug (in mass units) neutralized per volume serum with the caveat that these are arbitrary in vitro assay units and cannot be used to directly assess drug availability in vivo. Antibody levels reported in mass units based on interpolation of data from standard curves generated with a positive control standard antibody are generally less informative because interpretation is based on the specific control antibody.

2. Aspects of Assay Development

There are several important concepts to remember when using this multi-tiered approach to assess immunogenicity. First, the initial screening should be very sensitive. A low, but defined false positive rate is desirable because it maximizes detection of true positives. Other assays can be subsequently employed to exclude false positive results when determining the true incidence of immunogenicity.

Second, the assay should be able to detect all isotypes (particularly immunoglobulin M (IgM) and the different immunoglobulin G (IgG) isotypes).

Third, FDA recognizes that antibodies generated in patients may have varied avidities for the product, while the positive controls used to validate the assay and demonstrate data legitimacy may only represent a subset of potential avidities. Although this may be unavoidable, FDA recommends the applicant carefully consider the avidity of controls used to evaluate the assay.

A fourth consideration is how interference from biological materials (matrix, e.g., serum, plasma) will affect assay performance. The applicant should conduct assay performance tests in the same concentration of matrix as that used to assess patient samples. The applicant should also define the dilution factor that will be used for preparation of patient samples before validation studies assessing potential interference of matrix on assay results.

B. Screening Assay

1. Selection of Format

A number of different formats are available for screening assays. These include, but are not limited to, direct binding enzyme-linked immuno sorbent assay (ELISA), bridging ELISA, radioimmunoprecipitation assays (RIPA), surface plasmon resonance (SPR), Bethesda Assay (for clotting factor inhibitors, see section VIII, 3), and bridging electrochemiluminescence assays. Each assay has its advantages and disadvantages as far as rapidity of throughput, sensitivity, and availability of reagents. One of the major differences between each of these assays is the number and vigor of washes, which can have an effect on assay sensitivity. Epitope exposure is also important to consider as binding to plastic or coupling to other agents (e.g., fluorochromes) can obscure relevant antibody binding sites on the protein product in question.

2. Selection of Assay and Reagents

- Development of positive and negative controls

While many components of the screening assay may be standard (e.g., commercially available reagents) others may need to be generated specifically for the particular assay. The applicant should immunize animals (or hyperimmunize them with adjuvant) to generate a positive control. For the validation of immunogenicity assays, the positive control antibodies should be spiked into the matrix selected for routine assay performance (e.g., human serum diluted 1:10 in assay buffer). To prevent contamination of the assay matrix that could bias results, it is important to purify the positive control antibodies from the animal serum or plasma.

In addition, the applicant should carefully consider the selection of species when generating controls. For example, if an antihuman immunoglobulin reagent will be used to detect patient antibodies, the positive control and quality control samples should be detectable by that reagent (e.g., primate immune sera, humanized monoclonal). In some instances, the applicant may be able to generate a positive control antibody from patient samples. While such a reagent can be very valuable, such samples are generally not available in early trials. In addition, an applicant may not be able to generate such a reagent for therapeutic proteins with very low immunogenicity rates.

Once a source of antiproduct antibodies has been identified, the applicant should use it to assess assay validation parameters such as sensitivity, specificity, and reproducibility. FDA recommends the applicant generate and reserve specific dilutions of the sample for use as quality controls (QC). These dilutions should be representative of high, medium, and low values in the antibody assay. The applicant should use these samples for validation and patient sample testing to ensure the assay is operating within desired assay ranges at the time the assays are performed (system suitability testing).

FDA recommends the applicant establish a negative control for validation studies and patient sample testing. In this regard, a pool of sera from 5-10 non-exposed individuals can serve as a useful negative control. Importantly, the value obtained for the negative control should closely reflect the cut point determined for the assay in the patient population being tested. Negative controls that yield values far below that of the cut point may not be useful in ensuring proper assay performance.

For therapeutic monoclonal antibodies, the applicant should give special consideration to the selection of a positive control for the assay. If non-primate animals are immunized with a monoclonal antibody (mAb) containing a human immunoglobulin constant region (Fc) to develop a positive control, the antibody response is likely to be against the human Fc and not the variable region. Such a positive control may not be relevant for the anticipated immune response in human patients where the response to humanized mAb is primarily to the variable regions. Ideally, the positive control should reflect the anticipated immune response that will occur in humans.

- Detection reagent consideration

The nature of the detection agent is also critical. Reagents, such as Protein A are not optimal as they fail to detect all immunoglobulin isotypes. Although antibody bridging studies do not depend on isotype for detection, they can present specific concerns. In these assays, antigen is used to coat a surface, antibody containing samples are allowed to react with the antigen, and binding is detected by adding a labeled form of the antigen (product in question). Since multivalent binding of antiproduct antibody to the antigen on the plate can prevent binding of the detecting reagent, bridging assays are highly dependent on the product coating density and would be unable to detect lower affinity interactions. In these assays, the applicant should demonstrate that the labeling of the detection antigen does not significantly obscure critical antigenic determinants.

- Controlling nonspecific binding

Every reagent, from the plastic of the microtiter plate to developing agent, can affect assay sensitivity and non-specific binding. One of the most critical elements is the selection of the assay buffer and blocking reagents used to prevent non-specific binding to the solid surface. Since most assays require wash steps, the selection of specific detergents and concentrations is critical and should be optimized early. Similarly, the applicant should carefully consider the number of wash steps to reduce background noise, yet maintain sensitivity. A variety of proteins can be used as “blockers” in assays. However, these proteins may not all perform equivalently in specific immunoassays. For example, they may not bind well to the microtiter plate or may show unexpected cross reactivity with the detecting reagent. Therefore, the applicant may need to test several proteins to optimize results. Moreover, including uncoated wells is insufficient to control for non-specific binding. The capacity to bind to an unrelated protein may prove a better test of the binding specificity.

3. Interference and Matrix

Components in the matrix other than antibodies can interfere with assay results. Of greatest concern is the presence in the matrix of product or its endogenous counterpart. Specifically, if large quantities of product related material are present in sera/plasma, that material can prevent detection of antibodies in the test format. FDA recommends the applicant address such possibilities early (preclinical and phase 1 or early phase 2). The applicant should also examine this issue by deliberately adding known amounts of purified antibodies into assay buffer in the absence or presence of different quantities of the protein under consideration. This problem will also be minimized if the applicant collects patient samples at a time when the therapeutic protein has decayed to a level where it does not interfere with assay results. Data from pharmacokinetic studies are useful in establishing optimal sample collection times.

Other serum/plasma components may also influence assay results and it is usually necessary to dilute patient samples for testing to minimize such effects. The applicant should examine the effect of such interference by recovery studies in which positive control antibodies are spiked into serum at defined concentrations. Comparing results obtained in buffer alone with those in diluted serum can provide input on the degree of interference from matrix components and guide decisions on minimum starting dilutions recommended for sample testing.

4. Defining a Positive Result

One generally defines positive results by using a cut point (section IV, C). FDA recommends the applicant perform confirmation assays at the screening level. The applicant could also include additional titrations, antibody depletion, and antibody blockade with excess product (section V, C).

C. Neutralization Assay

1. Selection of Format

Two types of assays have been used to measure neutralizing antibody activity: cell-based biologic assays and non cell-based competitive ligand-binding assays. While competitive ligand-binding assays may be the only alternative in some situations, generally FDA considers that bioassays are more reflective of the in vivo situation and are recommended. Because the cell-based (bioactivity) assays are often based on the potency assay, historically, the format of these assays has been extremely variable. These bioassays are generally based on a cell's ability to respond to the product in question. For NAB assays, the bioassay should be related to product mechanism of action, otherwise the assay will not be informative as to the effect of NAB on clinical results.

The cellular responses potentially being measured in these bioassays are numerous and include outcomes such as phosphorylation of intracellular substrates, proliferation, calcium mobilization, and cell death. In some cases, the applicants have developed cell lines to express relevant receptors or reporter constructs. For many of these assays, there is a direct effect of neutralizing antibodies on the assay (e.g., inhibition of the cellular response). Alternatively, for monoclonal antibodies, the ability to block a response emanating from a receptor/ligand interaction may form the basis for a relevant potency assay. Therefore, the neutralizing assay may indirectly assess cell response by determining the "inhibition of inhibition." Generally, bioassays have significant variability and a limited dynamic range for their activity curves. Such problems can make development and validation of neutralization assays difficult and FDA understands such difficulties. Nonetheless, we will recommend such assays because they are critical to understanding the importance of patient immune responses to therapeutic proteins.

2. Activity Curve

The applicant should carefully consider the dose response curve (product concentration vs. activity) before examining other elements of neutralization assay validation. Assays with a small dynamic range may not prove useful for determination of neutralizing activity. Generally, the neutralization assay will employ a single concentration of product with different concentrations of antibody samples added to determine neutralizing capability. Consequently, the applicant should choose a product concentration whose activity readout is sensitive to inhibition. If the assay is performed at concentrations near the plateau of the curve; as shown in Figure 1, "No"; it may not be possible to discern neutralization. FDA recommends that the neutralization assay be performed at product concentrations that are on the linear range of the curve, as noted in Figure 1, "Yes." The assay should also give reproducible results.

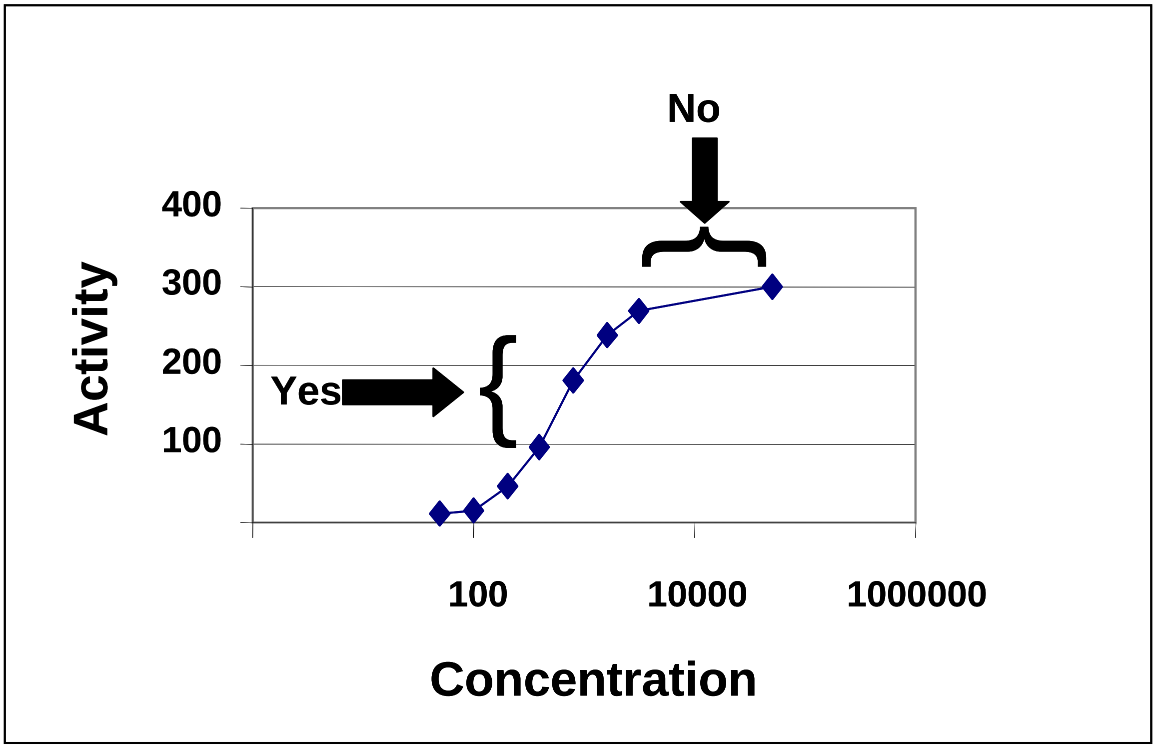

Figure 1. Activity Curve for a Representative Therapeutic Protein.

The X axis indicates a concentration of the therapeutic protein and the Y axis indicates resultant activity (e.g., concentration of cytokine secretion of a cell line upon stimulation with the therapeutic protein). The curve demonstrates a steep response to a product that plateaus at approximately 300. The "No" arrow indicates a concentration of a product that would be inappropriate to use in a single dose neutralization assay because it would represent a concentration relatively insensitive to inhibition by neutralizing antibodies. The "Yes" arrow represents an area on the linear part of the curve where the activity induced by that concentration of therapeutic protein would be sensitive to neutralization by antibody.

3. Interference

The matrix can also cause interference with neutralizing assays, particularly as sera or plasma components (apart from antibodies) may enhance or inhibit the activity of a therapeutic protein in bioactivity assays. For example, sera from patients with particular diseases may contain elevated levels of cytokines. These cytokines might serve to activate cells in the bioassay and obscure the presence of NAB. Therefore, the applicant should understand matrix effects in these assays. For some situations, approaches such as enriching antibodies from sera/plasma samples may be appropriate. However, this approach may result in the loss of antibodies. Consequently, such approaches will need to be thoroughly examined and validated by the applicant.

4. Confirmation of Neutralizing Antibodies

Because of the complexity of these biologic assays, confirmatory approaches are critical during assay development and validation and may be useful in determining whether patients have mounted a true neutralizing antibody response. The applicant should consider the following approaches:

a. As discussed above, performing antibody depletion assays to confirm the neutralizing activity is truly due to antibodies and not due to other inhibitory molecules could be useful.

b. In many instances, a cell may be responsive to multiple stimuli other than the product under study. In such cases, the presence of neutralizing antibodies can be examined in the presence of the product (which should be blocked by a specific NAB response) vs. alternative stimuli (which should not be blocked by a specific NAB response).

c. In some instances, serum may contain components that may yield false results in the NAB assay (soluble receptors, endogenous product counterpart). In such instances, adding test serum/plasma samples directly to the bioassay in the absence of product can be useful in understanding assay results.

d. Finally, confirmation of neutralizing activity may be achieved by examining neutralizing activity in the presence of additional product versus an irrelevant protein (immunocompetition). Reduced neutralization should be observed in the presence of the specific product but not with an unrelated molecule.

5. Cut Point of Neutralizing Antibodies Assays

Determination of assay cut point has historically posed a great challenge for NAB assays. Specifically, FDA recognizes the difficulty determining the degree of inhibition that is accurately indicative of neutralizing antibodies in a sample. As for all assays (see below), the determination should be statistically based and derived from assays using samples from patients not exposed to the product. If the degree of sample variation makes it difficult to assess neutralizing activity, other approaches may be considered but should be discussed with FDA before implementation. Alternatively, exploring other assay formats that lead to less variability and provide a more accurate assignment of cut point may be necessary.

6. Multiple Functional Domains

Some proteins possess multiple domains that function in different ways to mediate clinical efficacy. An immune response to one domain may inhibit a specific function while leaving others intact. In such cases, the applicant should develop several neutralization assays to truly evaluate the implication of a neutralizing antibody response.

IV. CLINICAL ASPECTS OF ASSAY VALIDATION

A. Critical Considerations and Caveats

An extremely important consideration for assay selection is whether the assay can perform adequately in the relevant clinical setting (e.g., with actual human samples representing the patient population under study). This fact is often not given adequate consideration early and leads to problems when assay validation studies are attempted. For example, patients with rheumatoid arthritis express appreciable amounts of rheumatoid factor (RF), IgM, anti-IgG. When the product under consideration possesses an immunoglobulin "tail," such as with monoclonal antibodies or Ig-fusion proteins, RF can interfere significantly with assay results. As a result, the applicant should carefully consider their ability to define reasonable assay cut points, problems with potential pre-existing antibodies, and the presence of analogous product/product-related material in the matrix early on in assay development.

B. Determining the Minimal Dilution

1. Importance

Matrix components can contribute to high assay background if undiluted, obscuring positive results. Therefore, there is almost always a need to dilute patient samples to maintain a reasonable ability to detect anti-product antibodies (sensitivity). Ideally, the minimum dilution is the dilution that yields a signal close to the signal of non-specific binding of assay diluent. However, there are exceptions where background remains high. Such a situation may necessitate careful analysis of pre-dose samples and determination of positivity as a significant increase over predose values. The applicant should carefully conduct statistical analyses that consider intersample variability to determine whether there has been a significant increase in antibody titer.

2. Approach

FDA recommends the applicant determine the minimum dilution from a panel of at least 10 samples from the untreated patient population (or healthy donors if these samples are not readily available). The minimum dilution also should involve the use of a dilution series for each of the samples. Greater numbers of samples may be recommended by FDA and will depend on the variability of the data.

3. Recommendation

While the minimum dilution ultimately selected by the applicant will depend on the assay design and patient population, FDA recommends that dilutions not exceed 1:100. Higher dilution may result in the spurious identification of a negative response when patients may actually possess low, but clinically relevant, levels of antibodies. However, we appreciate that in some instances greater initial dilutions may be required, and the overall effect on assay sensitivity and immunogenicity risk should be kept in mind.

C. Assay Cut Point

1. Definition

The cut point of the assay is the level of response of the assay at or above which a sample is defined to be positive and below which it is defined to be negative.

2. Determination

The cut point should be statistically determined by using negative control samples (e.g., samples from patients not exposed to product). A small number of samples (5-10 samples from untreated individuals) may initially be used during assay development. However, assay validation with a sample size of 50-100 is statistically more reliable for determining the variability of the assay to effectively define the cut point. By performing several runs of negative samples, the variability of the assay can be determined. It may also be necessary to determine the cut point for different populations of patients. Depending on disease states and interfering components in serum/plasma, the cut point value may vary.

When establishing the cut point, the applicant should also consider the removal of statistically determined outlier values. These values may derive from non-specific serum factors or the presence of pre-existing ("natural") antibodies in patient samples (see section VIII, 4-9). While such natural antibodies to a variety of endogenous proteins exist even in normal individuals, they can be much higher in some disease states. Using immunodepletion approaches, the applicant should identify those samples with pre-existing antibodies and remove them from the analysis. If the presence of pre-existing antibodies is a confounding factor, it may be necessary to assign positive responses or a cut point based on the difference between individual patient results before and after exposure. Through careful design consideration such as minimal dilution, removal of outliers from analyses and appreciation of the natural antibody incidence, arriving at a reasonable value to define assay cut point should be possible.

3. Recommendation

FDA recommends that the cut point have an upper negative limit of approximately 95 percent. While this value yields a 5 percent false positive rate, it improves the probability that the assay will identify all patients who developed antibodies. This sensitivity is particularly important in the initial screening assay as these results dictate the further analysis of the sample for NAB. Several approaches can be used. For example, parametric approaches using the mean plus 1.645 standard deviation (SD), where 1.645 is the 95^{th} percentile of the normal distribution may be appropriate. Other approaches include use of median and median absolute deviation value instead of mean and SD. Whatever approach is used, data must be presented to support the conclusion and the conclusion statistically justified. The specific approach employed will depend on various factors and FDA recommends that the method be discussed with FDA before implementation.

V. ASSAY VALIDATION

A. Validation of Screening Assay

1. Sensitivity

The applicant should determine the sensitivity of the assay to have confidence when reporting immunogenicity rates. A purified preparation of antibodies specific to the product should be used to determine the sensitivity of the assay so assay sensitivity can be reported in mass units/ml of matrix. Antibodies used to assess sensitivity can take the form of affinity purified polyclonal preparations, or monoclonal antibodies. FDA recognizes that the purification process may result in loss of low avidity antibodies. Therefore, the applicant should evaluate antibody avidity before and after purification as part of reagent characterization.

Assay sensitivity represents the lowest concentration at which the antibody preparation consistently produces either a positive result or a readout equal to the cut point (defined below) determined for that particular assay. As assessment of patient antibody levels will occur in the presence of biological matrix, testing of assay sensitivity should be performed with the relevant dilution of the same biological matrix (e.g., normal human serum, plasma). The final sensitivity should be expressed as mass of antibody detectable/ml of matrix. Based on data from completed clinical trials, FDA recommends that screening assays achieve a sensitivity of approximately 250 - 500 ng/ml as such antibody concentrations have been associated with clinical events.

2. Specificity

Demonstrating assay specificity is critical to the interpretation of immunogenicity assay results. This can be challenging because of the presence of product and process related impurities (such as host cell proteins) and serum factors. When the therapeutic protein represents an endogenous human protein, the applicant should asses cross reactivity with the native human protein. Similarly, when the therapeutic protein is a member of a family of homologous proteins, the applicant should assess cross reactivity with multiple family members. Demonstrating the specificity of antibody responses to monoclonal antibodies and Ig-fusion proteins poses particular challenges. The applicant should clearly demonstrate that the assay method specifically detects anti-monoclonal antibodies and not the monoclonal antibody product itself, non-specific endogenous antibodies, or antibody reagents used in the assay. Similarly, for patient populations with a high incidence of RF, the applicant should demonstrate that RF does not interfere with the detection method.

Perhaps the most straightforward approach to addressing specificity is to demonstrate that binding can be blocked by soluble or unlabeled purified product. Specifically, positive and negative control antibody samples should be incubated with the purified protein under consideration or an irrelevant protein. The reduction in response can then be determined. For responses to monoclonal antibody products, inclusion of another monoclonal with the same Fc but different variable region can be critical. If the assay is specific for the protein in question, the addition of specific soluble protein should reduce the response to background or the cut point whereas the addition of an unrelated protein of similar size and charge should have no effect. Conversely, addition of specific protein should have little effect on negative antibody control samples.

Other approaches to demonstrating specificity include the use of antibodies of irrelevant specificities to show that antibody binding is specific and not mediated by non-specific interactions with the substrate, blocking protein, or vessel. The issue of assay specificity is closely linked to the issue of assay interference from components in the matrix. Such interference can obscure the ability to detect samples that possess antibodies to the product. The presence of the drug itself or its endogenous counterpart in the matrix has the greatest potential to interfere with results.

The potential for interference by the drug present in the serum should be assessed by testing the effect of various concentrations of study drug on the high, medium, and low QC positive controls. Therefore, the applicant should dilute antibody samples with varying concentrations of drug to assess how much drug is required to eliminate or reduce detection in the assay. There should be a relationship between the quantity of antibody and amount of drug required for a specified degree of inhibition (e.g., the high positive control should be inhibited less by a given concentration of product than the low positive control). Further discussion on this important aspect of antibody testing is addressed below.

3. Precision

Demonstrating assay reproducibility (precision) is critical to the assessment of immunogenicity. This determination is particularly important when assessing changes in immunogenicity following changes in product manufacture, because such changes might only subtly alter immune response. The applicant should evaluate both intra-assay (repeatability) and inter-assay (intermediate precision) variability of assay responses. FDA recommends that inter-assay precision be evaluated on a minimum of three different days with a minimum of three replicates of the same sample in each assay. Intra-assay precision should be evaluated with a minimum of six replicates per plate. Samples should include negative controls and positive samples whose testing yields values in the low, medium and high levels of the assay dynamic range. The applicant should evaluate inter-operator precision when more than one operator will be running the assay.

FDA acknowledges that samples with a low concentration of antibodies are likely to have a higher variability than samples with high antibody concentrations. Nonetheless this determination for low concentration samples will be important for understanding patient samples that may truly possess low levels or low avidity antibodies vs. those that yield false positive results. Positional effects (e.g., location on the microtiter plate) are a major contributor to assay variability and the applicant should evaluate such effects in the course of evaluating assay precision.

4. Robustness and Sample Stability

The applicant should assess robustness as an indication of the assay's reliability during normal usage by examining the impact of small but deliberate changes in method parameters. For example, changes in temperature, pH, buffer, or incubation times can all impact results. FDA recommends storing patient samples in a manner that preserves antibody reactivity at the time of testing. Freezing and thawing patient samples may also affect assay results and those assay results should be evaluated. In addition, the applicant should examine other parameters affecting patient samples such as state of hemolysis and specific anticoagulants. Other considerations may include state of lipemia, presence of bilirubin, and presence of concomitant medications that a patient population may be using. The applicant should examine robustness during the development phase and if small changes in specific steps in the assay affect results, specific precautions should be taken to control their variability.

B. Validation of Neutralizing Assay

1. Sensitivity

The approach to demonstrating the sensitivity of the neutralization assay is similar to that of the binding assay. The applicant should report the sensitivity in mass units. FDA recognizes that not all anti-product antibodies are neutralizing and it can be difficult to identify positive control antibodies with neutralizing capacity. Nonetheless, such reagents are critical for demonstrating assay sensitivity.

The concentration of product employed in the neutralizing assay is also critical as discussed above. FDA recommends that the concentration of product used be on the linear region of the dose response curve for the product. FDA recognizes that while the use of low concentrations of product may lead to a neutralizing assay that is more sensitive to inhibition by antibodies, very low concentration of product may result in poor precision of the assay. Another feature of neutralizing assays is that they are often less sensitive than binding assay. While this limitation is noted, sponsors are encouraged to develop the most sensitive assays possible.

2. Specificity

Applicants should demonstrate assay specificity for cell based neutralizing assays. As mentioned above, for cells that may be responsive to stimuli other than the specific therapeutic protein, the ability to demonstrate that NAB only inhibit the response to product and not to other stimuli is a good indication of assay specificity. In such studies FDA recommends that the other stimuli be employed at a concentration that yields an outcome similar to that of the therapeutic protein. The applicant should also confirm the absence of alternative stimuli in patient serum.

3. Precision

Assay precision can also be more problematic for neutralizing assays than binding assays. Biologic responses can be inherently more variable than carefully controlled binding studies. Consequently, the applicant should perform more replicates for assessment of precision and assessment of patient responses than for the screening assay.

4. Other Elements of Neutralizing Assay Validation

The applicant should validate both specificity and robustness of the neutralizing assay during development. Approaches such as those described above for confirmatory approaches for neutralizing assays can support the specificity of the assay during validation. Many elements of assay validation of NAB are similar to those used for validation of the screening assay. The complexity of bioassays makes them particularly susceptible to changes in assay conditions and it is essential to control parameters such as cell passage number, incubation times, and media components.

C. Validation of Immunodepletion/Competitive Confirmatory Assay

While immunodepletion/competition assays are employed to confirm results of neutralizing assays, they are most often employed as an adjunct to antibody binding assays. While confirmatory assays need to be fully validated in a manner similar to binding and neutralizing assays (above), these assays raise some specific issues. In these assays, antibodies are specifically removed or competed[3] from patient samples and the loss of response is determined. The most difficult issue is identifying the degree of inhibition or depletion that will be used to ascribe positivity to a sample. In the past, 50 percent inhibition has been used, but this number is arbitrary and is unlikely to be relevant for all assays. FDA recommends that sponsors carefully address this issue during assay development and base determinations on meaningful data. In this regard, examining percent inhibition of QC samples (high, medium, and low) before and after immunodepletion/competition with specific vs. irrelevant proteins can help to identify meaningful values.

VI. IMPLEMENTATION OF ASSAY TESTING

A. Obtaining Patient Samples

FDA recommends the applicant obtain pre-exposure samples from all patients. The potential for pre-existing antibodies or confounding components in the matrix make it essential for one to understand the degree of reactivity before treatment. The applicant should then obtain subsequent samples, and the timing will depend on the frequency of dosing. Optimally, samples taken 7-14 days after exposure can help elucidate an early IgM predominant response. Samples taken at 4 to 6 weeks following exposure are generally optimal for determining IgG responses. For individuals receiving a single dose of product, the above time may be adequate. However, for patients receiving product at multiple times during the trial, the applicant should obtain samples at appropriate intervals throughout the trial.

The timing for obtaining these samples may be complicated and FDA recommends the applicant coordinate the sampling visits with visits to assess other aspects of the clinical trial. However, obtaining samples is essential and the applicant should obtain samples at a time when there will be minimal interference from product present in the serum. An applicant should consider the

[3] "Competed" refers to a competition assay where the ability of antigen specific antibodies to bind to either labeled or plate bound antigen is inhibited by unlabeled or soluble antigen.

product's half-life to help determine appropriate times for sampling. This is especially important for monoclonal antibody products because these products can have half-lives of several weeks or more and, depending on the dosing regimen, the therapeutic monoclonal antibody itself could remain present in the serum for months.

The level of product that interferes with the assay, as determined by immune competition, may also help define meaningful time points for sampling. If drug-free samples cannot be obtained during the treatment phase or the trial, then the applicant should take additional samples after an appropriate washout period (e.g., five drug half-lives). Obtaining samples to test for meaningful antibody results can also be complicated if the product in question is itself an immune suppressant. In such instances, the applicant should obtain samples from patients who have undergone a washout period either because the treatment phase has ended or because the patient has dropped out of the study.

B. Concurrent Positive and Negative Quality Controls

If the applicant completes the proper validation work and makes the cut point determinations, the immunogenicity status of patients should be straightforward to determine. However, FDA believes positive control or QC samples are critical and should be run concurrently with patient samples. We recommend that these samples span a level of positivity with QC samples having a known negative, low, medium, and high reactivity in the assay. More importantly, the samples should be diluted in the matrix in which patient samples will be examined (e.g., same percent serum/plasma). In this way, the applicant ensures that the assay is performing to its required degree of accuracy and that patient samples are correctly evaluated. For the low positive sample, we recommend that a concentration be selected that, upon statistical analysis, would lead to the rejection of an assay run 1 percent of the time. Such an approach would ensure the appropriate sensitivity of the assay when performed on actual patient samples.

FDA also recommends that these QC samples be obtained from humans or animals possessing antibodies that are detected by the secondary detecting reagent, to ensure that negative results that might be observed are truly due to lack of antigen reactivity and not due to failure of the secondary reagent. This issue is not a problem for antigen bridging studies (as labeled antigen is used for detection), although other considerations may apply in these assays.

C. Cut Point Normalization

FDA appreciates that there will be some degree of variability in an assay. Consequently, FDA recommends the applicant develop a predetermined method for normalization of data obtained at different times. During assay validation, the applicant should identify a negative or low QC sample and determine a normalization factor. The normalization factor is the difference in the readout value of the control and the value of the 95 percent limit obtained for the initial cut point determinations. The normalization factor can be added to the value obtained for the negative QC sample to normalize for the cut point of the assay performed at different times. Other approaches may also be appropriate such as normalizing all values against those obtained with a negative control sample or in extreme cases establishing plate specific cut points.

D. Reporting Patient Results

As discussed, unless a universally accepted and accessible source of validated antibody is available as a control and parallelism between the dilution curves of the control antibody and patient samples has been demonstrated, FDA believes it is neither necessary, nor desirable for the applicant to report patient antibody results in terms of mass units. Reporting in terms of titers (e.g., reciprocal of the dilution able to yield a background just at or above the cut point) is more appropriate and is well understood by the medical community. We believe attempts to convert such data into mass units by using standard curves or other data conversion methods are generally confusing and inaccurate.

E. Pre-existing Antibodies

The ability to test patient samples for antiproduct antibodies can serve as a critical safety assessment throughout clinical trial development. Early hints about risks of immunogenicity may be obtained from the measurement of pre-existing or natural antibodies. A growing body of evidence in the medical literature suggests that B cells and T cells with specificity for a number of self proteins exist naturally and may even be heightened in some disease states. For example, antibodies to IFN can be found in normal individuals (see section VIII, 7-9). Less surprisingly, pre-existing antibodies to foreign antigens, such as bacterial products, have also been found in normal individuals, possibly as a result of previous exposure to the organism or cross reactivity.

F. Specific Considerations

1. Monoclonal Antibodies

Some special considerations pertain to the detection of antibodies against monoclonal antibody therapeutics and in vivo diagnostics. Animal-derived monoclonal antibodies, particularly those of rodent origin, are expected to be immunogenic with the immune response directed primarily against the Fc portion of the molecule. In the early days of the therapeutic mAb industry, this was a primary reason for the failure of clinical trials.

Technologies reducing the presence of non-human sequences in monoclonal antibodies (chimerization and humanization) have led to a dramatic reduction but not elimination of immunogenicity. In these cases, the immune responses are directed largely against the variable regions of the monoclonal antibody. As immune responses against the variable regions of fully human monoclonal antibody are also anticipated, FDA does not expect that the use of fully human monoclonal antibodies will further reduce immunogenicity by a significant margin. Many of these concerns also pertain to Fc fusion proteins containing a human Fc region.

2. Rheumatoid Factor

Measuring immune responses to products that possess immunoglobulin tails (monoclonal antibodies, Fc fusion proteins) is particularly difficult when RF is present in serum/plasma. RF is generally an IgM antibody that recognizes IgG (although other Ig specificities have been noted). Consequently, RF will bind Ig regions, making it appear that specific antibody to the product exists. Several approaches for minimizing interference from RF have proven useful, including treatment with aspartame (see section VIII, 10) and careful optimization of reagent concentrations so as to reduce background binding. FDA recommends examining immune responses to Fc fusion proteins in clinical settings where RF is present to develop an antigenic moiety that corresponds to the non-Fc region of the molecule and assess whether patient serum binds the truncated product. For example, for a cytokine-Fc fusion protein, measuring antibody responses to the purified cytokine can help in assessing the specific immunogenicity of the fusion protein.

3. Fusion Proteins

Examination of immune responses to fusion proteins can be challenging and may require development of multiple assays to measure immune responses to both domains of the molecules as well as to the neoantigen formed at the junction of the components.

4. High Levels of Endogenous Protein in Sera

If serum/plasma possess high levels of protein that are analogous to the product under study, developing traditional antibody binding assays to measure relevant antibodies can be particularly challenging. For example, studies looking at immune response to albumin can be confounded by large quantities of serum albumin. In these instances, other approaches for measuring immunogenicity may be warranted, such as enzyme-linked immunosorbent spot (ELISPOT) or plaque type assays, to measure numbers of antigen-specific antibody secreting cells.

VII. OTHER ASPECTS OF IMMUNOGENICITY TESTING

A. Isotypes

While the initial screening assay should be able to detect all isotypes, in some circumstances the applicant should develop assays that discriminate between antibodies of specific isotypes. For example, for products that induce allergic responses, assays that can specifically measure levels of IgE may be important for helping predict and prepare for anaphylactic reactions in the clinic. In addition, the generation of immunoglobulin G4 (IgG4) antibodies has been associated with immune responses generated under conditions of chronic antigen exposure such as with factor VIII treatment. IgG4 antibodies have also been shown to be less pathogenic as they fail to fix complement and are associated with blocking of allergic responses (section VIII, 11). Consequently, determining if antibody responses occurring upon prolonged exposure to therapeutic proteins are associated with this isotype may be useful.

B. Epitope Specificity

FDA recommends the applicant direct initial screening tests against the whole molecule and its endogenous counterpart. However, for product development, the applicant should investigate the regions or “epitopes” to which immune responses are specifically generated. This determination may be particularly important with fusion molecules in which two proteins are genetically or physically fused. In these circumstances, the region where the two molecules join may form a neoantigen and immune responses to this region may arise. Because of epitope spreading, immune responses to other parts of the molecule may ensue, leading to generation of neutralizing antibodies to the product or its endogenous counterpart. For these products, FDA encourages sponsors to investigate the initiating event in the immune cascade. This knowledge may allow for modification to the protein to reduce its potential immunogenicity.

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