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Immunogenicity Issues in Drug Development

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Immunogenicity is an important factor that manufacturers must consider as they develop new protein therapeutics. It is important to understand the immunogenicity of new proteins both at the preclinical phase and in the clinical phase of development. This paper provides an overview of the issues that manufacturers should consider including some of the potential reasons that some proteins induce an immune response, a discussion regarding current methodology used to understand immunogenicity, and some examples of marketed protein therapeutics with immunogenicity issues. Given the increasing scrutiny from regulatory agencies around the way immunogenicity is assessed by manufacturers, the strategy of detecting and characterizing antibodies that are formed against protein therapeutics is becoming an important topic. Screening assays are typically performed first on all serum samples collected in the course of a trial to detect the presence of antibodies that can bind to the protein therapeutic. There are several platforms in use: radioimmune precipitation assays (RIP), enzyme linked immunosorbent assays (ELISA), electrochemiluminescent assays (ECL), and biosensor-based assays. Each has its advantages and disadvantages, and needs to be evaluated to identify the optimal platform for a specific therapeutic protein. Once antibodies are identified, a confirmatory assay is performed to verify and characterize the antibodies. A biological assay should be used next to test if these antibodies are capable of neutralizing the biological effect of the drug. Any sample that is positive for neutralizing antibodies, indicates that the antibody is probably having an impact on the patient's ability to derive full benefit from the therapeutic protein, and may be critical for patient safety.

Keywords Biacore, ELISA, immunogenicity, neutralizing antibody

INTRODUCTION

One of the challenges facing manufacturers of protein therapeutics is the potential that the recipient of that therapeutic may develop an immune response against the drug (Porter, 2001). Patients have been shown to raise antibodies against both proteins with non-native sequences (Thistlethwaite et al., 1988) and proteins purified from human blood products (Rosenberg et al., 1999). The consequences of developing antibodies can range from having no observable clinical effect (Cook-Bruns, 2001; Holmes et al., 2002), to having significant clinical consequences for the patient through neutralizing both the therapeutic protein and also the endogenous counterpart (Casadevall et al., 2002). One of the factors that determines if there is a clinical consequence of antibody formation is the biologic redundancy that exists for the functionality of the protein. That is, if there are several proteins capable of performing the biological function of the therapeutic, there is less risk that the biological function will not be performed. Other factors effecting clinical implications of an immune response are related to the magnitude of that immune response. There have been documented cases where different manufacturers of a therapeutic protein report very different rates of immunogenicity (Hanley, 1998; Lindsay et al., 2001). Some of the reasons for, and consequences of, an immune response are discussed herein as well as some of the issues surrounding the monitoring and understanding of the immune response. Since a complete understanding of immunogenicity of any given protein therapeutic requires an understanding of the methodology used, a review of the available technology and testing strategy is also provided.

CAUSES OF IMMUNOGENICITY

There have been several reported factors that can contribute to the immunogenicity of a therapeutic protein. Of importance in the list of possible risk factors for immunogenicity are the amino acid sequence differences between the therapeutic protein and the endogenous counterpart. Amino acid sequence differences can lead to an immune response either by direct recognition as a foreign entity if the different amino acids are on the surface of the molecule, or indirectly by altering the conformation of the molecule and exposing new epitopes. Either scenario can result in the immune system viewing the protein therapeutic as a foreign entity and triggering an immune response. One of the ways that a protein can induce an immune response is to have T-cell epitopes present. A T-cell epitope is a short amino acid sequence that is capable of reacting with specific T cells to stimulate them to produce cytokines that result in B-cell proliferation and production of specific antibodies.

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Abbreviations: ECL: electrochemiluminescent assays; ELISA: enzyme linked immunosorbent assays; RIP: radioimmune precipitation assays.

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In addition to sequence differences, there are a number of structural alterations that can result in triggering an immune response. In these cases, the changes allow the immune system to break the tolerance that exists for the endogenous counterpart and respond through production of antibodies. For proteins that mimic endogenous molecules, the immune system is normally tolerant to that protein and despite exposure, does not trigger an immune response. When there is a change to the molecule, the immune system now breaks through the tolerance and produces antibodies that are capable of interacting with the therapeutic as well as the endogenous molecule (Schellekens, 2003). Aggregation of proteins facilitates recognition by the immune system, which can lead to antibody production. Other structural modifications that have potential to increase immunogenicity include oxidation, deamidation, degradation, and conformational changes. Depending on the inherent stability of the molecule, certain storage conditions can lead to a structural change that could trigger an immune response. The inherent stability of the protein therapeutic is also influenced by its formulation as suboptimal formulations can lead to a more fragile protein. The production and purification of a protein can provide stresses that could have a negative impact on the protein's stability. These could either influence the protein directly or exert influence through some impurity or co-factor.

POTENTIAL CONSEQUENCES OF IMMUNOGENICITY

When an immune response occurs in a patient, there are several possible scenarios for the impact on the patient. In the majority of cases, there is no clinical effect determined. In some cases, the antibody may affect the pharmacokinetics of the therapeutic protein and cause the drug to be cleared either more rapidly or more slowly. Rapid clearance would decrease the efficacy of the drug as the drug would have less time to exert a biological effect. The decreased clearance observed with a sustaining antibody might have some benefit to the patient by keeping the drug in circulation longer, although the drug might not be active when bound to an antibody. The antibodies also might bind to the active region of the therapeutic protein and neutralize the biological effect of that protein. There could be a hypersensitivity reaction that is mediated by the antibodies. In the worst-case scenario for protein therapeutics that have a non-redundant endogenous counterpart, the antibodies would bind and neutralize not only the therapeutic protein but also the endogenous protein. When an endogenous protein is neutralized, the effect on the patient can be severe if the protein is an integral component of a non-redundant biological pathway such as erythropoietin is in erythropoiesis.

There are several components to the immune response that will determine whether or not there are clinical consequences for the patient. The amount of antibody that is produced, and whether the response is transient or prolonged, are both important risk factors. A sustained and robust immune response where high levels of antibody are produced is more likely to have an impact on the patient than a transient response where little antibody is produced. When helper T cells interact with the B cells and trigger maturation of the immune response, the consequences for the patient become more significant. A mature immune response is characterized by a switch from IgM to IgG antibodies being produced, an increase in the affinity for their target of the antibodies produced, and epitope spreading. Epitope spreading allows more regions of the target protein to be recognized by antibodies. In contrast to an immune response that does not generate T-cell help, the antibodies are present in higher concentrations, bind to their target with greater affinity, and can result in neutralizing antibodies. Another important risk factor that determines clinical relevance of an immune response is whether the antibodies are able to neutralize or block the biological effect of the drug. By assessing these qualities of the immune response, it can be determined how likely it is that the antibodies identified in a patient will cause clinical consequences.

One of the major consequences of anti-drug responses are hypersensitivity reactions that can be mediated by IgE or IgG via complement activation following formation of IgG immune complexes. The consequences of a hypersensitivity reaction can be quite severe (Porter, 2001).

ANALYTICAL METHODS FOR ASSESSING IMMUNOGENICITY

There are several platforms that have been established to detect the presence of antibodies against therapeutic proteins. Whatever assay is used, it is important that the characteristics of that assay be well understood. The assay should be evaluated for its sensitivity for detecting antibodies as well as its specificity. If the parameters of the assay are not well understood through validation, it is difficult to correctly interpret the data that comes from the assay. A publication by the AAPS Ligand Binding Assay Bioanalytical Focus Group describes industry recommendations for the design of these antibody assays (Mire-Sluis et al., 2004).

Screening immunoassays are generally used as the first test to detect antibodies. These immunoassays have the advantage of high throughput and rapid sample analysis time. The information provided by the screening immunoassay is very basic and is limited to whether there is a signal in the serum sample that is different from what is observed as background when a sample known not to contain antibody is tested. There are several platforms available for screening assays including radioimmune precipitation assays (RIP), enzyme linked immunosorbent assays (ELISA), electrochemiluminescent assays (ECL), and biosensor-based assays that use instruments such as the Biacore. None of these platforms are perfect, and each has advantages and disadvantages. When developing a screening immunoassay, it is beneficial to try different platforms and identify which is optimal for that therapeutic protein.

The steps for an RIP assay are shown in Figure 1. The advantages of this platform include that it is a liquid phase assay that

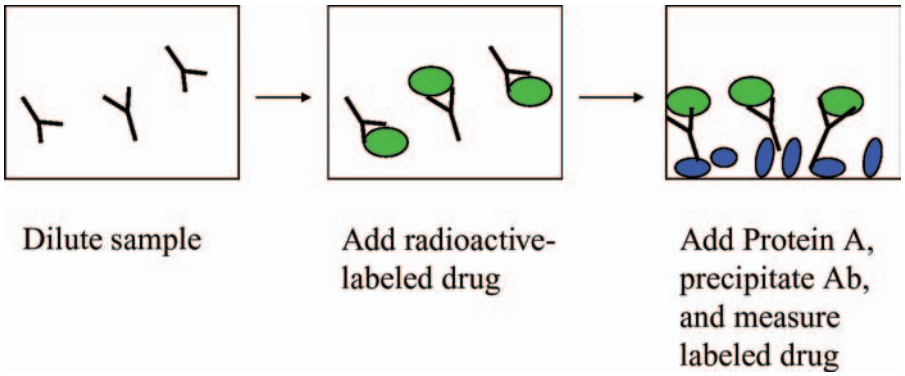

FIG. 1. Radioimmune precipitation assay. In a radioimmune precipitation assay, the sample is diluted, radiolabeled drug is added, and the resulting antibody-radiolabeled drug complexes are precipitated using protein A, protein G, or a combination. The radioactivity in the resulting pellet is proportional to the amount of antibody in the serum sample capable of binding the radiolabeled drug.

allows the antibodies to bind to a soluble form of the antigen (soluble therapeutic protein). This is ideal as it better models how the antibodies interact with the protein *in vivo*. Most of these RIP assays are very sensitive and allow detection of antibodies in the low ng/ml range. Despite these strong advantages, most of the industry is moving away from this type of assay due to two strong disadvantages. When precipitating the antibody-therapeutic protein complexes from the serum sample, the most common approach is to use protein A with or without combination with protein G.

Unfortunately, these precipitating agents are not very efficient at removing IgM antibodies from samples as reflected in the Pierce product information showing weak binding for protein A and protein G to human IgM (Pierce Biotechnology, Inc.). The weak binding to IgM translates to the possibility that not all product-specific IgM antibodies would be captured which could result in false negative results. In drug development, it is critical to be able to detect the early stages of an immune response, which translates into a requirement to detect IgM antibodies that can bind to the therapeutic protein. A second disadvantage is the additional burden of working with, and disposing of, the radioactivity used for this method. A third area of concern centers around the chance that the precipitation step will non-specifically trap labeled drug resulting in a false positive sample.

The ELISA shown in Figure 2 represents three forms of this method: the indirect, direct, and bridging methods. In the indirect

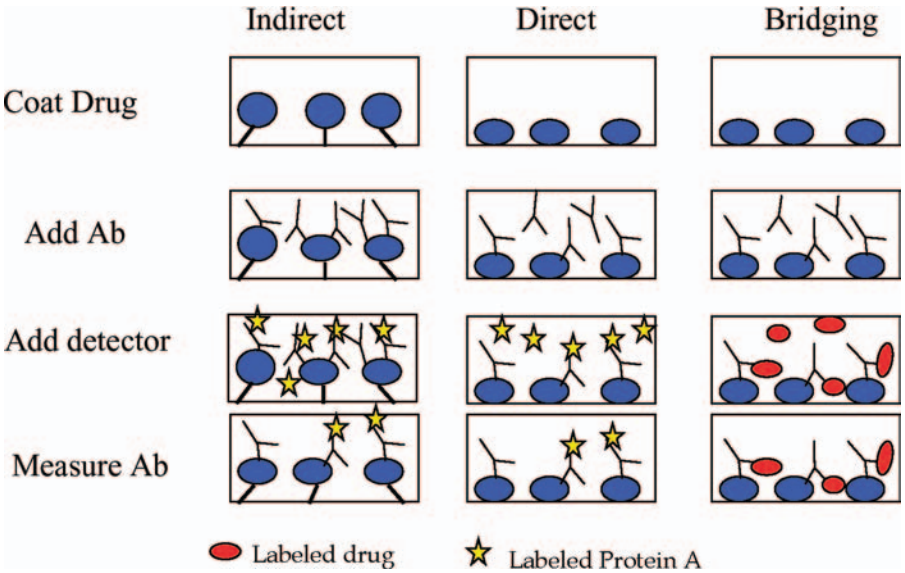

FIG. 2. ELISA platforms. Three forms of enzyme-linked immunosorbent assay (ELISA) are shown. The indirect form attaches the drug to the plate through some agent, and both the direct and bridging forms have the drug directly immobilized onto the microtiter plate. The bridging form takes advantage of antibody bi-valency (multi-valency for IgM) and detects the antibody that binds to the immobilized drug with a labeled form of the drug. The indirect and direct forms utilize agents such as labeled antispecies specific immunoglobulin or protein A to detect the captured antibody. The final step relies on a colorometric reaction where the resulting color intensity is proportional to the amount of antibody bound.

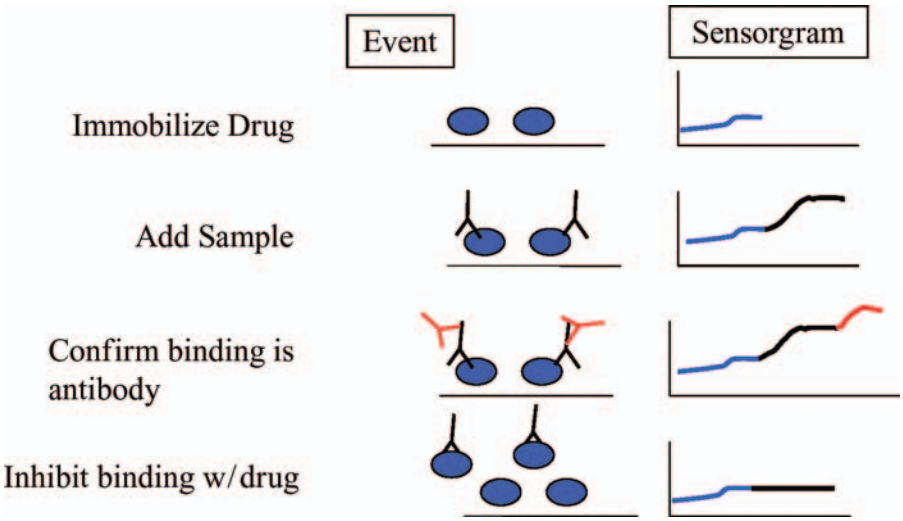

FIG. 3. Biacore assay. The steps for a Biacore assay are shown. The drug is covalently immobilized onto the sensorchip surface, sample is added, and the resulting binding is confirmed through addition of anti-species-specific immunoglobulin. At each step, the resulting binding is verified through inspection of the sensorgram. To test for specificity, the surface is regenerated and the sample is reapplied after preincubation with excess soluble drug. Inhibition by soluble drug confirms the specificity of the original binding. Note: IgM molecules are very difficult to completely inhibit.

method, the therapeutic protein is captured onto the microtiter plate with some agent whereas in both the direct and the bridging forms, the therapeutic protein is attached directly to the plate. Both the direct and indirect methods detect bound antibody with a labeled reagent such as anti-human immunoglobulin or protein A, while the bridging form uses labeled drug as the detecting reagent. All of the ELISAs share the advantage of very good sensitivity and the potential for automation and high throughput. ELISAs have been the most commonly used assay for detecting anti-therapeutic antibodies. In recent years, data has been shown that suggests ELISA platforms may be deficient in their ability to detect rapidly dissociating antibodies (Swanson et al., 1999). Initially, this was not thought to be of great concern because the perception was that these rapidly dissociating antibodies would be of no clinical significance. There are now data in the literature suggesting that these rapidly dissociating antibodies can neutralize the biological effect of the drug, which makes them potentially very clinically relevant (Swanson et al., 2004). It is important for developers of ELISAs to test the ability of their assay to detect these rapidly dissociating antibodies, sometimes referred to as low affinity antibodies. The bridging ELISAs are most prone to missing the rapidly dissociating antibodies because they rely on both arms of the antibody to remain simultaneously bound to the drug, one arm to the immobilized drug and the other to the labeled form, to allow detection.

ECL assays are similar to ELISAs but utilize electrochemiluminescent detection rather than colorimetric detection. This provides a greater dynamic range and generally greater sensitivity than ELISAs. One concern with ECL assays is that there can be a hook effect, especially in liquid phase assays, that causes a decrease in signal as the antibody concentration increases. In assays with a hook effect, increasing concentrations of the ligand (in this case an antibody) cause a plateau in the signal, but instead of the plateau remaining, the signal then begins to go downward with further increases in the ligand. The resulting curve resembles a hook. The danger is that a sample containing very high concentrations of antibody could score negative on the test if the hook of the curve reaches below baseline. To avoid misinterpretations of results due to a hook effect, multiple dilutions of the sample should be analyzed.

A fourth category of immunoassays includes biosensor-based assays that use instruments such as the Biacore. Figure 3 shows the steps from a typical Biacore assay. A sensorgram from a Biacore assay is shown in Figure 4. Some of the advantages of this platform are that the data from the binding interaction are all captured in real time; that there is typically no need for an amplification step; that both low and high affinity antibodies can be readily detected; and that it is very straightforward to confirm that observed binding is a result of immunoglobulin binding. Briefly, the instrument allows immobilization of up to four independent ligands onto a carboxymethyl dextran surface using covalent binding (Mason et al., 2003).

A diluted serum sample is then allowed to bind to the immobilized surface(s) and the resulting sensorgram displays any resulting binding. This platform is better at detecting rapidly dissociating (low affinity) antibodies because they are monitored for binding as the binding event occurs as shown in Figure 5. This contrasts with the other platforms where there are multiple wash and incubation steps before the detection phase of the assay. These wash and incubations allow some of the bound antibody to dissociate from the drug and thus avoid detection. If enough of the initially bound antibodies dissociate away from the surface and are undetected, a sample that contains antibodies might falsely be considered negative (Swanson

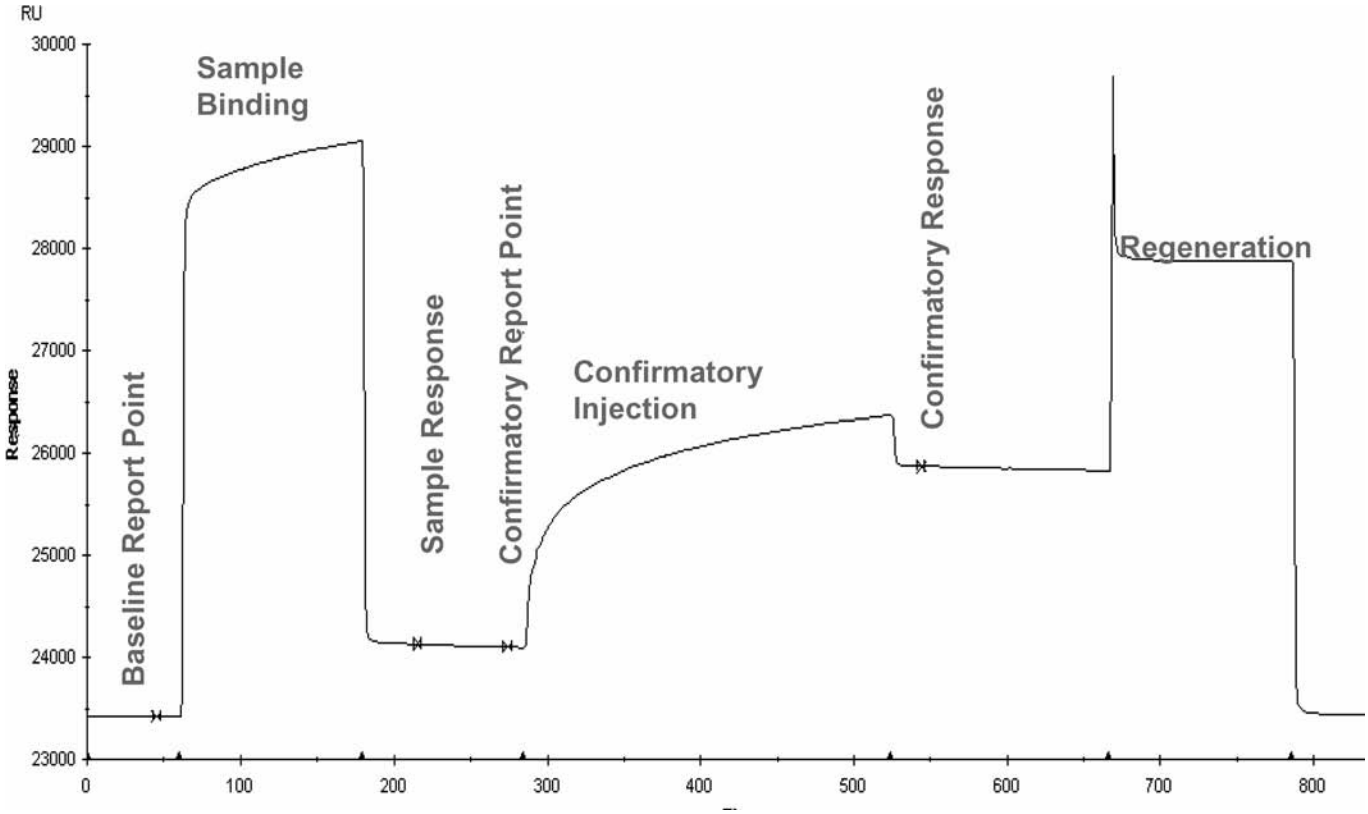

FIG. 4. Biacore sensorgram. A typical Biacore sensorgram is shown with labeled report points.

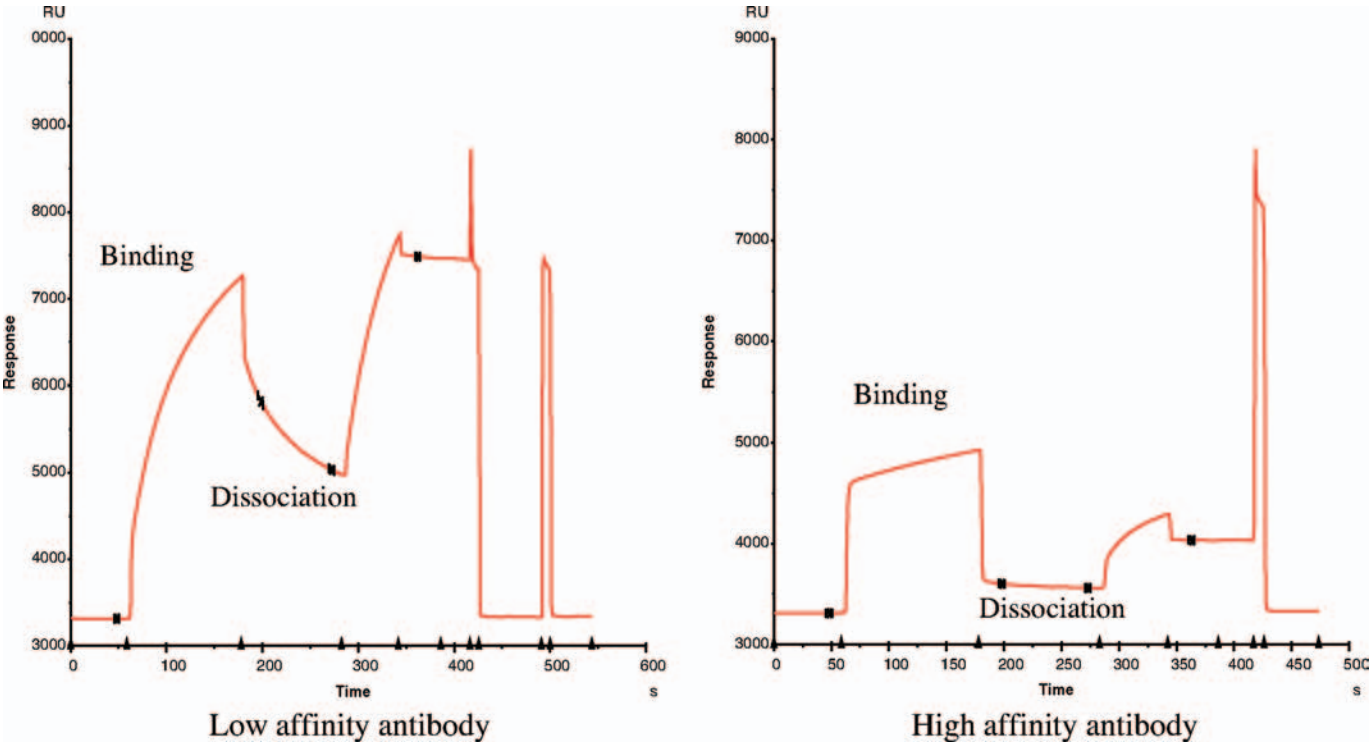

FIG. 5. High and low affinity antibodies. Biacore sensorgrams from low affinity (rapidly dissociating) and high affinity (slow dissociating) antibodies are shown. The rapid dissociation of some antibodies is a characteristic that makes these antibodies very difficult to measure using more traditional assay platforms including ELISA.

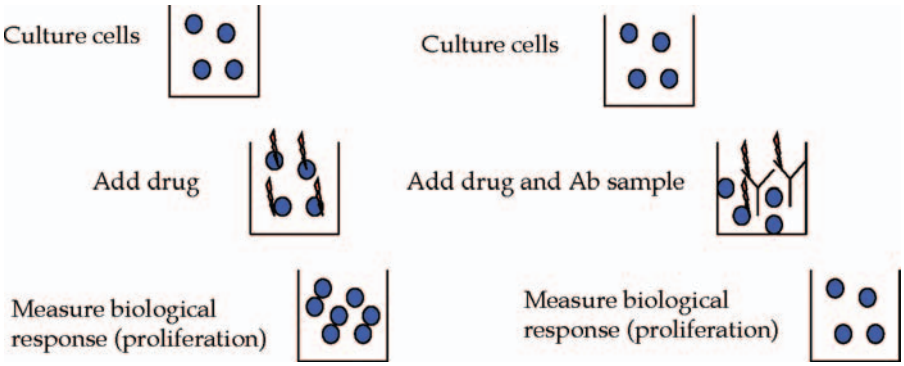

FIG. 6. Biological assay. The steps for a typical biological assay for neutralizing antibodies are shown. Cells are cultured, the protein therapeutic is added, and the biological response from the cells is measured. This response could include proliferation, cytokine release, mRNA production, or some other measurable activity. A duplicate set of cells is also used where the serum sample to be tested for neutralizing antibodies is added in conjunction with the protein therapeutic. The resulting measured biological activity is measured and compared to the cells where the negative control serum sample was added. A reduction in measured biological response indicates neutralization has occurred.

et al., 2002). Another advantage of the Biacore assay is that it allows for a thorough characterization of any antibodies that are detected. This characterization can include concentration, isotype, relative affinity, and epitope determination (Swanson, 2005).

Disadvantages of the Biacore-based assays include the high cost of the instrumentation, the need for a dedicated analyst to use the instrument, the relatively low throughput that the instrument supports, and typically less-than-average assay sensitivity. Once antibodies are identified by a screening immunoassay, a confirmatory immunoassay can be used to verify the screening assay result. This confirmatory test can be performed in several ways. A second assay platform can be used to verify that the screening test was measuring antibodies specifically bound to the therapeutic protein. Another approach is to treat the serum sample with an agent to remove all immunoglobulin and re-test in the screening assay.

With this example, a sample that originally scored positive and then scores negative when immunoglobulins are removed, would be considered positive for antibodies. In contrast, if the sample still scored positive after immunoglobulin removal, the original binding that remained after removal of immunoglobulin would be considered non-specific and the sample would be considered negative. A third approach for confirmatory testing involves adding excess soluble drug to the sample followed by re-analysis in the screening assay. In this case, if the original test was positive for binding and the sample is negative in the presence of excess soluble drug, the sample is considered positive for antibodies against the therapeutic protein because the soluble drug competed with the immobilized drug. If the original sample is positive for binding and remains positive after addition of excess soluble drug, the sample is considered negative because the original binding could not be blocked with the excess soluble drug as would be expected with a true antibody-antigen interaction. The one caveat to this interpretation is that if the antibody being detected is an IgM, it may be very difficult if not impossible to fully inhibit this binding by the addition of excess soluble drug.

A sample that tests positive by immunoassay for antitherapeutic antibodies should next be tested in a biological assay to determine if these antibodies are capable of neutralizing the biological effect of the drug. A typical example of a biological assay for neutralizing antibodies is shown in Figure 6. The biological assay is designed around a biological effect that the therapeutic protein has on a living cell. The cells are preferably from an immortal cell line to allow assay reproducibility and consistency, but when necessary, primary cells could be used. The biological effect mediated by the therapeutic protein must be measurable and can include parameters such as proliferation, apoptosis, cytokine release, or mRNA production. When looking for neutralizing antibodies, the cells are exposed to the therapeutic protein administered in conjunction with a serum sample. If there are neutralizing antibodies in the serum sample, the cells will not respond to the therapeutic protein in the same magnitude as cells administered the protein in the absence of neutralizing antibodies.

WHY BIOASSAYS FOR NEUTRALIZING ANTIBODIES ARE IMPORTANT

A challenge in testing for antitherapeutic antibodies is understanding if the detected antibodies have any clinical relevance for the patient. One factor that certainly helps determine if an antibody has relevance, is whether the antibody is causing a change in pharmacokinetics of the therapeutic protein. This is not typically performed during all phases of clinical studies or in the case of postmarketing experiences. Typically, pharmacokinetic analyses are performed on single exposures and in longer Phase 3 trials, the late stage pharmacokinetics are not routinely examined. One test that can be performed however, is the biological assay to determine if the antibodies neutralize the biological effect of the drug. If the biological assay for neutralizing antibodies

is positive, it is highly likely that the antibody is having an impact on the patient's ability to derive full benefit from the therapeutic protein. The effect is even more pronounced when the biological activity being monitored in the bioassay is critical for the efficacy of the therapeutic protein.

There are many challenges in establishing a relevant biological assay and in some cases the challenges are insurmountable. In these cases, a surrogate to a biological assay is utilized that relies solely on binding and does not include a cellular response to that binding. An example is a receptor-binding assay where the success of the assay depends only on the ability of the therapeutic to bind to its receptor. While these assays can be quite informative, especially in the case of therapeutic monoclonal antibodies, they are inferior to a true biological assay that measures a response by the cell after interaction with the protein therapeutic. The closest model to what is happening *in vivo* is by examining the full biological response, not just a binding phenomenon.

EXAMPLES OF IMMUNOGENICITY WITH MARKETED PRODUCTS

A recent review by Koren et al. (2002) describes the immunogenicity profile of 52 therapeutic proteins. While most of these proteins had little impact from immunogenicity, the two most notable exceptions were thrombopoietin and erythropoietin. The increase in antibody-mediated pure red cell aplasia described by Professor Casadevall and attributed to the use of Eprex® is an example of neutralizing antibodies that cross-react with the endogenous protein (Casadevall et al., 2002). Although the cause for these cases of antibody-mediated PRCA has not been fully determined, the consequences for the patient were significant. The antibodies blocked both the therapeutic and the endogenous erythropoietin, and resulted in cessation of red blood cell production as demonstrated by the absence of red blood cell precursors in the bone marrow. The neutralizing antibodies responsible for pure red-cell aplasia in some of the patients have been characterized as predominately IgG_1 and IgG_4 with concentrations ranging from 4 to 44 micrograms/ml (Swanson et al., 2004).

SUMMARY

When developing a protein therapeutic, it is important to understand the immunogenicity of the drug. This requires that appropriate assays be developed and validated that have sufficient sensitivity to detect the presence of antibodies capable of binding to the therapeutic, and that have sufficient specificity to minimize false positive results. The assay testing strategy should include a biological assay to determine if any antibodies detected by the immunoassays are capable of neutralizing the biological effect of the drug. Fully characterizing antibodies generated in response to treatment with a protein therapeutic makes it easier to assess if the immune response has clinical relevance for the patient.

The cases of antibody-mediated pure red-cell aplasia described by Casadevall underscore the importance of fully understanding the immune response to a therapeutic protein. The fact that while theories have been proposed for these cases, the cause has not been convincingly identified is troubling. It points out that causes for immunogenicity remain poorly understood, and that the only reliable method for understanding the immunogenicity and the consequences of that immunogenicity remains closely controlled clinical studies. Only when there exists a body of data from controlled clinical studies where the protein therapeutic is tested in a sufficient number of subjects for a period of at least one year can any conclusions be made about the protein's immunogenicity. One of the important aspects in demonstrating the safety of any protein therapeutic includes appropriate immunogenicity assessment. Even though the effects of antitherapeutic antibodies may not be as significant as demonstrated with antibody-mediated pure red-cell aplasia, the importance of understanding the immunogenicity as it relates to the efficacy of the drug is significant.

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