

Review

Recommendations on risk-based strategies for detection and characterization of antibodies against biotechnology products

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Abstract

The appropriate evaluation of the immunogenicity of biopharmaceuticals is of major importance for their successful development and licensure. Antibodies elicited by these products in many cases cause no detectable clinical effects in humans. However, antibodies to some therapeutic proteins have been shown to cause a variety of clinical consequences ranging from relatively mild to serious adverse events. In addition, antibodies can affect drug efficacy. In non-clinical studies, anti-drug antibodies (ADA) can complicate interpretation of the toxicity, pharmacokinetic (PK) and pharmacodynamic (PD) data. Therefore, it is important to develop testing strategies that provide valid assessments of antibody responses in both non-clinical and clinical

Abbreviations: ADA, anti-drug antibodies; ELISA, enzyme-linked immunosorbent assay; Epo, erythropoietin; GDNF, glial cell derived nerve growth factor; GLP, Good laboratory practice; Ig, immunoglobulin; MGDF, megakaryocyte growth and differentiation factor; OECD, Organization for Economic and Development Co-Operation; PD, pharmacodynamic; PK, pharmacokinetic; PRCA, pure red cell aplasia; r-Hu, recombinant human; TNF, tumor necrosis factor; Tpo, thrombopoietin.

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studies. This document provides recommendations for antibody testing strategies stemming from the experience of contributing authors. The recommendations are intended to foster a more unified approach to antibody testing across the biopharmaceutical industry. The strategies proposed are also expected to contribute to better understanding of antibody responses and to further advance immunogenicity evaluation.

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1. Introduction

Evaluation of the immunogenicity of therapeutic proteins and peptides remains an important part of product development. There is increasing evidence that immune responses to biotechnology products can have a wide variety of effects on non-clinical and clinical studies. In some non-clinical studies, anti-drug antibodies may affect the pharmacokinetics, pharmacodynamics, bioavailability and efficacy of the product. Therefore, antibodies can alter the activity of the product in a way that leads to altered exposure in toxicology studies, by reducing or extending half lives, altering the rate at which a product reaches its target etc. Anti-drug antibodies can also cause their own toxicity by cross-reacting with endogenous molecules in animal models.

Antibody-related clinical sequelae that can occur in humans range from no apparent or mild side effects to altered efficacy, immune complex mediated symptoms, and allergic reactions. In the most severe examples, autoimmune syndromes, such as thrombocytopenia (Zipkin, 1998; Li et al., 2001) or pure red cell aplasia (PRCA) (Casadevall et al., 2002) may develop in patients with drug-induced antibodies capable of neutralizing the biological activity of both the drug and its endogenous counterpart (i.e. thrombopoietin or erythropoietin respectively). Thus, it is necessary to collect appropriate data regarding the appearance and characteristics of antibodies induced over time and assess how these findings may be associated with clinical outcomes.

A previously published paper (Mire-Sluis et al., 2004) describes the design and optimization of immunoassays used to determine if a sample contains antibodies that bind to the product. It discusses in detail important assay parameters such as assay cut point, sensitivity, precision, specificity and relative antibody level. Validation of immunoassays for detection of antibodies directed against therapeutic antibodies has been described in a recently published paper (Geng et al., 2005) and recommendations for the design optimization and qualification of cell-based assays for detection of neutralizing antibodies have also been published recently (Gupta et al., 2007).

This manuscript provides recommendations for the host antibody testing and characterization strategies for non-clinical and clinical studies based upon risk assessment of the drug and the study conditions in which the antibodies were generated. Such an approach is primarily intended to encourage proactive antibody-monitoring strategies in order to minimize patients' safety risks. However, the authors also assume that sponsors will establish the most appropriate antibody screening schemes as well as the extent of antibody characterization on a case-to-case basis, and in agreement with regulatory agencies (Rosenberg and Worobec, 2004).

2. Antibody monitoring based on risk assessment

The premise of the risk assessment strategy is to consider the severity of consequences of an antibody

response to a protein therapeutic if it is induced. The critical factors to be considered are related to the biological function of the product or its endogenous counterpart(s) the target of the product, the mode of administration and the health status of the subject. Because of its complexity, the risk assessment should be carried out in a collaborative fashion between toxicologists, clinicians, PK and assay experts. Timely consultations with regulatory agencies and/or clinical safety monitoring boards may be necessary as well. The identification of the risk level will affect the ADA testing scheme in terms of timing and frequency of sampling, neutralizing activity assessment, qualitative, semi-quantitative or quantitative measurement, as well as characterization of a positive response.

In some non-clinical studies, one can encounter relatively high incidence and high levels of antibodies to human, humanized, or chimeric proteins and peptides. As pointed out above, an antibody response could lead to difficulties in interpretation of toxicity, PK, PD and bioavailability data. Limiting antibody testing to detection only, especially in the non-human primate studies, may not always be sufficient. Some antibody characterization such as titer or relative concentration may be recommended. Evaluation of the neutralizing activity may be based on the assessment of PK/PD or biomarker data, if available, or on the results of functional cell-based bioassays or ligand-binding assays.

In clinical studies, the extent of characterization inevitably depends on the potential risk of antibody-related sequelae that can occur in humans because patient safety is of primary concern. An example of factors to consider during risk assessment is summarized in Table 1 where multiple factors such as potential immunogenicity of the protein, its biological role, indication of use, route of administration, duration of treatment as well as health status of the patient (e.g. immune competence) are taken into account. A single applicable factor within the “High Risk” category may be sufficient to establish a High Risk situation.

The type of drug should be also taken into account when establishing the antibody testing methodology and specificity. If the biopharmaceutical is a fusion molecule of multiple components, with at least one component representing a potentially high risk of adverse events, a method to determine to which component the antibodies bind is recommended.

The greater the assessed risk, the more extensive and more frequent antibody testing and characterization should be applied. The frequency of sample analysis should be based on the timing and incidence of antibody

Table 1

Examples of factors to be considered in assessing the risk of antibody-related clinical sequelae

Higher risk	Lower risk
Product: <ul style="list-style-type: none"> • Endogenous version exists • Endogenous version unique 	Product: <ul style="list-style-type: none"> • No endogenous version • Endogenous version redundant
<ul style="list-style-type: none"> • Replacement therapy • Repetitive treatment • Non-intravenous route of administration^a 	<ul style="list-style-type: none"> • Not a replacement therapy • Single dose treatment • Intravenous route of administration^a
Target: <ul style="list-style-type: none"> • Endogenous version exists • Endogenous version unique 	Target: <ul style="list-style-type: none"> • No endogenous version • Endogenous version redundant
<ul style="list-style-type: none"> • Subject/health status • Sole therapy • Life threatening disease 	<ul style="list-style-type: none"> • Subject/health status • Other therapies exist • Not a life threatening disease
<ul style="list-style-type: none"> • Not immunosuppressed • Autoimmune/inflammatory disease 	<ul style="list-style-type: none"> • Immunosuppressed • No autoimmunity/inflammation

^a The following immunogenicity of the administration route has been claimed but exceptions may exist: inhalation > subcutaneous > intraperitoneal > intramuscular > intravenous. Specific testing of individual products is encouraged.

response as well as occurrence and severity of clinical sequelae.

3. Examples of risk-based antibody characterization

There are compelling reasons to design antibody-monitoring strategy in accordance with the risk-based approach. The utility of such an approach became quite apparent during development of recombinant human thrombopoietin (r-HuTpo) analogues. In one development program, r-HuTpo was shown to be highly immunogenic and was deemed to be a high-risk molecule. This assessment was initially made based on the non-clinical immunogenicity data. Severe thrombocytopenia in animals positive for neutralizing anti-Tpo antibodies was observed in several species even after dosing with respective autologous Tpo molecules. Furthermore, non-clinical studies in mice demonstrated a direct causative relationship between neutralizing anti-Tpo IgG antibodies and thrombocytopenia. Injections of neutralizing IgG isolated from the Tpo immunized and thrombocytopenic animals caused thrombocytopenia in Tpo naïve healthy animals (Koren, 2000). Criteria outlined in Table 1 also classify Tpo into the high immunogenicity risk. This molecule is an endogenous growth factor that regulates a vital physiological function, a loss of which can be life threatening.

During the clinical development, administration of r-HuTpo was, in this program, limited to immunocompromised cancer patients only. In several clinical studies r-HuTpo was dosed exclusively via the intravenous route. This dosing scheme was also combined with an extensive antibody-monitoring strategy. An ELISA for screening antibodies that bound to the full-length Tpo molecule, an ELISA for antibodies binding to the amino-terminal (bioactive) portion of the molecule, and an ELISA for antibodies that inhibit binding of Tpo to its receptor were used sequentially in a contingent fashion. In addition, samples positive in all three ELISAs were analyzed in a human megakaryocyte proliferation assay for antibodies capable of neutralizing Tpo's biological activity. These assays were carried out in real time (weekly) to provide clinicians with timely information on the occurrence and the type of antibodies so that Tpo administration could be stopped if the trend towards the development or presence of neutralizing antibodies was detected. No neutralizing antibody-related adverse effects were observed in any of the clinical studies utilizing the intravenous r-HuTpo administration (Koren, 2000, 2002). Only in one clinical study, r-HuTpo was subcutaneously administered to patients with gynecological cancer treated with carboplatin (Vadhan-Raj et al., 2000). During the sixth treatment cycle, anti-Tpo IgG antibodies were detected in one of the 28 treated patients. The serum from this patient partially inhibited Tpo binding to its receptor in the ELISA assay as well as proliferation of megakaryocytes *in vitro*. Epitope mapping experiments revealed antibody(ies) directed against the "site 1" of the Tpo receptor-binding domain whereas no reactivity with the other receptor-binding site was detected (Koren, 2000). It should be pointed out that Tpo exerts its biological activity by dimerizing receptor molecules utilizing two distinct binding sites (Pearce et al., 1997, Park et al., 1998, Hou and Zhan, 1998). After six cycles, this patient's chemotherapy as well as Tpo treatment was stopped and the chemotherapy-induced thrombocytopenia subsequently returned to normal platelet counts within a few months. The above antibody specificity data suggested a trend towards the neutralizing antibody response in this patient. However, the anti-Tpo repertoire apparently did not spread enough to cover the other receptor-binding site judged by the inability to completely block Tpo/receptor interactions *in vitro* and by relatively quick recovery of the chemotherapy-induced thrombocytopenia. In fact, no antibody-related thrombocytopenia was observed during the entire clinical development of this Tpo molecule. It would, therefore, appear that the described antibody-monitoring and characterization strategy as well as dosing regimens

and targeted patient population were commensurate with the assessed high Tpo immunogenicity risk.

In another development program, a truncated form of Tpo (megakaryocyte growth and development factor, MGDF) was subcutaneously administered to immunocompromised cancer patients, platelet donors and healthy human subjects. Neutralizing anti-Tpo antibodies were detected in 0.6%, 0%, and 4% of these subjects, respectively. Long lasting severe thrombocytopenia was associated with IgG anti-Tpo neutralizing antibodies in some subjects (Zipkin, 1998; Li et al., 2001). This clinical outcome underscores the importance of having robust and reliable assays to support clinical development of therapeutic proteins.

It has been shown that neutralizing anti-Epo antibodies can induce PRCA (Casadevall et al., 2002; Bennett et al., 2005). In a number of PRCA patients, an SPR-based biosensor assay combined with the bioassay for neutralizing activity showed that all studied PRCA patients had developed a mature immune response as judged by the high concentration of high affinity, neutralizing IgG4 and IgG1 antibodies (Swanson et al., 2004). In contrast, low-affinity, non-neutralizing IgM anti-Epo antibodies have been detected in patients with no signs of PRCA (Amgen, data on file).

In another study, occurrence of neutralizing antibodies directed against glial cell derived nerve growth factor (GDNF) coincided with the isotype switching from IgM to IgG and with an increase in antibody level in patients dosed with recombinant human GDNF (Tatarewicz et al., 2005).

Monitoring of the IgA response may be of relevance as well. IgA ADA were observed in addition to IgG ADA in the one subject who developed a detectable ADA response after nebulizer administration of an aerosolized therapeutic humanized antibody directed against IgE (Fahy et al., 1999). The IgA ADA response is likely to be more frequently observed as the administration of inhaled therapeutic proteins expands (Thippawong, 2006). Furthermore, it has been also shown that psoriatic patients respond to various antigens with the IgA as a predominant isotype (Lapadula et al., 1992, Borg et al., 1994, Rantakokko et al., 1997, Lindquist et al., 2002). While the overall ADA rate was low for psoriasis patients dosed with a humanized therapeutic antibody (Mortensen et al., 2004), IgA responses were observed in a subset of patients who developed a measurable antibody response (Genentech, data on file).

The importance of determining antibody level was also described in patients with Crohn's disease treated with infliximab, a chimeric monoclonal antibody to TNF-alpha. The median therapeutic response to this drug was

significantly shorter in patients with higher ADA levels (Baert et al., 2003), demonstrating the value of utilizing ADA responses in the overall evaluation of a study outcome and product efficacy. Furthermore, patients who became positive for ADA to infliximab were approximately 2–3-fold more likely to have an infusion reaction than those without ADA (Package Insert, Centocor, Inc.). Additional examples that demonstrate immunogenicity of therapeutic antibodies and ADA related side effects include adalimumab, a human anti-TNF- α antibody. Anti-adalimumab antibodies can reduce efficacy and cause exantema (Package Insert, Abbott Laboratories, Bender et al., 2007). Antibodies to natalizumab, a humanized anti-VLA4, can cause loss of efficacy and infusion reactions (Package Insert, Biogen-IDEC Inc.) whereas ADA directed against abciximab, a chimeric anti-GIIb/IIIa F(ab'), have been associated with thrombocytopenia and anaphylaxis in some cases (Package Insert, Centocor, Inc.).

The above examples demonstrate that the sensitive detection assays combined with antibody characterization in terms of neutralizing activity, level and isotype do provide helpful information directly related to patient safety and treatment efficacy as well as overall understanding of the immune response to therapeutic proteins. Thorough information on ADA response is necessary for a proper risk versus benefit assessment of therapeutic proteins.

4. Antibody testing

On the basis of the above risk assessments we recommend a two-step antibody testing and character-

ization strategy as well as reporting scheme that is applicable to both non-clinical and clinical studies.

Fig. 1 illustrates the first step where all pre- and post-dosing samples are analyzed for antibodies. Two essential tests here are the screening assay and confirmation by drug inhibition and/or immunoglobulin depletion. Mire-Sluis et al. (2004) have recommended the use of the upper bound of a one sided 95% reference interval for the assay cut point (threshold) with a probability of 5% false positive results in the screening assay. The rationale for such an approach is to err on a conservative side and to minimize the probability of false negative results. Because of this precaution, it is important to carry out a drug-inhibition test in all samples above the cut point in order to confirm the specificity of the antibody signal and to distinguish between the true and false positive samples. Although the specificity confirmation by the drug-inhibition test is preferable, an immunoglobulin depletion step or testing of the antibody binding in presence and absence of the immobilized drug could be used to evaluate ADA specificity. ADA screening is recommended for multi-dose preclinical non-human primate (or surrogate species) safety studies and for single and multiple dose clinical studies and should be continued until the safety profile of the drug has been established which is typically at the time of regulatory approval. It is also important to note that post-approval testing may be warranted. Whenever possible, ADA analysis should be performed on samples collected after a suitable drug washout period in order to reach conclusive results.

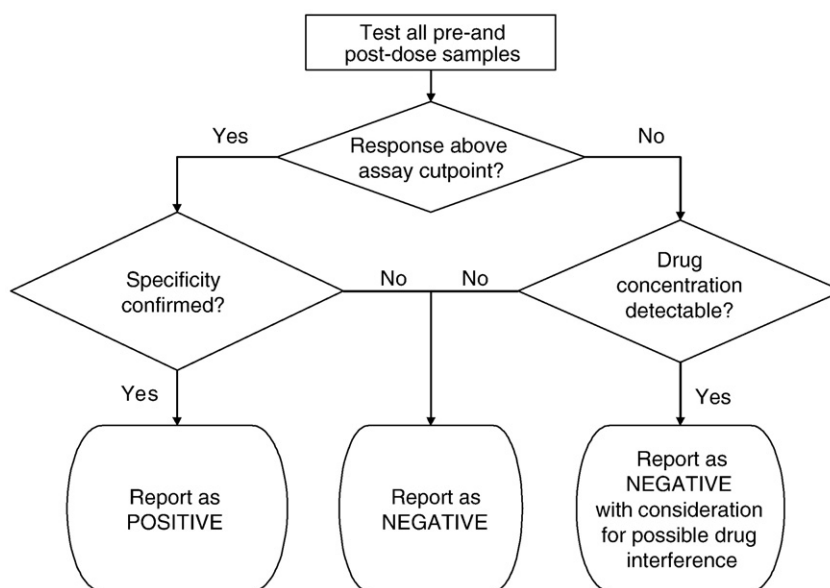


Fig. 1. STEP 1: Recommended testing strategy for detection of anti-drug antibodies.

Alternatively, the assay may be developed using techniques that remove or mask interfering drug; or may be developed in a manner such that it is not influenced by the presence of drug in the sample. Sample pretreatment techniques, such as acid dissociation of drug–antibody complexes with subsequent antibody detection (Patton et al., 2005, Moxness et al., 2005, Smith, 2006) or drug removal (Lofgren et al., 2006) have been reported to minimize drug interference. The relative sensitivity of ADA assays in the presence of drug is difficult to characterize due to the lack of true positive ADA samples with known ADA concentrations to evaluate in during assay development. For this reason, the sensitivity and drug tolerance reported for an assay should be carefully considered relative to the positive control(s). When a sample contains drug levels that are below the quantification limit of the PK assay, and the antibody result is below the assay cut point, the sample may be reported as “negative” for ADA. However, if a sample contains quantitative levels of drug, and the ADA result is below the assay cut point, the role of drug interference must be considered. For ADA assays that are affected by the presence of drug, if the sample is reported as “negative”, a statement of possible drug

interference should accompany the result. Additionally, if the ADA result is above the cut point of the assay and the drug concentration is higher than the tolerance threshold, the positive titer reported for that sample may be undervalued due to drug interference and a statement of possible drug interference should be also made. Similar consideration should be made during performance of the confirmatory assay.

It is important to emphasize here that the reporting of ADA values is only a part of an immunogenicity assessment. A positive result means that antibody was detected and a negative result means that antibody was not detected under the conditions of the analysis. The detection of ADA, or lack thereof, should be considered with other study parameters such as pharmacokinetics, pharmacodynamics, and adverse event data to determine if immunogenicity is of concern to the patient population. If the ADA result is “negative” in parallel with declining PK and/or PD values, the ADA may have been undetectable due to sensitivity, specificity, or interference factors not controlled for in the assay. Additional sample treatment may be necessary to clarify ADA status. Alternatively, if the ADA result is “positive” in parallel with unchanged PK/PD profile

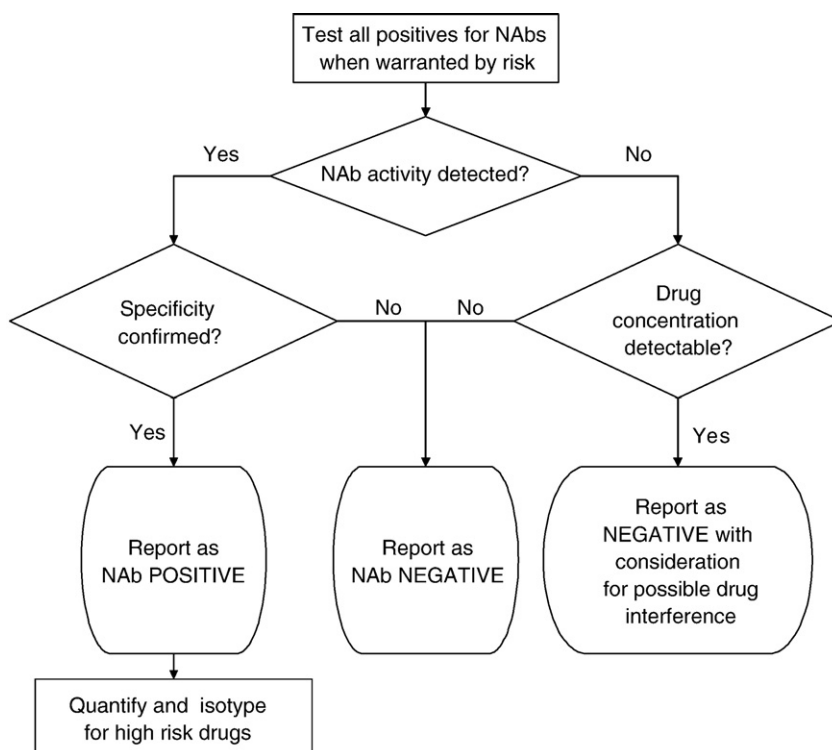


Fig. 2. STEP 2: Recommended strategy for characterization of anti-drug antibodies based on risk assessment.

and absence of related adverse events, the safety assessment of the presence of ADA will again rely upon the risk-based approach. Obviously, these considerations apply when appropriate PK/PD data are available.

Fig. 2 summarizes the strategy proposed for the characterization of anti-drug antibodies in non-human primate (or surrogate species) and clinical studies. Only samples positive in the screening assay (STEP 1) should be characterized as described in STEP 2, when warranted by the risk assessment. In high-risk cases, measurement of neutralizing antibody (NAb) activity should be carried out using functional cell-based assays as the most preferable methodology. If no appropriate cell lines are available, the ligand-binding NAb assays could be used. Evaluation of the PK/PD or biomarker data may be helpful as an indirect assessment of the neutralizing antibody activity. Generally, detection assays are more sensitive than NAb assays. However, sometimes a NAb assay may be more sensitive than a detection immunoassay. In the case of high-risk drugs, this situation would necessitate analysis of all clinical samples with the NAb assay.

Determination of NABs may be considered on a case-to-case basis for non-human primate (or surrogate species) study samples. If the antibody incidence is low and toxicity is well defined, there would be little justification for intensive antibody characterization and NAb analysis because drug exposure would not be questioned. In contrast, if the antibody incidence is high and toxicity is poorly defined, the presence of NABs should be considered to evaluate whether animals were adequately exposed to the active drug and that the study provided an adequate assessment of potential toxicity. In such cases, assessment of neutralizing antibody activity should be carried out following the above recommendations described for clinical studies. In lower species, NAb assays would not be recommended unless the study served as the primary study supporting the safety of the product for humans.

If NAb activity is detected, samples should be confirmed for specificity by the drug inhibition and/or immunoglobulin depletion test. Again, NAb results should be reported with consideration of the sample drug concentrations as described for ADA screening. In cell-based assays for NABs, minimizing the interference of the drug in sample is likely to be more challenging compared to the immunoassays. An additional NAb analysis following drug washout may be the only approach for reaching conclusive results unless a method of drug removal is available (Lofgren et al., 2006). Samples showing neutralizing activity in

Table 2
Recommended antibody testing during drug development

Drug development stage	Recommended testing
Non-clinical studies	<ul style="list-style-type: none"> ● Develop ADA screening assays for the lead molecule and, if applicable, for the surrogate molecule in relevant species and matrices (e.g. serum, plasma, CSF, milk). Determine the level of drug tolerated in the ADA assays. ● Fully validate ADA screening assays for GLP/OECD studies. ● Determine ADA screening frequency: <ul style="list-style-type: none"> – Test at baseline and appropriate intervals throughout the study based on risk assessment. – Test at the end of recovery phase, if applicable. – and, if necessary, test after the drug washout. ● Risk-based optional characterization activities: <ul style="list-style-type: none"> – Determine testing for NABs in non-human primates on the basis of risk assessment and toxicity. – If necessary develop and validate NAb assays for non-human primate studies.
Clinical studies	
Phase 1	<ul style="list-style-type: none"> ● Develop and validate ADA screening assays in relevant human matrices prior to dosing. ● Determine ADA screening frequency on the basis of risk assessment. <ul style="list-style-type: none"> – For low-risk molecules, screen for ADA at baseline and the end of study and/or after drug washout. – For high-risk molecules, screen for ADA at baseline and bi-weekly or monthly intervals throughout the study, and/or after drug washout. ● When warranted by risk assessment, develop and validate NAb assay. ● Determine NAb testing frequency on the basis of risk assessment. <ul style="list-style-type: none"> – For lower-risk molecules, test at baseline and the end of study, and/or after drug washout. – For high-risk molecules, test at baseline and bi-weekly or monthly intervals throughout the study, and/or after drug washout. ● If NAb response develops, test until subjects are negative on a monthly or bimonthly basis.
Phase 2 and 3	<ul style="list-style-type: none"> ● Based on Phase 1 immunogenicity testing results such as ADA incidence, level and neutralizing activity, develop an appropriate and adequate testing strategy. <ul style="list-style-type: none"> – For the low-risk/low immunogenicity molecules, testing at baseline and end of study is recommended. – For high-risk/immunogenic molecules, at least monthly testing is recommended in real-time.
Phase 4	<ul style="list-style-type: none"> ● Post-marketing antibody-monitoring strategy should be established according to risk assessment. ● New indications may require different strategies (e.g. for inflammatory and autoimmune disease more frequent testing, and for cancer, less frequent testing may be needed).

the initial NAB assay can still be reported as NAB-negative if their specificity cannot be proven by the confirmatory assay(s). Such samples are reported as

NAb-negative with a non-antibody-related inhibitory activity.

Additional characterizations of ADA+/NAb+ samples may be warranted for high-risk drugs or for mechanistic understanding of the ADA response. The determination of antibody level (titer or relative antibody concentration) and major immunoglobulin isotypes (IgG, IgM, IgA) for the high-risk drugs in humans may assist in evaluating how far the immune response had advanced, especially if neutralizing antibodies are present. It should be noted that ADA quantification results might be incorrectly interpreted if circulating levels of drug are present and interfere with the assay.

In case of hypersensitivity, detection of drug-specific IgE may be performed. However, it can present a considerable methodological challenge due to its low concentrations in serum and uncertainty about optimal timing of the test. A serum mast cell beta-tryptase (Payne and Kam, 2004) test could be helpful if drug-specific IgE assay is not available. A skin test with appropriately formulated drug might be considered, although with caution, because of possible further stimulation of the immune response. Table 2 summarizes recommendations for antibody testing during the non-clinical and different stages of clinical drug development.

5. Concluding remarks

The first paper in this series described common approaches for developing and optimizing immunoassays for antibodies to biotechnology products (Mire-Sluis et al., 2004). The second describes development and optimization of cell-based assays for neutralizing antibodies (Gupta et al., 2007). This paper recommends a tiered, risk-based strategy to evaluate antibody responses to therapeutic proteins and peptides. The proposed strategy for clinical studies is based on risk assessment that factors in the clinical indication, the nature of the target as well as potential antibody effects on safety and efficacy. For the non-clinical studies, the antibody testing strategy is based on assessment to what extent antibody results are needed for the correct interpretation of the exposure and toxicity data. These recommendations are intended to facilitate a standardized approach for assessing the immunogenicity of therapeutic proteins across the biopharmaceutical industry.

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