



Review

Immunogenicity assessment of biotherapeutic products: An overview of assays and their utility



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ABSTRACT

Biotherapeutic products (BTPs) are the fastest growing medicines in the pharmaceutical market. Despite their clinical success, the immunogenicity of BTPs continues to be a major concern. Assessment of immunogenicity as well as appropriate interpretation of immunogenicity data is therefore, of critical importance for defining safety profile of these products for the purpose of their licensure and use. In the past decade, much progress has been made towards how immunogenicity should be studied. This article reflects the content of the brief presentation on principles of methods used for immunogenicity assessment and their merits and limitations given at the first World Health Organization (WHO) implementation workshop on rDNA derived biotherapeutic products held in the Republic of Korea in May 2014 to support the case studies on immunogenicity presented and discussed during the workshop. The purpose of this article is to provide an overview of the methods used for assessing immunogenicity of biotherapeutic products (BTPs) and the most important considerations in interpreting results in the context of regulatory overview of these products.

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1. Introduction

Since approval of the first recombinant biotherapeutic product (BTP), insulin in 1982, several BTPs have been successfully approved for treatment of various indications. Despite their success, BTPs when administered to patients can elicit immune responses that may directly impact product safety and efficacy. For example, antibodies that develop in response to a BTP may alter the product's pharmacokinetic profile, diminish its pharmacodynamic effect and compromise clinical efficacy [1–6]. In some instances, they can cause undesirable effect(s), such as hypersensitivity reactions or severe adverse events (for example, profound anaemia) that can even be fatal [7–11]. Well-known examples are thrombocytopenia in patients treated with thrombopoietin (TPO) and pure red cell aplasia in chronic renal disease patients treated with Eprex, an approved erythropoietin (EPO) product, following induction of neutralizing antibodies which cross-reacted with the functionally non-redundant endogenous EPO [7,8]. Since immunogenicity has

been reported with various products including monoclonal antibodies [9,11–14], immunogenicity continues to receive significant attention from regulatory bodies, industry and clinicians.

Immunogenicity assessment is one of the regulatory requirements for BTP approval which includes the review of immunogenicity studies and the interpretation of the results. As stated in WHO Guidelines on BTPs [15], immunogenicity should be investigated in the target population since animal testing and in vitro models cannot predict immune response in humans. In addition, immunogenicity has a role in demonstrating product comparability following manufacturing changes and similarity in the context of biosimilar development [15,16]. Even minor changes can potentially affect the bioactivity, efficacy or safety including immunogenicity of a BTP. Consequently, guidance has been provided by regulatory agencies – European Medicines Agency (EMA) and Food and Drug Administration (FDA), United States Pharmacopoeia and white papers published by industry with the expectation that these guidance documents will facilitate immunogenicity assessment [17–21]. For a biosimilar, demonstration of similarity in terms of immunogenicity of a biosimilar in a head-to-head comparison to the reference product is a critical parameter for defining its safety profile [16,17].

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A myriad of factors, some product related but also patient and disease related influence immunogenicity [22,23]. The structural characteristics of the therapeutic product for example, native amino acid sequence or variant, any chemically modified forms, presence of aggregates, process related impurities for example, host cell proteins, formulation changes, primary packaging – container, the functional properties of the protein, the patient's genetic predisposition and immune status, the type of disease(s), concomitant medication, frequency and route of administration, single/multiple use and previous exposure are some of the contributory risk factors for immunogenicity (Table 1). While glycosylation does not appear to play a major role, non-human or non-mammalian glycosylations within the product due to the expression system used can induce immune responses. An example is cetuximab which contained a terminal galactose- α -1, 3-galactose because of the expression system used and caused anaphylaxis in patients with pre-existing cross-reactive antibodies, but did not induce any immune response in naïve individuals. All factors need to be considered in the risk-based approach when designing and planning studies for immunogenicity assessment for BTP including multiple domain therapeutics [24]. It is critical to consider the risk of generating an immune response, the potential severity of the induced response and the risk/benefit in the target population [22,23].

Unfortunately, the prediction of unwanted immunogenicity of a therapeutic product, its characteristics and clinical significance, is difficult. As methodologies mature (in silico, in vitro and possibly in vivo models), the ability to assess or closely predict and mitigate immunogenicity risk for particular patient populations may be enhanced considerably however, currently the utility of these procedures and how they correlate clinically remains to be seen.

2. Immunogenicity assessment and clinical relevance

Currently, the most technically feasible approach for testing a product's immunogenicity involves measuring antibodies specifically generated against the product. In clinical studies, therefore, detection and characterization of antibodies is important to understand the efficacy and safety of a BTP. To date, no single assay can provide all the necessary information on the immunogenicity profile of a biotherapeutic. Therefore, a well devised bioanalytical strategy involving a panel of assays is required (Fig. 1). For a majority of BTPs, a typical strategy involves a screening assay for detection based on the ability of the antibodies to bind to the protein complemented with a confirmatory step (e.g., adsorption with excess antigen) followed by an assay for assessing the neutralizing capacity of the antibodies. Such immunogenicity data are generally analyzed in the context of their relevance to

pharmacokinetics and/or pharmacodynamics and clinical effects in patients [2,25,26]. An understanding of the kinetics of antibody development, whether transient or persistent, non-neutralizing or neutralizing and how it relates to clinical impact is important. As outlined in the WHO Guidelines on BTPs [15], a carefully selected sampling collection plan with appropriate sampling points (baseline, sequential sampling during treatment, wash-out/follow-up sample) should be integrated in the strategy. For high-risk products, more frequent sampling and real-time testing is recommended with a sampling post study in some cases (for example, for patients that are antibody positive until they test negative in two sequential samples). For low-risk products, retrospective monitoring may be considered but collection of samples at appropriate time-points is essential. Additionally, a screening assay is pivotal in the immunogenicity assessment of a therapeutic and should have adequate sensitivity and must be appropriate for the intended use. For example, since mAb therapeutics persist and have a long half-life, the assays developed should be capable of detecting antibodies in the presence of levels of the therapeutic expected to be present in patient samples and sampling plans should include sample collection following a wash-out period. Other confounding factors include pre-existing cross-reactive antibodies, heterophilic antibodies, soluble targets, or ligands, rheumatoid factors, concomitant medication, etc. and the assessment of these incorporated and appropriate measures implanted to minimize any interference.

Apart from evaluation of the presence of binding antibodies, further characterization of positive samples in terms of titre determination, assessment of ability to neutralize the biological activity of the therapeutic and elucidation of the immunoglobulin class(es) and subclass(es) may enable a better understanding of the immunogenicity of the BTP and its clinical impact.

3. Current methods for antibody detection and characterization

3.1. Screening assays

These determine the presence (or absence) of antibodies based on the ability of the antibodies to recognize the relevant antigenic determinants in the therapeutic protein. The aim of the assays is to detect in patient samples polyclonal antibodies – these are unique to each patient and differ in characteristics (for example, isotype and affinities etc.) between patients and also within the same patient following multiple administrations of the product. This scenario makes characterization of an antibody response difficult and association with clinical impact challenging. However, since these assays are the first step in any immunogenicity

Table 1

Some factors which influence the unwanted immunogenicity of therapeutic proteins.

Factors determining the immunogenicity of therapeutic proteins

Product-related (Intrinsic)

- molecular structure – primary amino acid sequence or variants, novel epitopes;
- aggregates, degradation products, oxidized or deamidated forms;
- host cell DNA/proteins;
- formulation;
- primary packaging.

Product-related (Extrinsic)

- dose, route, frequency of administration, episodic/continuous treatment, duration of treatment, previous exposure;
- cellular or soluble target;
- biological properties of the therapeutic;
- endogenous counterpart, redundant or non-redundant.

Host-related

- genetic profile;
- immune status, disease state, medication.

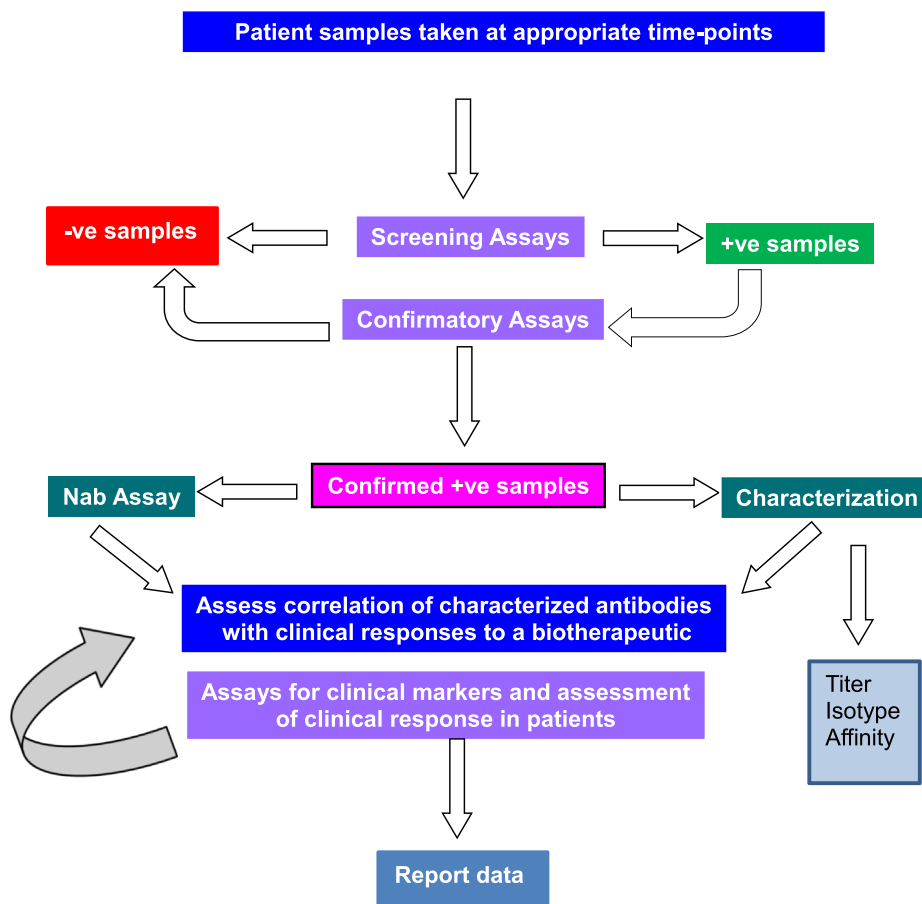


Fig. 1. A simple strategy for detection of antibodies in patients receiving biotherapeutic products.

evaluation, they are generally designed to detect false positives (typically 5%) which are confirmed for specificity using confirmatory assays prior to titre determination or any further characterization.

New platforms for antibody screening are continually being exploited to increase the ability to detect antibodies (Table 2A) but there are numerous issues which need to be considered prior to development of a certain assay platform. These are given below:

- selection of an appropriate assay format and design for the therapeutic. Assay formats vary between different proteins and likely to depend on the therapeutic protein class. For a non-immunoglobulin recombinant protein, all platforms listed in Table 1 are viable options for antibody determination. However, if the protein is a mAb, the choice is limited to some extent due to secondary antibody cross-reactivity. A direct ELISA is not suitable unless it is modified. Although a therapeutic mAb (often have k light chain) to capture and enzyme-labelled anti-lambda to detect or mAb-Fab or F(ab')₂ to capture and labelled anti-IgG, anti-IgM as detector can be used, these assays usually have a high background due to reactivity of serum of healthy subjects with capture reagents [27];
- assay sensitivity is dependent on the positive control used. There are generally no reference standards or human polyclonal antibodies available as calibrators and so assays are quasi-quantitative. Assays are developed using a surrogate positive control antibody, often produced by hyperimmunisation of a non-human species or by phage display;

- ability of assay to detect antibodies with desired specificities (IgM, IgG subclasses etc);
- potential interference from the therapeutic itself, particularly with mAb therapeutics which are dosed at high levels and persist as they have a long half-life, interference from co-medications and/or disease-specific issues (for example, rheumatoid factor (RF), drug target. For minimizing therapeutic interference, implementation of an appropriate strategy such as acid dissociation [28,29], use of wash-out samples can be incorporated to enable antibody detection. Likewise additional steps will need to be introduced to address drug target interference [30].

All assays should be optimized and validated for their intended purpose using samples from a similar patient population. Validation of antibody assays is an essential pre-requisite for obtaining results that are reproducible, accurate and meaningful. It is necessary to develop validation criteria for assays in use within various laboratories. For screening, methods such as enzyme-linked immunosorbent assays (ELISAs), radio-immunoprecipitation assays (RIPA) and newer technologies such as electrochemiluminescence (ECL), bead-based assay (AlphaLISA Perkin Elmer), Gyrolab system (Gyros AB), surface plasmon resonance (SPR, Biacore), bio layer interferometry (Octet, ForteBio) can be used (Table 2A). Although most have moderate throughput and are automated, in most clinical immunology laboratories, ELISAs are a popular choice due to their robustness, reliability, sensitivity, ease of automation and the requirement of a generic platform which is readily available. When applied to clinical

Table 2A

Commonly used screening assays.

Type of Assay	Advantages	Disadvantages
Direct/Indirect ELISA	Easy to use and automate High through-put High therapeutic tolerance Inexpensive Generic reagents and instrument	May bind non-specifically High background May fail to detect low-affinity antibodies Requires species specific secondary reagent
Bridging ELISA	Easy to use and automate High through-put Low background, High therapeutic tolerance in solution phase High specificity (dual-arm binding) Generic reagents and instrument	Antigen labelling required May fail to detect low-affinity antibodies Highly susceptible to interference by therapeutic, serum components e.g., anti-human Ig molecules, multivalent targets May not detect IgG4
Electrochemiluminescence	High through-put, large dynamic range Minimally affected by matrix High tolerance to therapeutic in solution phase Detection signal consistent during life of TAG conjugate	May require two antigen conjugates Antigen labelling required Susceptible to interference by therapeutic, serum components e.g., anti-human Ig molecules, multivalent targets May not detect IgG4
Radioimmunoprecipitation assay	Moderate through-put High sensitivity Can be specific Inexpensive	Vendor-specific equipment & reagents Can be isotype specific May not detect low-affinity antibodies. Requires radiolabelled antigen. Decay of radio-label may affect antigen stability
Surface plasmon resonance	Automated Determines specificity, isotype, relative binding affinity Enables detection of both 'low-affinity' and high affinity antibodies. Detection reagent not required	Antigen immobilization may alter therapeutic. Regeneration step may degrade antigen. Sensitivity often less than binding assay. Expensive vendor-specific equipment & reagents

samples such as sera or plasma, they are subject to variable matrix effects and interference due to various substances including RF, complement and circulating therapeutic. However, they may not detect low affinity (for example, IgM) or rapidly dissociating antibodies which, in some instances, may be neutralizing due to the wash steps included in the procedure. Replacement of enzyme conjugate with a fluorescent conjugate, such as europium–streptavidin, provides the basis for assays, which require a fluorimeter but may exhibit greater specificity than 'traditional' ELISA. Other readout methods, for example, the electrochemiluminescence (ECL) platform which is now gaining tremendous popularity, dissociation-enhanced lanthanide fluorescent immunoassay, time-resolved fluorescence resonance energy transfer, are also available. Table 2A lists advantages and limitations of commonly used assay methods which are briefly described here.

- i. ELISAs – Different assay formats for example, direct, indirect, bridging or competitive ELISA can be employed. In the direct assay, serum or plasma samples are incubated with the immobilized antigen and the bound antibody detected using an enzyme-labelled anti-immunoglobulin reagent of appropriate specificity conjugated to an enzyme (for example, alkaline phosphatase, horseradish peroxidase), or a small molecule, (for example, biotin), which acts to amplify the signal following binding of an enzyme conjugate, (for example, streptavidin–alkaline phosphatase). The final colour due to enzyme substrate addition is directly proportional to the antibody concentration in test samples and is measured spectrophotometrically. ELISAs rely on antigen immobilization on a plastic surface but immobilization can alter the antigen conformation and mask epitopes such that antibodies specific to the masked epitopes may not be recognized [31]. To overcome this problem, an indirect format in which a capturing agent (for example, a monoclonal antibody specific to the antigen or streptavidin to capture antigen conjugated to biotin) is immobilized to

anchor the antigen allowing exposure of all available epitopes as in the case of IFN- β can be used [31]. These assays require species specific secondary antibodies for detection and this poses a problem when using an animal serum, as a positive antibody control when evaluating human sera for the presence of antibodies in the assay. The issue of species specific secondary antibody is overcome in a bridging assay and this along with other advantages means that bridging assays are a preferential choice and are widely used by industry as opposed to direct or indirect ELISA formats.

- ii. The bridging ELISA uses the antigen both for capturing the antibody and for detection. The antigen is appropriately conjugated or tagged such that a colorimetric signal is developed. Sensitivity can be enhanced by using streptavidin-coated plates to capture biotinylated antigen and a differently labelled (for example, Digoxigenin) antigen to complete the bridge followed by addition of enzyme-labelled antidigoxigenin antibodies for the colorimetric readout as shown for Erythropoietin antibodies [32]. Such assays are claimed to have high specificity (dual arm binding), an acceptable sensitivity, are highly tolerant to the presence of therapeutic and do not require species-specific reagents. The disadvantage is that they may fail to detect low affinity or functionally monovalent antibodies such as IgG4 and are susceptible to interference from serum factors including drug target and RF. Nevertheless, these assays are in use for detecting antibodies directed against monoclonal antibody therapeutics and showed superiority over other platforms in detecting antibodies when combined with acid-dissociation procedures to dissociate immune-complexes [33]. This approach also utilizes generic reagents and instrumentation and removes heavy reliance on specialized equipment from a single vendor [33].
- iii. ECL – The ECL platform (MSD) employs a ruthenium-conjugated protein instead of the enzyme conjugate. Ruthenium labels are stable, non-radioactive and offer a

- choice of convenient coupling chemistries. An oxidation–reduction reaction of ruthenium ions in the presence of tripropylamine generates an ECL reaction under appropriate voltage stimulation. As ruthenium ions are recycled, the electrochemical signal is amplified to yield increased sensitivity. Since the ECL instrument (MSD) uses carbon electrode plates, available as standard, high bind or precoated with streptavidin or avidin, ECL based assays may be developed in either conventional formats or bridging assay protocols as used for ELISAs. This platform offers distinct attributes – a greater dynamic assay range, high tolerance to matrix and circulating antigen [34] and in certain instances may allow detection of low affinity antibodies depending on the format used (solid-phase/solution phase). However, this may need to be evaluated on a case by case basis if wash steps are included in the procedure. Although this platform is widely used for detecting antibodies directed against monoclonal antibody therapeutics, it suffers from the same limitations as the bridging ELISA and may not detect the IgG4 isotype and is susceptible to RF interference [35]. It is critical that conjugated antibody reagents, particularly the detection reagent for ELISA or ECL bridging assays should be carefully monitored for stability and aggregation to maintain the quality and robustness of assay performance over time [35].
- iv. **Bead-based assay:** A no-wash alternative to the ELISA is a simple bead-based approach, offered by AlphaLISA (Perkin Elmer) which relies on chemiluminescence and can be set up either as sandwich or competition assays. In the former, the analyte is captured by a biotinylated antibody bound to streptavidin-coated donor beads and a second antibody conjugated to AlphaLISA acceptor beads. Binding of the two antibodies to the analyte brings the beads into proximity. Laser irradiation of donor beads triggers a cascade of chemical events resulting in a chemiluminescent signal. In the competitive format, a biotinylated analyte bound to streptavidin donor beads is used with an antibody conjugated to the acceptor beads. These assays exhibit remarkable sensitivity, have a wide dynamic range and are suitable for miniaturization and automation.
 - v. **Surface Plasmon Resonance (SPR):** These assays measure antigen–antibody interaction in ‘real-time’ and provide a continuous signal of the events when the sample flows over an antigen immobilised sensor chip and generates a signal due to a change in the refractive index caused by a difference in mass as the analyte binds to the ligand. This change in refractive index is directly proportional to the amount (mass) of binding antibody in the sample being tested [36]. Sequential injection of a species-specific antibody can be used to confirm specificity and to enhance signal. Generally, SPR assays are low throughput and less sensitive compared with ELISAs, however, they have an advantage as they are likely to detect early immune responses characterized by low affinity antibodies, which may be neutralizing and clinically significant and are not detected by other assays [37,38]. Lofgren and coworkers [38] showed that the SPR despite its low tolerance to a therapeutic was more effective in detecting low affinity antibodies against a therapeutic mAb than an ELISA that incorporated an acid dissociation step to reduce interference from the mAb [38]. The ELISA was more sensitive for detecting high affinity antibodies and had a high tolerance to the mAb relative to SPR [38]. In addition, since SPR can be used for isotyping and kinetics, it is more suited for antibody characterization. Until now, the Biacore technology (Biacore TM, GE Healthcare) has predominantly been used for antibody detection by SPR but other instruments (for example, ProteOn XPR36, Bio-Rad Laboratories) are available and may even offer advantages over existing technology.
 - vi. **Biolayer interferometry:** Another technology which is in use for immunogenicity applications is based on the principle of biolayer interferometry (Octet, ForteBio). Like SPR, it offers label-free quantitation and kinetic analysis of antibodies in real time and detects low affinity antibodies. From limited application to date, it appears that it also has a higher tolerance to the residual therapeutic in the sample in comparison with the ECLA and a step-wise bridging ELISA [39].
 - vii. **Radioimmunoprecipitation assays (RIPA):** These assays are highly sensitive but the requirement of a radiolabel and the low throughput limits the utility of these assays in a clinical setting. In these assays, serum is incubated with a radio-labelled antigen and the resulting antigen–antibody complex precipitated using polyethylene glycol or immobilized protein A/G or antiglobulin and the precipitated radioactivity assessed. This technique is used for EPO antibodies [7,40]. More recently for antibodies directed against adalimumab, an antigen-binding assay based on protein A/G to capture Ab & labelled mAb-Fab or F(ab')₂ to detect has been used. RIPAs may not detect certain antibody isotypes (for example, IgM) but detect IgG4 antibodies and low-affinity antibodies which can, in some cases, be clinically significant [12,27,41].
- ### 3.1.1. Interpretation of results
- The importance of the appropriate interpretation of immunogenicity data in the context of regulatory oversight of these products is explained in the WHO Guidelines on BTP and SBP [15,16]. In addition to the pre-licensure evaluation of BTP and SBP, it is also important to address a need for post-marketing surveillance. Several considerations that regulators need to apply are summarized below as starting points for reviewing the immunogenicity data.
- Prior to evaluation of samples in antibody assays, it is necessary to clearly define the criteria for interpretation of results for example, antibody-positive or antibody-negative. The approach commonly adopted by industry uses a ‘cut-point’ (threshold) strategy. Recommended procedures for determination of cut-points are described in several publications [25,42]. Simplistically, samples that generate signals below a cut-point are considered negative and those that are at or above the cut-point value are positive. Often the screening cut-point is determined statistically from the level of binding seen with the use of negative controls (~50 therapeutic-naïve normal human serum samples; 3–4 assay runs) during assay validation and is based upon a targeted false positive rate of 5%. So, the cut-point = mean (negative controls) + 1.645 SD (negative controls). In some cases, there may be outliers (due to assay background and matrix effects or due to pre-existing anti-therapeutic antibody) which are excluded. If there is variability between runs, alternative approaches for example, a floating cut-point which requires a correction factor can be used. This situation is likely to occur due to differences in the biology of the samples of healthy subjects as opposed to the disease population [42].
- ### 3.1.2. Confirmatory assays
- These are necessary for elimination of false positive samples identified during the initial screen using any of the procedures stated above. A commonly adopted approach is to add excess antigen to the sample and test both the treated and untreated sample in the same assay; signal reduction in the treated versus the untreated sample confirms the presence of antibody in the sample [25,42]. Therefore, a confirmatory assay rules out the false positive

Table 2B
Methods for detection of neutralizing antibodies.

Type of assay	Advantages	Disadvantages
Bioassay	Functional assay which distinguishes antibodies with neutralizing potential Assesses neutralization capacity May correlate with clinical response	Relatively time-consuming Often variable – affected by serum (matrix) effects and interfering factors e.g., inhibitors, soluble receptors etc. Susceptible to interference by therapeutic Validation difficult e.g., cell-lines, reagents etc.
Competitive ligand binding assay	Rapid; sensitive Relatively easy to use Not affected by matrix Does not require cell-lines Easy to develop and validate	Antigen labelling may alter/denature antigen Susceptible to interference by therapeutic May not represent true functional read-out May not correlate with clinical response

samples detected in the screening assay from further evaluation. The confirmatory cut-point has been defined as the level of signal inhibition at or above which a sample is judged to have specific antibody and is statistically determined by testing negative controls (for example, drug-naïve samples) in the absence and presence of therapeutic. Confirmatory cut-point = mean inhibition +3.09 SD; where mean inhibition is mean percent change of negative controls spiked with the excess of a therapeutic before assay, from an unspiked sample (inhibition). Alternatively, protein A or G can be used to capture all immunoglobulin from a sample followed by re-testing of the sample, which should be negative if an antibody in the original sample caused the positive signal.

3.1.3. Neutralizing antibody (Nab) assays

Determination of the neutralizing potential of the induced antibodies is an essential element of immunogenicity evaluation. Neutralizing antibodies inhibit the biological activity of a therapeutic by binding to epitope(s) within the active site(s) of the molecule. Because Nabs can trigger clinical effects, specific and sensitive *in vitro* methods are needed for detection. Two types of Nab assays are mainly used; a cell-based bioassay or a non-cell-based competitive ligand binding (CLB) assay (Table 2B). The former assesses some functional aspects of the protein or mechanism of action but since these assays are time-consuming, variable, difficult to establish and validate, the use of a non-cell-based CLB assay is gaining momentum at least for some therapeutics depending on the mechanism of action.

Understanding the mode of action, the target and effector pathways of the therapeutic are critical for identification of a suitable Nab assay. Additionally, the risk of developing Nabs and the impact on clinical sequelae also needs to be considered. For a high risk product with an endogenous counterpart, a cell-based assay which is sensitive and has the capability of determining Nabs with a high specificity is required. While cell-based assays are often employed for agonistic therapeutics, CLB assays are being considered for antagonistic molecules (for example, anti-IgE) with humoral targets and are allowed by some regulatory agencies. However, for antagonists such as monoclonal antibody therapeutics with effector functions for clinical efficacy, cell-based assays are recommended as the mechanism of action (MoA) cannot be adequately reflected in the non-cell-based CLB assay. The preference by regulators of cell-based assays since they represent the physiological situation often makes them a logical first choice; however, bioassays may not be sufficiently sensitive or measure neutralization effectively. In such cases, alternative functional methods including non-cell-based CLB assays could be explored to assess Nab and advice sought from regulators if considering their use for clinical studies.

In a cell-based Nab assay, the sample is preincubated with the therapeutic and if Nabs are present, the therapeutic is unable to

bind to its target resulting in an inhibition of assay response. Various assay types can be used for cell-based Nab assays [43]. These include cell proliferation, apoptosis, phosphorylation of intracellular substrates, chemokine/cytokine release, and gene expression. Cell-based assays have inherent technical challenges – are often influenced by the sample matrix and require significant validation. For example, the assay should differentiate the antibody from other sample components for example, complement, coagulation factors, soluble receptors, lipids, concomitant medications and an administered product. Use of novel approaches such as quantitative PCR assays or reporter gene assays using chemically treated division-arrested cells can overcome some of the problems seen with cell-based assays [44,45].

Depending on the mechanism of action of the therapeutic and how it interacts with cells, the assay format can vary. A therapeutic acting directly on the cells via cell surface receptors (for example, cytokines, mAbs directed against cell surface determinants) to induce a response requires a simple assay format. However, if the therapeutic blocks the ligand from interacting with its receptor and interferes with the biological activity of the ligand (for example, mAbs against soluble factors), the format is rather complex and the assay technically challenging. Approaches for the design, optimization and qualification of Nab cell-based bioassays have been published and are a useful starting point for assay development and understanding the potential challenges that may be encountered [46]. Validation of Nab bioassays is important [47]; validation of some cell-based methods is published and provides useful guidance [48,49]. As for screening assays, a cut-point approach can be used to determine whether a sample is positive for neutralizing activity.

Non-cell-based CLB Nab assays are a simple and useful platform for Nab assessment as they do not suffer from some of the technical limitations of cell-based assays. As for ligand binding assays, any of the available detection systems (for example, enzymatic, radiochemical, fluorometric, chemiluminescence, electrochemiluminescence or SPR technology) can be used. However, CLB assays should only be used if relevant to the MoA of the product. For example, a CLB assay is appropriate in a scenario where a therapeutic mAb acts by binding to a soluble ligand thereby blocking it from interacting with its receptor thus inhibiting the biological action of the ligand. Since the assay procedure measures binding to the target and inhibition of the binding activity if Nabs are present, it is reflective of the therapeutic's MoA.

Two formats can be used for CLB assays. In the simple direct assay, the therapeutic at a defined concentration in a microtiter plate (for capture) is incubated with the labelled target/ligand (the detector) and the resulting binding generates a signal, which is abolished if Nabs are present in the sample. Alternatively, the ligand or the receptor can be used for capture and the labelled therapeutic as detection reagent, however, this approach is claimed

to be more susceptible to interference from the therapeutic. In the complex indirect format, the ligand is immobilized and the therapeutic inhibits the labelled receptor from binding to the ligand so there is very little or no signal generated. However, if Nabs are present, these will bind to the therapeutic and prevent it from blocking the binding of the labelled receptor to the ligand resulting in an increased signal. A reverse format is also feasible. Based on limited information available, CLB assays seem promising in terms of performance and sensitivity at least for some therapeutics but not all. Therefore, the utility of these assays in the clinical setting is certainly a viable option [50,51].

Evidence regarding neutralizing antibodies and clinical impact has mainly been amassed from data derived from cell-based Nab assays. An association of neutralizing antibodies with a loss of efficacy and relapse in interferon-beta treated multiple sclerosis (MS) is well documented [4,52]. Clinical trials in MS with Natalizumab (Tysabri) also showed that neutralizing antibodies were associated with loss of efficacy in patients [53]. Similarly, in GM-CSF treated patients, results from neutralization assays correlated with the effect of antibodies on clinical response [54]. However, there are also examples in which Nabs appear to have no impact on drug efficacy, pharmacodynamics or adverse events. Therefore, when interpreting data from antibody assessments, it is important to ensure that the results of Nab assays are evaluated in the context of other clinical endpoints to determine their significance in terms of clinical outcome.

3.1.4. Comparative immunogenicity

As stated in the WHO Guidelines on SBPs [16], immunogenicity of similar biotherapeutic products and the reference product should be evaluated in head-to-head comparative clinical trials in the most sensitive patient population and employ the same clinical protocols (for example, routes of administration, treatment schedules, sampling procedures, sampling time points including baseline/pre-treatment sample and storage conditions) and antibody assays for the generation of valid comparative data. A homogeneous population for each arm of the study is important. The amount of immunogenicity data needed is dependent on experience gained with the reference product and/or the product class. Although data for products used chronically should usually be collected over a 12 month period, a shorter duration may be justified based on the immunogenicity profile of the reference product. For example, if antibody development for a reference product occurs within six months of treatment initiation, collection of immunogenicity data for less than one year pre-licensing may be justified with submission of data for the additional time-period (up to one year) post licensure. It should be noted that antibody assays should be conducted using the product given to the patients and data reporting should include antibody incidence, kinetics and onset of response, transient or persistent, relative titers and characteristics of the induced antibodies. Levels of a therapeutic in the samples should also be determined. Following this, a meaningful evaluation of antibody data with clinical data (PK, PD, other endpoints) is needed to determine the significance of antibodies in terms of clinical outcome [16,17]. If significant differences in an immunogenicity profile of the two compared products are detected, it can be concluded that the products are significantly different. While a lower immunogenicity of the biosimilar as opposed to the reference product does not preclude approval as a biosimilar, all data needs to be examined in the context of totality of evidence available for the biosimilar product. Reduced development of neutralizing antibodies in the case of a biosimilar may erroneously suggest that the biosimilar is more efficacious than the reference product. In such a situation, further subgroup analysis of patients, both antibody positive and antibody negative for the two products

may be helpful to establish that the efficacy of the two products is similar [18]. It should be noted that comparison of immunogenicity data of the biosimilar with historical data of the reference product obtained in clinical trials in other studies is invalid.

4. Conclusions

A systematic evaluation of immunogenicity is necessary for approval of all BTPs and biosimilar medicines. Assessment of immunogenicity should be included in post-marketing monitoring and considered in risk management and risk mitigation plans for all products. With such safeguards and pharmacovigilance in place, the risk of immunogenicity of a biotherapeutic should be minimized resulting in better and safer products.

The importance of having a good understanding of immunogenicity as an important characteristic of biotherapeutic products is an essential requirement for reviewing the data generated for product approval. In reality, a specific problem encountered by regulators globally is limited information that they have when they are reviewing manufacturers' dossiers some practical considerations for using immunogenicity assays for evaluation of monoclonal antibodies and interpretation of results were discussed in WHO workshop held in Seoul 2014 and the case studies from that workshop are available [55]. Immunogenicity assays are part of product development and the expertise and experience with assay selection, assay execution and interpretation of results of particular assays are best understood by the developers of these assays. Although there are well resourced regulatory agencies with appropriate specialists for immunogenicity assessment of BTPs, in many countries this assessment is creating a problem when it comes to the regulatory decision making process. Sometimes, it is also a missed opportunity for regulators to address a need for post-marketing surveillance with a specific focus on the immunogenicity aspects of a product. Therefore, this article is intended as a first step in bringing the complex issue of immunogenicity of biotherapeutic products including biosimilars for the attention of regulators who are in charge of regulatory oversight of products of great importance for global public health.

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Conflict of interest

The authors have disclosed no potential conflicts of interests.

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