

Review

Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biologicals

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Abstract

An important aspect of evaluating the safety of therapeutic biologicals is the assessment of the unwanted immunogenicity of such biologicals in recipients. Properly planned immunogenicity studies with appropriately devised strategies are critical if valid conclusions concerning the unwanted immunogenicity are to be derived. Such studies need to be conducted using carefully selected and validated procedures. Several techniques are available for detection and measurement of immunogenicity including immunoassays, radioimmunoprecipitation assays (RIPAs), surface plasmon resonance (SPR) and bioassays. A combination of methods for characterization of the induced antibodies is usually necessary for a detailed understanding of the type(s) of antibodies generated against a therapeutic product. This review considers the benefits and limitations of the various techniques available for antibody detection and outlines a strategy for the assessment of unwanted immunogenicity of therapeutic products.

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1. Introduction

Unwanted immunogenicity of biological products is now recognised as a serious problem (Porter, 2001; Stein, 2002). Such immunogenicity can be expected with nonhuman sequence proteins of sufficient size (Chance et al., 1976; Thistlethwaite et al., 1988; Niaudet et al., 1993), but it is now established that

antibody formation can occur in immunocompetent recipients after treatment with products derived from human sera and tissues (Richards et al., 1993; Rosenberg et al., 1999) and also with rDNA-derived products that are identical or nearly identical in sequence to native human proteins (Kaplan et al., 1986; Antonelli et al., 1991, 1999; Steis and Longo, 1994; Wadhwa et al., 1996, 1999; Prummer, 1997; Casadevall et al., 2002). An additional consideration for patients with genetic disorders is that the endogenous protein may be absent, mutated or produced in a conformation that renders it nonfunctional. Consequently, the administered therapeutic protein could be recognised as for-

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Table 1
Examples of biological proteins reported to be immunogenic in humans

Protein category	Protein	Type and producer cells	Indication	Binding antibodies	Neutralizing antibodies	Clinical consequences
Nonhuman proteins	Staphylokinase	Natural	Acute myocardial infarction	Yes	Yes	Anaphylactic and anaphylactoid reactions, further therapy precluded
	Calcitonin	Natural	Hypercalcemia	Yes	Yes	Loss of efficacy
	Insulin	Natural	Diabetes	Yes	Yes	Loss of efficacy uncommon
Human proteins	Glucocerebrosidase ^a	Natural	Type I Gaucher disease	Yes	Yes	Loss of efficacy
	Factor VIII ^b	Natural	Haemophilia A	Yes	Yes	Loss of efficacy
	Chronic gonadotrophin releasing hormone ^c	Natural	Infertility	Yes	Yes	Loss of efficacy
Homologous to native proteins	Follicle stimulating hormone ^c	Natural	Infertility	No	No	–
	IFN- α 2a	rDNA-derived (<i>E. coli</i>)	Hairy cell leukemia, Kaposi's sarcoma, chronic myeloid leukemia, hepatitis	Yes	Yes	Loss of efficacy
	GM-CSF	rDNA-derived (<i>E. coli</i>) ^d	Myeloregeneration, immunostimulatory therapies	Yes	Yes	Loss of efficacy
		rDNA-derived (<i>E. coli</i>) ^d		Yes	No	No loss of efficacy
	G-CSF	rDNA-derived (<i>E. coli</i> , CHO)	Myeloregeneration, neutropenia	No	No	No loss of efficacy
	IFN- β	rDNA-derived (CHO)	Multiple sclerosis	Yes	Yes	Loss of efficacy not reported
Sequence variants	Epo	rDNA-derived (CHO)	Anaemia	Yes	Yes	Cross-reacted with endogenous protein and caused adverse effects
	Insulin	rDNA-derived (<i>E. coli</i>)	Diabetes	Yes	Yes	Loss of efficacy not reported
	IL-2 ^e	rDNA-derived (<i>E. coli</i>)	Renal cell carcinoma	Yes	Yes	Loss of efficacy associated with both types of antibodies
	IFN- β	rDNA-derived (<i>E. coli</i>)	Multiple sclerosis	Yes	Yes	Loss of efficacy
	GM-CSF	rDNA-derived (yeast)	Immunostimulatory therapies	Yes	Yes	Not studied
Chemically modified	IFN- α Con 1	rDNA-derived (<i>E. coli</i>)	Hepatitis C	Yes	No	Loss of efficacy not reported
	Pegylated MGDF	rDNA-derived (<i>E. coli</i>)	Cancers/normal volunteers	Yes	Yes	Cross-reacted with endogenous protein and caused adverse effects
Hybrid molecules	GM-CSF/IL-3 hybrid (PIXY 321)	rDNA-derived (yeast)	Chemotherapy-induced leukopenia in cancer patients	Yes	Yes	Clinical efficacy abrogated
	TNFR2-Ig	rDNA-derived (CHO)	Rheumatoid arthritis	Yes	No	No correlation with clinical responses or adverse effects

^a rDNA-derived preparation less immunogenic than the natural product (rDNA-derived versions licensed).

^b rDNA-derived product showed higher rate of antibody formation which was associated with loss of efficacy (rDNA-derived versions licensed).

^c rDNA-derived product non-immunogenic (rDNA-derived versions licensed).

^d Two preparations from different manufacturers.

^e Naturally derived IL-2 less immunogenic.

eign and elicit an immune response (Dietrich et al., 1979; Jacquemin and Saint-Remy, 1998).

The incidence and characteristics of the induced antibodies are variable and dependent on several criteria which include the product (the type of product—the structural or sequence difference from the native protein, impurity profile, formulation, etc.), the dose and duration of treatment, the frequency of dosing, the route of administration, the immune status and/or genetic profile of the patient, the disease type and the functional activities of the protein itself (Gribben et al., 1990; Prummer, 1997; Runkel et al., 1998; Wadhwa et al., 1996; Ullenhag et al., 2001; Porter, 2001; Stein, 2002). Some biologicals such as granulocyte colony stimulating factor (G-CSF) and interferon- γ (IFN- γ) appear to be non-immunogenic, whereas other proteins such as insulin (Scherthaner, 1993), growth hormone (Moore and Leppert, 1980; Kaplan et al., 1986; Buzi et al., 1989; Massa et al., 1993), factor VIII (Jacquemin and Saint-Remy, 1998; Scandella, 2002) as well as cytokines such as interferon- α (IFN- α), interferon- β (IFN- β), interleukin-2 (IL-2) and granulocyte macrophage colony stimulating factor (GM-CSF) have been reported to induce antibody formation (Table 1) (Gribben et al., 1990; Antonelli et al., 1994, 1999; Ragnhammar et al., 1994; Scharenberg et al., 1994; Steis and Longo, 1994; Von Wussow et al., 1994; Oberg and Alm, 1997; Prummer, 1997; Khan and Dhib-Jalbut, 1998; Antonelli and Dianzani, 1999; Wadhwa et al., 1999). Some therapeutic antibodies (Breedveld, 2000) are also associated with immunogenic responses (Stein, 2002; Baert et al., 2003), and attempts are continuing to design variant molecules with a reduced immunogenic profile in humans (Table 2).

The effects of antibody development can range from no apparent adverse effects to significant adverse reactions and impaired clinical responses to treatment.

Table 2
Antibody responses following therapy with antibody products

Antibody product	Incidence of antibody formation (%)
Murine	
Whole antibodies	55–80
Fab or Fab' fragments	<1–8
Chimeric	1–13
Humanized	<1–8

Data taken from Stein (2002).

In certain cases, some patients develop antibodies which neutralize the biological activity of the therapeutic product and become unresponsive to treatment. The first reports of such neutralizing (or 'inhibitor') antibodies followed factor VIII replacement therapy in patients with haemophilia A (Roberts and Cromartie, 1984); these patients subsequently require factor VIII 'bypass' therapies (e.g., immunosuppressive therapy in combination with high doses of factor VIII, plasmapheresis, etc.) to overcome the effects of the induced antibodies (Saint-Remy, 2002). Recombinant staphylokinase treatment in patients with myocardial infarctions can also rapidly induce neutralizing antibodies which may reduce its efficacy or induce allergic reactions (Declerck et al., 1994; Vanderschueren et al., 1994, 1995). The development of neutralizing antibodies that appear to compromise therapy has also been reported for some cytokines, e.g., GM-CSF (Wadhwa et al., 1996, 1999) and IFN- α (Steis et al., 1988). In other cases (e.g., IL-2), both neutralizing and non-neutralizing antibodies may affect the clinical response (Hjelm Skog et al., 2001). In some instances, as reported recently for megakaryocyte growth and differentiation factor (MGDF) which is a truncated version of thrombopoietin, and for erythropoietin (Epo), the antibodies neutralize the endogenously produced biological protein, which can result in serious clinical consequences (Li et al., 2001; Casadevall et al., 2002).

The complexity of unwanted immunogenicity requires careful selection, validation and interpretation of procedures used for its evaluation. It is normally necessary to use a combination of methods for characterization of the induced antibodies. It is clear that use of different methods can produce differing data, and this review considers the techniques available and their advantages and shortcomings. A strategy for the assessment of unwanted immunogenicity of cytokine products is also suggested.

2. Current methods for detection and characterization of antibodies

A prerequisite for studies on immunogenicity of therapeutic proteins is the ability to detect and characterize antibodies using suitable and appropriate methods that can provide accurate and precise data. In theory, a range of techniques exists for investigating

the presence of antibodies generated against therapeutic proteins in biological fluids (Table 3). These include immunochemical methods such as binding assays and immunoblotting or biophysical methods such as surface plasmon resonance (SPR) which are mainly employed for detection of binding antibodies. Bioassays which are imperative for assessing the functional characteristics of the antibodies, i.e. distinguishing whether they are neutralizing or non-neutralizing, are also essential in most cases (Swanson et al., 1999, 2002; Wadhwa et al., 1999; Indelicato, 2003).

2.1. Assays which measure binding

Binding assays use the specific interaction of antibody to antigen to provide quantitative informa-

tion about antibody (or antigen) concentration in unknown samples. Several types of assay formats can be used for detection of binding antibodies; some of these are briefly described.

2.1.1. Solid phase binding immunoassays

These assays are most commonly used for antibody detection. The serum or plasma samples are incubated with the antigen which has been previously immobilized directly onto microtitre plate well surfaces. Bound antibody is then detected using a radiolabelled or an enzyme-labelled anti-immunoglobulin reagent of appropriate specificity, the latter method termed enzyme-linked immunosorbent assay or ELISA (Fig. 1A).

In general, such immunoassays are fairly sensitive for detection of antibodies. However, there are many

Table 3
Methods used for detection of antibodies

Type of assay	Parameter measured	Advantages	Disadvantages
Binding assay	Identifies antibodies capable of binding to the antigen preparation.	Rapid. Relatively easy to use. High throughput assay—often used as a ‘screening assay’ for antibody detection. Sensitivity is good.	Can give misleading results due to spurious binding and/or nonspecific ‘matrix effects’. May fail to detect ‘low-affinity’ antibodies. Appropriate detection reagents necessary. Some formats require immobilization of the antigen which may alter the conformation of the native protein.
Immunoblotting	Assesses the specificity of the antibodies for protein in antigen preparations.	Dissects the specificity of the antibodies. Provides profile of reactivity against subcomponents of the product. Sensitivity is good.	Nonquantitative. Relatively low throughput. May fail to detect antibodies which do not recognize antigen after SDS-PAGE separation. Appropriate detection reagents necessary.
Surface plasmon resonance	Shows antigen–antibody interaction in real time.	Automated. Provides information on the specificity, isotype, relative binding affinity and relative concentration. Enables detection of ‘low-affinity’ antibodies.	Expensive. Requires dedicated equipment. May require immobilization of the antigen which may alter the conformation of the native protein. Sensitivity usually less than binding assay.
Bioassay	Assesses the neutralizing potential of the antibodies.	Functional assay which distinguishes antibodies with neutralizing potential. Quantitates antibodies which neutralize the biological activity of the antigen. May correlate with clinical response.	Relatively time-consuming. Often variable. Can be affected by nonspecific serum (matrix) effects and non-antibody neutralizing factors, e.g., inhibitors, soluble receptors, etc. Validation can be difficult because of the reagents, e.g., cell-lines, etc.

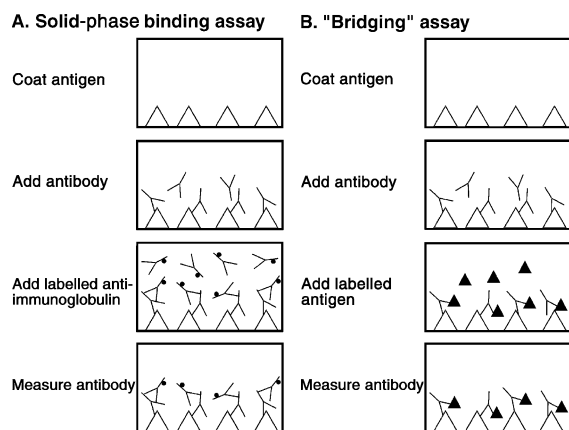


Fig. 1. A diagrammatic representation of two different immunoassay formats.

drawbacks associated with these types of assays (described in detail elsewhere in this review; refer to Table 3). Nonspecific binding is often a major concern and alternative strategies may need to be considered. In some cases, at least adopting a 'bridging antibody' format in which antibody is captured by solid phase bound antigen and detected using liquid phase labelled antigen (Fig. 1B) can produce binding assays with high specificity (antigen must be recognised twice by antibody for detection) and acceptable sensitivity.

Another inherent difficulty with any immunoassay that requires multiple reagent additions followed by wash cycles is that antibodies of low affinity, especially those antibodies with rapid dissociation rates, may not be detected. Extensive washing of microtitre plates during the assay procedure may remove low-affinity antibodies. Such low-affinity antibodies, however, may have a high antigen binding capacity and/or ability to neutralize the bioactivity of the therapeutic protein, and it is important, therefore, that these be detected. Therefore, alternative techniques such as surface plasmon resonance (SPR) should be included in the screening exercise as these assays are capable of detecting antibodies that may have been missed using other assay types (Swanson et al., 1999).

2.1.2. Surface plasmon resonance (SPR) assays

In these assays, interactants can be detected directly without the use of labelling techniques by measuring the binding of ligands (e.g., antibodies) to

immobilized molecules (e.g., antigens) on a sensor chip surface which consists of a carboxymethyl dextran layer covalently attached to a gold film coated onto a glass slide. Ligands can be covalently bound to the matrix layer of the sensor chip, and subsequent binding to the immobilized ligand can be quantitatively measured using biospecific interaction analysis (BIA) technology (Fagerstam et al., 1992; Malmqvist, 1993). Several instruments, e.g., BIA-CORE 3000™ (and others with different formats), are available for studying molecular interactions in real time. As polarized light enters across the metal film on the sensor chip surface under conditions of total internal reflectance, a signal is generated as a dip in reflective light intensity at a specific angle. This signal is produced as a sensorgram and represents a plot of the SPR angle as a function of time. The data are reported in response units and corresponds to the amount of sample bound to the immobilized ligand.

The use of SPR provides significant advantages in comparison with traditional binding immunoassay methods and has been used to detect and characterize antibodies to several cytokines including IL-10 (Swanson et al., 1999) and PEG-IFN α 2b (Takacs et al., 1999). SPR is a direct assay that does not require the use of secondary labelled reagents and is very specific. In addition, SPR uses covalent attachment of the ligand to the carboxymethyl layer (which results in more predictable effects on conformation) rather than the less defined passive adsorption onto the solid plastic surface of a microtitre well used in many immunoassays. Moreover, results can be obtained rapidly, within only a few minutes, since long sample incubations are not required. An important attribute of this methodology is that the antibodies can be characterized easily and rapidly in terms of determination of their relative affinity because the dissociation of the antibody from the immobilized surface can be directly observed (Fig. 2). As long as a sufficient amount of data is collected during the dissociation phase, a gross interpretation regarding the relative affinity of the interaction can be made. SPR can also detect populations of antibodies that have lower affinity and rapid dissociation rates and have been missed in binding assays. This detection is a result of the real-time sample analysis that allows monitoring of sample association and then

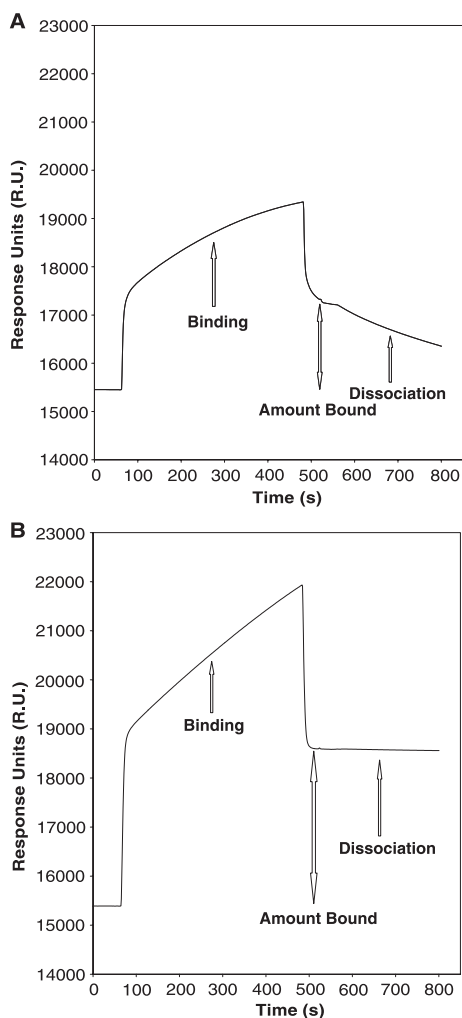


Fig. 2. SPR sensorgrams showing binding and dissociation of two samples on a sensor chip immobilized with GM-CSF using a BIACORE 2000. A serum sample from a patient treated with GM-CSF (A) and human anti-GM-CSF plasma (B) which was used as a positive control (available from NIBSC—coded 97/538). The arrows indicate binding of the antibodies, the amount bound and the dissociation of the antibodies. The data show that the two samples differ in their dissociation profile. Panel A shows a rapid dissociation indicating the presence of 'low-affinity antibodies' as compared with Panel B which has a slow dissociation indicative of 'high affinity antibodies'.

dissociation. By using SPR to screen the sera of patients for the presence of antibodies to a therapeutic product, all antibodies capable of binding to the product can be detected. Proper assay validation is crucial for avoiding misinterpretation of results

(Swanson et al., 1999, 2002; Wong et al., 1997). It should be noted, however, that the antibodies detected require further characterization in a biological assay for determination of their neutralization potential (Swanson et al., 1999; Wadhwa et al., 1999).

2.1.3. Immunoblotting

The methods discussed above can provide valuable information concerning the presence (or absence) of antibodies which recognise the relevant antigen(s). Quantitative and sensitive data can be generated if an appropriate assay format is adopted. However, none of the procedures directly address the specificity of the antibodies detected. Unless the antigen preparation used is completely homogeneous, it cannot be assumed that the antibodies detected have specificity for particular components. In some cases, minor components in a product can be highly immunogenic which can cause data to be misinterpreted. For example, very low levels of expression system-derived bacterial proteins in rDNA-derived products can cause significant antibody development, whereas the major protein present (the active principle) may be much less immunogenic. Assessment of antibody specificity normally requires the use of a method which separates components present in the antigen and then determines which of these is recognised by the antibodies. Immunoblotting is usually ideally suited for this, although other procedures, e.g., radioimmunoprecipitation assays (RIPAs), can be used.

For immunoblotting, SDS-PAGE run under reducing or nonreducing conditions is most commonly used to separate protein components in the antigen. Following transfer of protein (normally electrophoretically) from the SDS-PAGE gel to a supporting membrane (usually nitrocellulose-based), the blot is probed with patient's serum followed by appropriately labelled anti-immunoglobulin reagent (Wadhwa et al., 1999). Sensitivity can be maximised by the use of enhanced chemiluminescence (ECL)-based detection systems. Immunoblotting must be carefully controlled to assess the validity of the data generated, and it must be remembered that some antibodies will fail to bind to antigenic components after they have been subjected to SDS-PAGE.

2.1.4. Radioimmunoprecipitation assays (RIPAs)

RIPAs can be used to assess serum or plasma samples for the presence of antibodies against relevant antigens. For this, serum is incubated with a radio-labelled antigen and antigen–antibody complexes precipitated by addition of an appropriate reagent, e.g., immobilized protein A or G or antiglobulin. The precipitate is assessed for antibodies specific for the antigen by counting the radioactivity present in it. The technique is often coupled with antigen analysis in the precipitate to allow assessment of the antigen components bound by antibody, e.g., by SDS-PAGE/autoradiography. The procedure is very difficult to automate, and sample throughput is normally slow. RIPAs can be prone to artefacts, and the radiolabelling process can mask/denature epitopes recognised by antibodies. However, in some cases, these assays can be very sensitive and useful for antibody detection as has been shown for erythropoietin (Casadevall et al., 2002).

2.2. Assays which assess neutralizing capacity

These assays are essential for establishing and/or quantifying the neutralizing activity of antibodies that have developed following therapy with a product. Assessment of neutralization potential requires testing of samples in an assay which assesses the biological activity of the product (Wadhwa et al., 1999). This is usually a bioassay since such assays alone are capable of measuring bioactivity of biologicals (Wadhwa and Thorpe, 1998a,b; Thorpe et al., 1999). Bioassays are often associated with potential problems which must be controlled. For example, bioassays can be sensitive to nonspecific serum (matrix) effects and prone to interference with non-antibody inhibitory factors such as soluble receptors, binding proteins, etc. (Wadhwa and Thorpe, 1998a,b; Thorpe et al., 1999). It is crucial to establish that any inhibitory activity detected in the bioassay is essentially attributable to the neutralizing activity of the specific antibodies present in samples in order to prevent misleading conclusions about the immunogenicity of the therapeutic product. This can only be accomplished by conducting experiments using spiked sera to ensure confidence in the assay used and by using assays that have been properly validated. Proper validation can be achieved by using strategies which involve inclusion of carefully select-

ed samples known to be negative and/or positive for relevant antibodies (Indelicato, 2003).

3. Problems associated with the detection and characterization of antibodies

Several factors need to be considered when setting up assays for antibody detection. These relate to the nature of the sample, the nature of the therapeutic product and the assay format used for antibody detection.

3.1. Nature of sample

The nature of the biological fluid undergoing evaluation for the presence of antibodies can have a considerable impact on the performance of the assay. Samples for antibody analysis are often serum or plasma, but other biological fluids may also be used. Biological fluids usually contain a complex milieu of components (other than the antigen), albeit in variable amounts which can affect the assay in a nonspecific manner. In some instances, biological fluids may contain naturally occurring antibodies or antigen–antibody complexes which can pose problems for antibody determination.

3.1.1. Matrix effects

Matrix effects, i.e. phenomena influencing assays which are unrelated to the analyte and are primarily due to the sample composition, can considerably affect some immunoassays (Thorpe et al., 1997; Wadhwa and Thorpe, 1998a,b; Banks, 2000). These effects are a major concern as serum or plasma often used for antibody detection assays can influence assays and yield ‘false positive’ results. Rheumatoid factors and/or heterophilic antibodies present in serum or plasma may cross-react with antibodies in the assay format and provide high background values contributing to antigen nonspecific binding (Hennig et al., 2000). In other instances, soluble receptors or binding proteins may impair antigen–antibody interaction and produce ‘false negative’ results. Certain serum samples (sometimes called ‘sticky’ samples to reflect the unknown nature of the interference) may contain nonspecific substances which interfere with antibody assays (Wadhwa et al., 2000). Therefore, it is essential

to include appropriate controls in antibody detection assays (Thorpe et al., 1997). A pretreatment sample taken from the same individual as the posttreatment sample is often the best control for inclusion in antibody assays. If the pretreatment sample gives a positive result, the nature of the cause (specific or nonspecific) should be established.

3.1.2. Naturally occurring antibodies

In addition to various interfering substances, biological fluids may also contain preexisting naturally occurring apparently specific antibodies to a variety of proteins, e.g., cytokines and adhesion molecules. Such antibodies have been reported for some cytokines in sera of both healthy and diseased individuals, al-

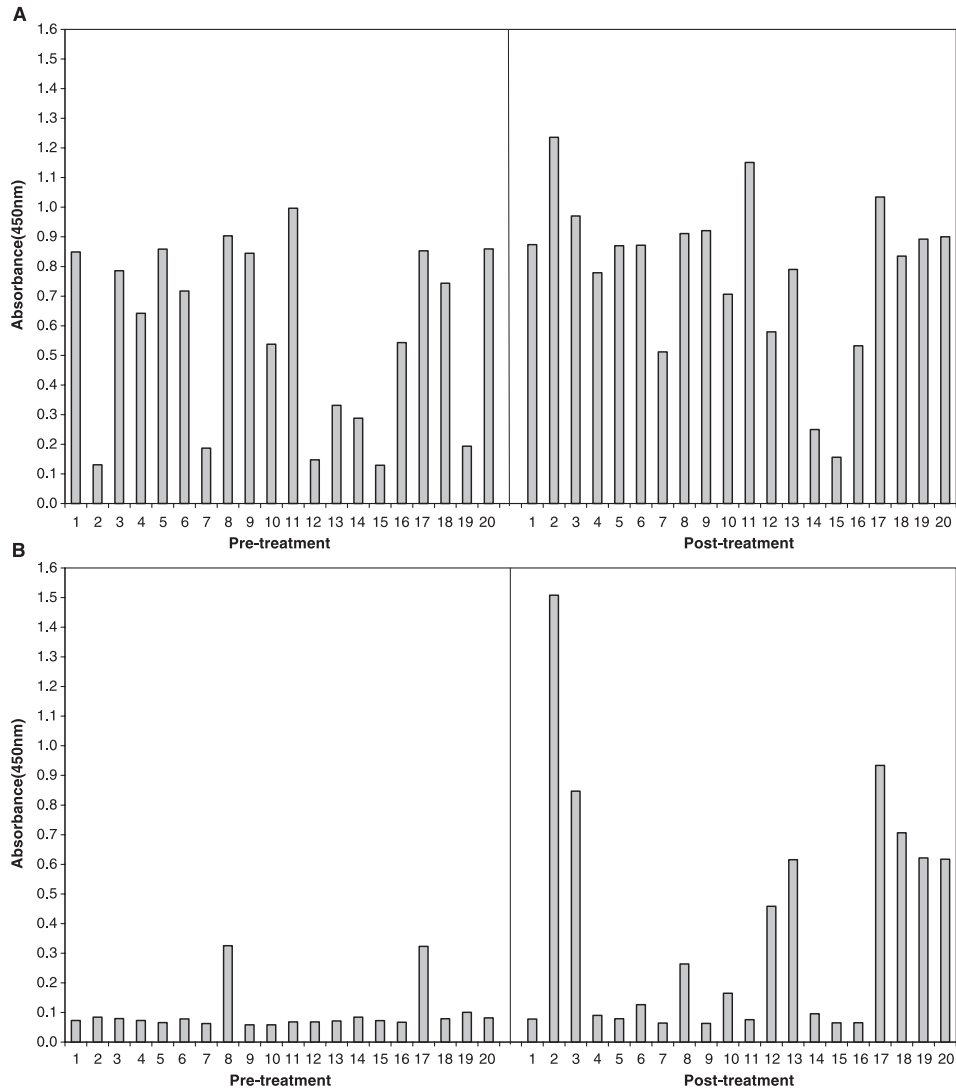


Fig. 3. ELISA data showing binding profile using sera from a cohort of cancer patients (numbered 1–20) pre- and posttreatment with yeast-expressed GM-CSF (in combination with a tumor antigen). Plates were coated with GM-CSF expressed in yeast (Panel A) or *E. coli* (Panel B). Serum samples were diluted 1/20 in all assays. Human anti-GM-CSF plasma (97/538; available from NIBSC) obtained from a patient known to contain antibodies which bind GM-CSF was used as a positive control in all assays. This gave an absorbance value of 0.91 and 1.12 in binding assays using yeast- or *E. coli*-expressed GM-CSF preparations, respectively.

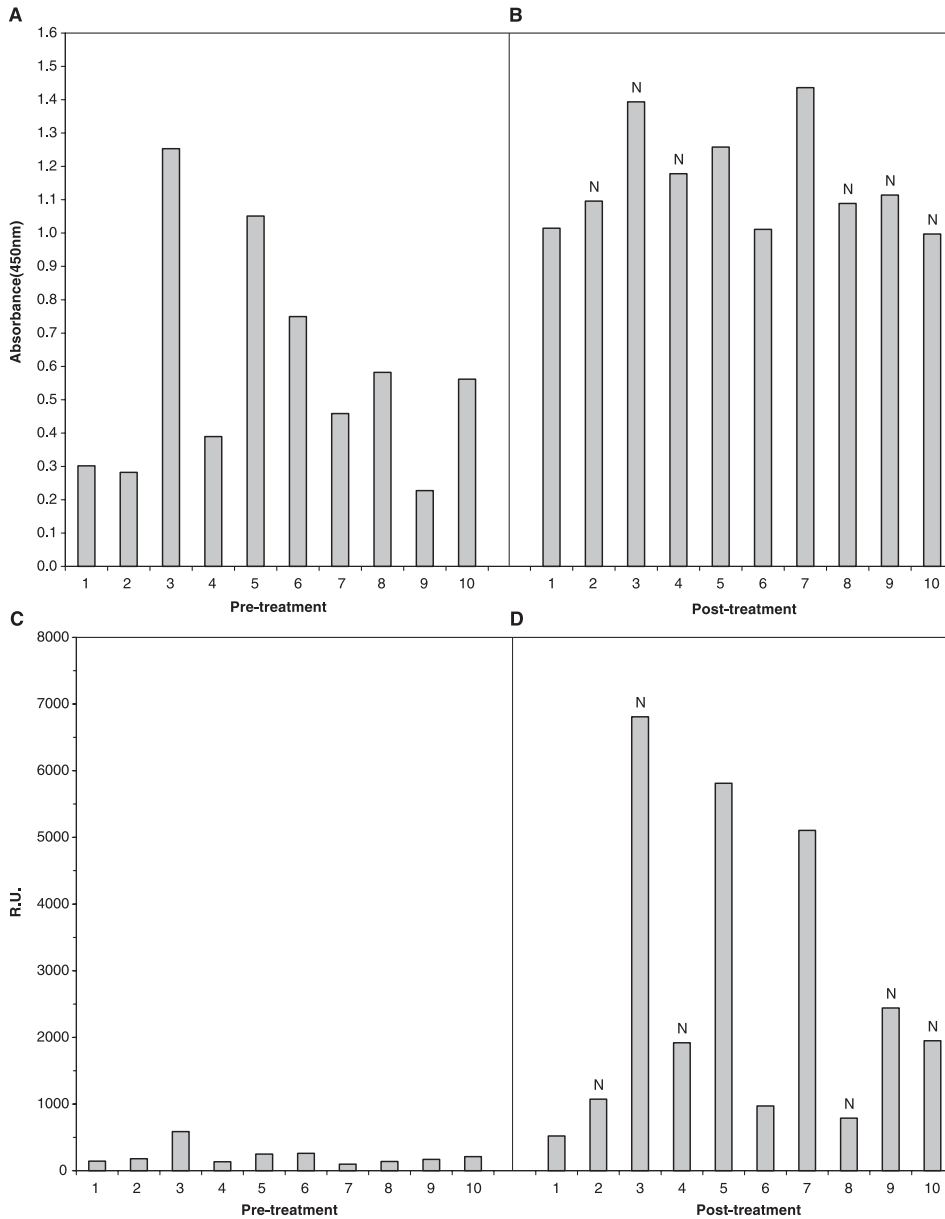


Fig. 4. Data showing binding profile using sera from a group of patients (numbered 1–10) pre- and posttreatment with yeast-expressed GM-CSF alone in ELISAs (Panels A and B) and SPR assays (C and D). In these experiments, serum samples were diluted 1/20 for ELISAs and 1/5 for SPR assays. The yeast-expressed GM-CSF product was used as the antigen in all assays. For SPR assays, GM-CSF was immobilized onto a sensor chip surface according to the manufacturer’s instructions and bound ~ 2900 response units. N indicates that the sample neutralized the biological activity of GM-CSF in a bioassay. Human anti-GM-CSF plasma (97/538; available from NIBSC) obtained from a patient known to contain antibodies which bind GM-CSF was used as a positive control in all assays. This gave an absorbance value of 1.14 in ELISA procedures and a response unit of 2340 in SPR assays. This figure also indicates that serum samples showing similar high binding responses may or may not neutralize the bioactivity of GM-CSF.

though the levels may differ between normal and patient populations (Ross et al., 1990; Hansen et al., 1993; Amiral et al., 1996; Jouvenne et al., 1996; Menetrier-Caux et al., 1996; Meager et al., 1997, 1999). As stated earlier, inclusion of relevant pre-therapy samples in antibody detection assays should be considered for a proper interpretation of the results obtained.

3.1.3. Antigen–antibody complexes

In some instances, samples for antibody analysis from individuals or animals may contain immune complexes or even large amounts of the product itself depending on the pharmacokinetics of the product. Appropriate validation of methods with particular types of biological fluids is therefore very important to assure appropriate assay performance and ensure confidence in the results obtained. If the assay format is such that it does not recognise antibodies that are bound to the antigen, a strategy to dissociate the complexes may be considered. Chromatographic separation of dissociated complexes may sometimes be necessary for confirming the presence of antibodies (Wadhwa et al., 2000).

3.2. Characteristics of the biological preparation

The characteristics of the therapeutic biological against which antibodies are being assessed can significantly influence the type of assay(s) used for antibody detection. For example, in studies using ELISAs for detection of antibodies against yeast-expressed GM-CSF, pretreatment serum samples from some of the patients showed reactivity with the yeast-expressed GM-CSF product (when immobilized) and also with another yeast-derived protein, IL-3, indicating that the binding to the yeast-expressed GM-CSF was unrelated to GM-CSF itself and spurious. This reactivity was, however, not evident in ELISAs in which GM-CSF preparations derived from other expression systems (e.g., *Escherichia coli* or CHO cells) were used as the antigen (Fig. 3), or when SPR was employed for detection of GM-CSF antibodies (Fig. 4). In certain cases, antibodies may not only be raised against the active biological substance in a product, but also against the excipients that have been used for the formulation of the product. In some instances, antibodies may be generated against nonproduct-re-

lated contaminants such as host cell-derived proteins or other minor constituents which may have been co-purified with the active component of the product. For instance, patients treated with a GM-CSF product expressed in *E. coli* developed antibodies against GM-CSF and also against *E. coli* proteins which were present as trace contaminants within the product. This was confirmed by conducting immunoblotting experiments with lysates of the *E. coli* strain used for expression of the GM-CSF protein as shown in Fig. 5 and also adsorption studies with the relevant bacteria (Wadhwa et al., 1999).

Enhanced immunogenicity of a product may also be related to modifications induced within the product during preparation, formulation and storage (Cleland et al., 1993; Konstek et al., 1999). Oxidation of amino acid residues (Konsstek et al., 1999), formation of aberrant forms such as aggregates (Moore and Lepfert, 1980; Palleroni et al., 1997; Braun et al., 1997; Konstek et al., 1999), degradation products (Josic et al., 1999) or clipped or deamidated forms in the product can also induce an immunogenic response. An example of this type of response is illustrated in

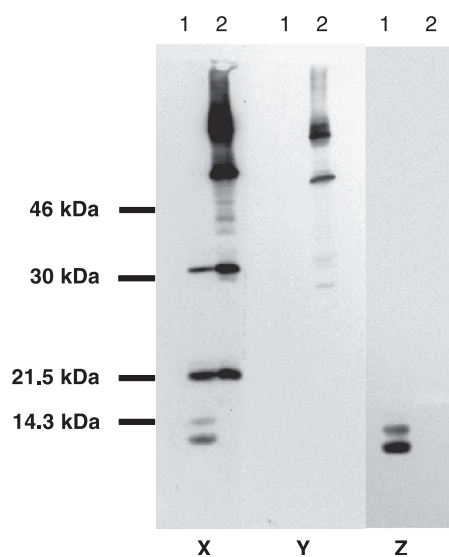


Fig. 5. A comparison of the binding pattern observed by immunoblotting of an *E. coli*-expressed GM-CSF preparation (lane 1) and an *E. coli* lysate used for expression of GM-CSF (lane 2) using serum from a patient pre- (Panel Y) and posttreatment (Panel X) with the same GM-CSF preparation. A specific sheep anti-GM-CSF serum (Panel Z) was used as a positive control (Wadhwa et al., 1999).

Fig. 6. These moieties, however, may not necessarily be detected if they do not occur in sufficient amounts or only appear transiently.

3.2.1. Antigen conformation

During the process of immobilization of the antigen in binding assays, it is essential that the native conformation of the antigen is maintained. If the conformation of the antigen is altered in a manner that entails masking/blocking of the epitopes, the antibodies specific to that epitope may not be recognised. In most ELISAs, the antigen of interest is coated directly on plates as described earlier. In some cases, this approach may alter antigen conformation and subsequently result in an underestimation of samples that contain antibodies. In such situations, the use of alternative strategies, e.g., SPR assays or

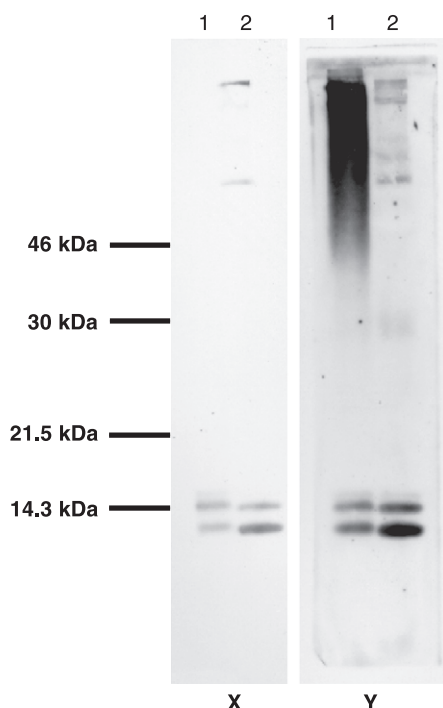


Fig. 6. Results obtained by immunoblotting of two GM-CSF products, A (lane 1) and B (lane 2) with sera from a patient treated with product A (Panel Y). A specific sheep anti-GM-CSF serum was used as a positive control (Panel X). Strong recognition of GM-CSF protein which migrated as three close bands at approximately 15 kDa is evident in both panels. In Panel Y (lane 1), there is also evidence of a heterogeneous smear indicating the presence of high molecular weight aggregates in the GM-CSF product.

different types of ELISA formats, may overcome such problems. In the case of IFN- β , it has been shown that ELISAs in which the antigen was captured by a specific monoclonal antibody, or in which biotinylated antigen was captured by streptavidin (coated on plates), permitted detection of antibodies that were capable of recognising the antigen in its native state. Some of the antibodies that were detected using these strategies were not recognised in direct ELISAs (Brickelmaier et al., 1999).

4. Interpretation and quantitation of results of antibody assays

One of the most difficult issues associated with assays for antibody detection is the interpretation and evaluation of the results obtained. The usual approach for evaluation of results is the identification of whether the sample contains antibodies (based on data-related criteria) followed by the quantification of the amounts of antibodies (if present) in the relevant biological sample. Obviously, the results obtained and the analysis will depend on the type of assay, the assay design used and the issue being considered, e.g., a preliminary screening assay for distinguishing samples that are positive for antibodies or a careful and thorough quantitation of antibody levels in the unknown sample. It is very important to ensure that adequate control samples have been included to assure confidence in the results obtained with use of a particular assay. Standardization of antibody assays is difficult. In some instances, hyperimmune serum from animals immunised with the product can be used as a positive control if other appropriate reference reagents or antibody standards are not available. This approach of using a positive control serum in assays is very valuable as a performance indicator and for assay validation. However, the use of an antibody standard for quantitation of antibody levels (rather than as a performance indicator for assays) raises important scientific issues. Polyclonal antibodies are a heterogeneous mixture of antibodies with differing characteristics. Furthermore, the amounts of the antibodies present in different samples may vary considerably. Use of an appropriate assay design should also be considered if antibody levels are to be correctly determined.

4.1. Antibody binding assays

These assays are generally used for screening and identifying positive samples. In these assays, the general procedure adopted for establishing the background of the assay is by testing a number of appropriate negative control samples in the assay for determination of a threshold or 'cutoff value' for the assay. Any sample with a value below the threshold is considered to be negative. For identification of a 'positive' sample, the use of at least $2 \times$ the mean value obtained for the background is commonly used in many laboratories (Mire-Sluis, 2002). In ELISAs, results are normally expressed as an absorbance value. A high absorbance value indicates that the sample has high capacity for binding to the antigen and is positive for the presence of antibodies.

For studies on antibody binding by SPR, the procedure used for establishing the threshold value for binding assays can also be applied. Details on this have been described by Swanson et al. (2002). In these assays, the data are expressed in response units and correspond to the amount of sample that is bound to the immobilized ligand. Typical results obtained using this assay are shown in Fig. 4. Relative antibody concentration and binding affinity may be determined by comparison of the sample with a positive antibody control.

4.2. Bioassays

After a sample has been identified as containing binding antibodies, normally, the next step is to determine whether the antibodies have the ability to neutralize the biological activity of the product. This is because data obtained from a bioassay may correlate with reduced clinical response to therapy, whereas results from binding assays may not. For example, in GM-CSF studies, it has been shown that sera, which showed high binding and, in some cases, even identical binding responses in ELISAs, differed in their ability to neutralize the biological activity of the cytokine. Similarly, sera showing high relative response units in SPR experiments, which were identical in some instances, also differed in their neutralization profile (Fig. 4). However, this is not always the case.

Following determination of samples that contain neutralizing antibodies using a bioassay, the issue of

how to express the levels of antibodies in the samples remains problematical. In several published studies, the amount of antibody is expressed as a 'titre' value. However, the 'titre' value can vary between assays and especially between laboratories. Alternatively, one can express the neutralizing activity of a serum sample (containing antibody) by the amount of serum required to neutralize the biological activity induced by a constant amount of the antigen in the assay used. For example, for GM-CSF, the volume of serum required to neutralize the activity of 10 IU of cytokine can be calculated using serum ED50 responses obtained by fitting common asymptotes and slope for all sera analysed. This approach can also be used to analyse responses to different GM-CSF preparations/products (Wadhwa et al., 1996, 1999) and can be applied to other biologicals (Wadhwa et al., 2000).

5. Assay validation and standardization

Significant improvements in obtaining reliable and reproducible data are possible when basic principles of assay design and specificity are established and implemented via validation studies. In addition, standardization of such assays should also be considered if meaningful and valid results are to be obtained.

5.1. Assay specificity

Understanding the discriminability (or functional sensitivity) of an assay (i.e. the ability to discriminate between samples with genuine antibodies as 'positives' and those without antibodies as 'negatives') is fundamental to improving accuracy and reliability of the results the assay produces. Matrix effects are an issue with most assays, as previously discussed, and strategies are needed to eliminate or reduce the risk of detecting false positives and/or false negatives. It is equally important to identify samples that cause interference in the assays and also to implement procedures that overcome at least some of the problems that are encountered with these assays. In ELISAs, a simple approach involving the coating of ELISA plates with other non-antigen-related molecules and detecting positive binding is indicative of nonspecific binding. Experiments which involve spiking of patient samples with an antibody preparation of

known content can be conducted to establish percentage recovery over a range of concentrations to ensure appropriate detection of antibodies. Where feasible, the specificity of samples which are recognised as 'positive' should be assessed using alternative procedures such as immunoblotting which provides information on the real specificity of the antibodies, i.e. those directed against the product or against some other moieties present in the product (Fig. 5). However, immunoblotting may, in some instances, show spurious binding depending on the antigen and the type of sample used.

Additionally, SPR assays may be used for determining the presence of the antibodies since these can often discriminate between specific and nonspecific binding. Fig. 4 demonstrates that the spurious binding of patient sera (taken prior to treatment with GM-CSF) seen with yeast-expressed GM-CSF in ELISAs (Panel A) can be eliminated by use of SPR assays (Panel C). In these assays, assay specificity can be determined by analysing the serum samples in the absence and presence of a predetermined concentration of the antigen. Abrogation of the positive signal is indicative that the criterion of assay specificity has been accomplished.

5.2. Choice of appropriate control samples for assays

It is essential to select appropriate negative control samples, e.g., pretreatment samples for inclusion in assays, and to ensure that the samples are available for determining the nonspecific background of the assay and also as an indication of samples which contain preexisting antibodies against antigen. The choice of the samples is very important and problems such as matrix effects should be considered during sample selection. It must be noted that samples from normal individuals and/or patients may differ due to the nature of the disease and/or concomitant therapy, and therefore assays should be validated using samples from the most appropriate and relevant individuals. During assay development, an antibody positive human sample is often unavailable. Therefore, polyclonal antibodies raised in animal species can be employed as positive controls for indicating assay performance and can be used on a routine basis. In general, inclusion of appropriate standards and positive controls routinely in assays is

the best strategy for assurance of valid results (Figs. 3 and 6).

5.3. Validation and standardization of antibody assays

On the basis of the scientific issues involved in antibody assays, it is necessary to develop validation and standardization criteria that would be appropriate for the antibody assays under development in various laboratories. It should be realized, however, that the parameters (requiring validation) are unique to each method and its intended use and therefore must be carefully determined on a case-by-case basis.

At present, the lack of standardization of antibody assays and antibody reference preparations makes it difficult or even impossible to draw conclusions on immunogenicity of different products by comparing results from assays that have been conducted using different methods and/or in different laboratories. A myriad of factors can be responsible for variation in the immunogenicity profile of different biological products, and so data on relative immunogenicity should be interpreted with caution. Such data can only be valid if the products have been evaluated in the same trials (using the same clinical protocols) and the sera analysed for antibodies using the same assay procedures. Consideration should be given to validation of antibody assays for obtaining meaningful and reproducible results. Towards this goal, some antibody reference preparations are now available from NIBSC. These include a preparation of a spontaneously occurring GM-CSF specific antibody from a myasthenia gravis patient with thymoma (Meager et al., 1999), an IFN- β specific antibody preparation derived from two multiple sclerosis patients treated with IFN- β (99/606) and an IFN- α specific antibody preparation (00/490) from a patient with red cell aplasia.

6. Strategy for studies on immunogenicity

It is very important to devise an appropriate strategy which includes a range of complementary assay methods for the assessment of unwanted immunogenicity of biological products. Careful prospective

planning of immunogenicity studies is critical if valid conclusions concerning unwanted immunogenicity are to be derived. This includes identification of appropriate sampling points (including pretreatment controls) and correlation of antibody assays with clinical data on safety and response to the therapeutic biological (see Fig. 7).

Experience with cytokines has shown that antibodies may be induced only after several doses of product have been given and that neutralizing antibodies follow the production of non-neutralizing responses (Wadhwa et al., 1996, 1999; Ullenhag et al., 2001). In some cases, only a small percentage of patients produce antibodies and only a subpopulation of them develop neutralizing antibodies (Wadhwa et al., 1996, 1999; Ullenhag et al., 2001). This implies that long-term immunogenicity studies involving analysis of samples from a large number of treated patients may be necessary if a complete assessment of immunogenicity (and its consequences) is to be obtained. Details of strategies to be adopted will depend on the product, patient status, duration of therapy and desired clinical response.

7. Conclusions

The detection, measurement and characterization of antibodies against biological therapeutics is a significant task and achieving valid, useful results involves more than simply ensuring that appropriate tests are performed. Thus, the assays used for assessment of immunogenicity of a biological product (and the criteria used for distinguishing positive from negative or levels of magnitude) have to be carefully selected and validated. The assay design, specificity, inclusion of appropriate controls and performance of the assay are all important considerations if reproducible, accurate and meaningful data are to be obtained. There is no single available method that can provide full information on detection and characterization of antibodies, and therefore implementation of a strategy which incorporates use of various methods for the assessment of samples is essential. The use of this approach will provide a detailed understanding of the profile and significance of antibody responses generated against a therapeutic product.

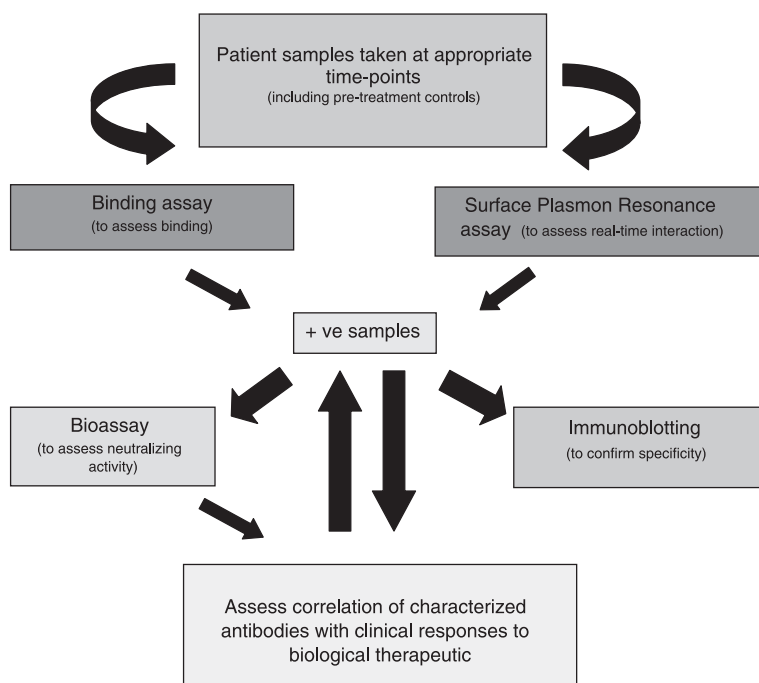


Fig. 7. Strategic overview of antibody detection in patients receiving biological products.

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