



Strategies and Assays for the Assessment of Unwanted Immunogenicity

Meenu Wadhwa & Robin Thorpe

To cite this article: Meenu Wadhwa & Robin Thorpe (2006) Strategies and Assays for the Assessment of Unwanted Immunogenicity, Journal of Immunotoxicology, 3:3, 115-121, DOI: [10.1080/15476910600845534](https://doi.org/10.1080/15476910600845534)

To link to this article: <https://doi.org/10.1080/15476910600845534>



Published online: 09 Oct 2008.



Submit your article to this journal [↗](#)



Article views: 256



View related articles [↗](#)



Citing articles: 21 View citing articles [↗](#)

Strategies and Assays for the Assessment of Unwanted Immunogenicity

Meenu Wadhwa and Robin Thorpe

Biotherapeutics Group, National Institute for Biological Standards and Control, Hertfordshire, EN6 3QG, UK

The assessment of unwanted immunogenicity associated with biological products continues to be a major issue for the biotechnology industry. Monitoring of unwanted immunogenicity during the development of the product from pre-clinical stage through clinical trials is now a regulatory expectation with extended post-marketing commitments required for at least some of these products. Prospective planning of immunogenicity studies incorporating appropriately devised strategies is critical if valid conclusions concerning the immunogenicity profile of a product are to be derived. An important consideration of such studies is the selection of optimized, rigorously validated and standardized methodologies for detection and characterization of antibodies with emphasis on desired assay design, assay controls, and performance criteria. Binding assays with different formats and detection systems, radioimmuno-precipitation assays or surface plasmon resonance procedures are often used as the basis for a screening assay. For assessing the neutralizing capacity of the antibodies, however, a neutralization assay is an absolute requirement. Therefore, a panel of methods is usually necessary for a detailed understanding of the type(s) of antibodies induced against a therapeutic product. This manuscript considers briefly the benefits and limitations of the different techniques available for antibody detection and characterization. A strategy that can be adopted for the assessment of unwanted immunogenicity of therapeutic products is also suggested.

Keywords immunogenicity, strategy, neutralization assays, immunoassays, validation

INTRODUCTION

Immune responses directed against biological therapeutics have been recognized as a major concern limiting the long-term use of these medicines (Porter, 2001; Koren et al., 2002; Stein, 2002). Such immune responses are often benign, but in some instances, they can have undesirable effect(s), ranging from

mild to severe adverse anaphylactoid reactions or in some cases, reduced clinical efficacy, or sometimes even worse, severe clinical consequences in recipients of such therapies (Steis et al., 1988; Rosenberg et al., 1999; Wadhwa et al., 1999; Hjelm Skog et al., 2001; Li et al., 2001; Casadevall et al., 2002; Saint-Remy, 2002; Baert et al., 2003; Bertolotto, 2004; Francis et al., 2005).

The issue is complex as the incidence of antibody formation varies greatly between proteins and also between products based on the same protein, in some cases, even with the same amino acid sequence (Antonelli et al., 1991; Wadhwa et al., 1999; Casadevall et al., 2002; Bertolotto et al., 2004). Antibody induction is also associated with variable clinical consequences depending on the product (Steis et al., 1988; Wadhwa et al., 1999; Casadevall et al., 2002). Awareness of such product related immunogenicity has been highlighted by the increased development of pure red cell aplasia (subsequent to induction of neutralizing antibodies) in chronic renal failure patients treated with the particular erythropoietin product EPREX (Casadevall et al., 2002). This incident provoked serious concern among manufacturers, regulatory organizations, clinicians and patients. As a consequence, regulatory agencies are urging manufacturers to build-in program for management of risks associated with immunogenicity during product development and use including the post-registration period.

Unfortunately, the prediction of unwanted immunogenicity of a therapeutic product, its characteristics and clinical significance, is currently difficult. This is because the immune response to a therapeutic protein is determined by a myriad of factors that converge to result in an immune response in the first instance, and the clinical consequences (if any) of that response thereafter. These include the structural properties of the therapeutic product (and its composition, formulation), the therapeutic regimen, the functional properties of the protein, as well as patient-related factors such as the genetic predisposition, the immune status of the population being treated and the type of disease(s) under treatment (Table 1). As a consequence of this multifactorial response, the relevance of specific antibodies can only be determined in clinical use. Therefore, studies involving immunogenicity need to be conducted early during product development and further correlated with parameters such as pharmacokinetics

Received 27 February 2006; accepted 3 May 2006.

Address correspondence to Meenu Wadhwa, Cytokine and Growth Factor Section, Division of Immunology and Endocrinology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, England EN6 3QG; e-mail: mwadhwa@nibsc.ac.uk

TABLE 1

Some factors that influence the unwanted immunogenicity of therapeutic proteins

Factors determining the immunogenicity of therapeutic proteins
Product-related (Intrinsic)
• Molecular structure—variation in amino acid sequence and/or glycosylation pattern relative to native protein, novel epitopes, • Product impurities—aggregates, degradation products, oxidized or deamidated forms, host cell proteins • Formulation • Biological properties of the therapeutic
Product-related (Extrinsic)
• Dose, route, frequency of administration and duration of treatment • Cellular or soluble target • Endogenous counterpart
Host-related
• Genetic profile • Immune status • Disease state

and/or pharmacodynamics and clinical effects for assessment of the clinical significance of the induced antibodies.

Assessment of Unwanted Immunogenicity

The EMEA Guideline on Comparability of Medicinal Products Containing Biotechnology-derived Proteins as Active Substance: Nonclinical and clinical issues (CPMP, 2004) clearly states that “Immunogenicity must always be addressed by clinical data, unless clinically relevant immunogenicity can be excluded by other means.” In reality, alternative options do not exist and therefore direct assessment of immunogenicity is always necessary. The guideline recommends that “the assessment of immunogenicity requires validated antibody assays, characterization of the observed immune response, as well as evaluation of the correlation between antibodies, pharmacokinetics/pharmacodynamics and efficacy and safety” and that the role of immunogenicity in events related to adverse events such as infusion reactions and loss of efficacy should be considered, a view that is also reflected in The International Conference on Harmonization Guideline S6 (1997). While both guidelines recommend the determination of incidence and titer of antibody responses to the biotherapeutic prior to and post-treatment, the EMEA guideline emphasizes post-marketing programs for monitoring of antibodies in recipients. Neither guideline suggests use of any particular methodologies and antibody-testing strategies. The EMEA guideline further states that the screening assays should be sensitive enough to detect low titre antibodies as well as antibodies to conformational and linear epitopes and that an

assay for detection of neutralizing antibodies should be available. Further guidance is needed on antibody assays, validation and standardization issues and strategies to be adopted when conducting immunogenicity programs.

The complexity of unwanted immunogenicity requires careful prospective planning of studies if valid conclusions are to be derived (Wadhwa et al., 2003). The most common approach for testing unwanted immunogenicity of a product is by measuring antibodies specifically generated against the product (Thorpe and Swanson, 2005). The characteristics of the antibodies differ in terms of their immunoglobulin class, affinity, epitope recognition and also in their ability to neutralize the activity of the therapeutic protein. Use of carefully selected and validated assays capable of providing accurate and precise data on detection and measurement of antibodies is necessary for evaluation of the unwanted immunogenicity of a therapeutic product (Mire-Sluis et al., 2004).

Current Methods for Detection and Characterization of Antibodies

Different techniques are available for investigating the presence of antibodies generated against therapeutic proteins in biological fluids. Each of the currently available methods have distinct advantages and limitations that are considered in Table 2. Such methods include immunochemical procedures such as solid or liquid immunoassays, radioimmunoassays (RIPA) and biophysical methods such as surface plasmon resonance (SPR), which are mainly used for detection of binding antibodies (Wadhwa et al., 2003; Thorpe and Swanson, 2005). Binding assays can be conducted using a variety of formats. These include direct, indirect, bridging, and competitive platforms using radioligand, enzymatic, fluorescent, chemiluminescent, or electrochemical luminescence (ECL) detection systems (Mire-Sluis et al., 2004). These assays provide valuable information concerning the presence (or absence) of antibodies which recognize the relevant antigen(s) and are often used as “screening assays” for distinguishing antibody-positive samples from those that are antibody-negative. Quantitative and sensitive data can be generated from such assays if an appropriate format is adopted (Wadhwa et al., 2003; Thorpe and Swanson, 2005).

An inherent difficulty with most immunoassay formats is that they require multiple reagent additions followed by wash cycles. Extensive washing of microtitre plates during the assay procedure, however, may remove low affinity antibodies, especially those antibodies with rapid dissociation rates, and so these antibodies may not always be detected. Such rapidly dissociating antibodies, however, may in some instances, have a high antigen binding capacity and/or ability to neutralize the bioactivity of the therapeutic protein and it is important to ensure that these antibodies are detected. Recently, the use of ECL reporters has been shown to offer distinct attributes over conventional ELISA formats. A greater dynamic assay range, higher tolerance to sample matrix and circulating antigen and ability

TABLE 2
Methods used for detection of antibodies

Type of assay	Parameter measured	Advantages	Disadvantages
Binding assay—Direct Format (Coating with antigen and detecting with labeled anti-Ig)	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. Good sensitivity	Prone to spurious binding and matrix effects May fail to detect low-affinity antibodies. Immobilisation of antigen may mask or alter epitopes Species specificity and isotype detection determined by secondary reagent Detection reagent may differ between control and sample
Binding assay—Indirect Format (Coating with a specific mAb or biotin etc followed by antigen)	Identifies antibodies capable of binding to the antigen preparation	Coating with a specific mAb keeps antigen in oriented position Consistent coating and maintains antigen conformation	Extensive studies required to demonstrate that the coating mAb does not mask or alter epitopes May fail to detect low-affinity antibodies. Species specificity and isotype detection determined by secondary reagent
Binding assay—Bridging Format (Coating with antigen and detecting with labeled antigen)	Identifies antibodies capable of binding to the antigen preparation	High through put Dual arm binding ensures high specificity Can detect all isotype responses	Immobilisation of the antigen may alter the conformation of the protein Requires labelled antigen. Labelling may alter/denature antigen/mask epitopes May fail to detect rapidly dissociating antibodies
Electrochemiluminescence—Bridging Format	Measures antigen-antibody binding in liquid phase	High through put; Liquid phase Detects total antibody and isotypes Allows use of high concentrations of matrix Tolerates interference from antigen Detection signal consistent during life of TAG conjugate	Requires two antigen conjugates (biotin and TAG) Antigen labelling may alter/denature antigen, mask/alter epitopes Equipment & reagents are vendor-specific
Radioimmuno-precipitation assay	Measures antigen-antibody binding in liquid phase	Moderate to high throughput Good sensitivity Can be specific	Can be isotype specific, may fail to detect rapidly dissociating antibodies Requires radiolabelled antigen Radio label may alter/denature antigen, decay of radio-label may affect antigen stability
Surface plasmon resonance	Shows antigen-antibody interaction in liquid phase in real time	Automated Provides information on the specificity, isotype, relative binding affinity and relative concentration Enables detection of both low-affinity and high affinity antibodies	Expensive Requires dedicated equipment, reagents are vendor specific May require immobilisation of the antigen that may alter the conformation of the native protein Regeneration step may degrade antigen

(Continued on next page)

TABLE 2
Methods used for detection of antibodies (*Continued*)

Type of assay	Parameter measured	Advantages	Disadvantages
Immunoblotting	Assesses the specificity of the antibodies for protein in antigen preparations	Detection reagent not required; Not species specific	Sensitivity often less than binding assay
		Dissects the specificity of the antibodies	Non-quantitative
		Provides profile of reactivity against subcomponents of the product	Relatively low throughput
Bioassay	Assesses the neutralizing potential of the antibodies	Good sensitivity	May fail to detect antibodies which do not recognize antigen after SDS PAGE separation
		Functional assay that distinguishes antibodies with neutralizing potential	Appropriate detection reagents necessary
		Quantitates the neutralization capacity (in vitro) of antibodies capable of neutralizing the biological activity of the antigen	Relatively time-consuming
		May correlate with clinical response	Often variable. Can be affected by non-specific 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.

to detect low affinity antibodies are some of the features of this technology.

SPR technology has gained popularity over the last few years for detection of binding antibodies. SPR assays are unique as they show antigen-antibody interaction in “real-time” and allow monitoring of sample association and then dissociation facilitating detailed antibody characterization e.g., determination of relative affinity and isotype. Results can be obtained rapidly, within only a few minutes, since long sample incubations are not required. Furthermore, by using SPR for detection of antibodies to a therapeutic product, all antibodies capable of binding to the product including rapidly dissociating antibodies (which may have been missed in other assay formats) can be detected (Swanson et al., 2002).

Confirmatory assays are necessary for elimination of false positive samples identified during the initial screen using any of the procedures stated here. Approaches such as competitive immunoassays, SPR can be used for confirming antibody positivity although an assay based on a different scientific principle from that used for the screening assay should usually be considered. It is also necessary to select confirmatory assays taking into account the limitations and characteristics of the screening assay. Use of the immunoblotting procedure, which provides information concerning the specificity of the antibodies detected, is ideal as the antibodies may have specificity for other components (e.g., contaminants) in the product and can cause data to be misinterpreted (Wadhwa et al., 1999). For example, very low

levels of expression system derived bacterial proteins in rDNA products can cause significant antibody development whereas the major protein present (the active principle) may be much less immunogenic (Wadhwa et al., 1999). However, other procedures, such as analytical radioimmunoprecipitation assays, can also be used for confirmation.

For a majority of biotherapeutics, a typical assessment of immunogenicity usually involves use of a two-tiered approach; a screening assay complemented with a confirmatory step (e.g., immunoblotting, antigen adsorption, etc., as described above) which is then followed using a bioassay for assessment of the neutralizing capacity of the antibodies. While screening assays determine the relative amount of antibodies by assessing ability to bind to the protein, they cannot discriminate between neutralizing and non-neutralizing antibodies, an important feature of bioassays. Inclusion of a bioassay in immunogenicity studies is essential for most biotherapeutics as it is the only method that is capable of distinguishing whether the induced antibodies are neutralizing or nonneutralizing. Moreover, results from bioassays can often be correlated with the effect of antibodies on clinical response (Wadhwa et al., 1996, 2003; Bertolotto, 2004).

Bioassays assess some functional aspect of the protein or mechanism of action (rather than the intended clinical effect) and measure the potency of the protein. These assays often provide the basis for construction of a neutralizing antibody assay and usually require primary cultures of cells (e.g., bone marrow cells of human or animal origin) or continuous

cell-lines. Bioassays using primary cells are often inconsistent and prone to problems. In contrast, state-of-art assays using continuously growing factor-dependent (cytokine dependent) or factor-independent cell-lines and/or transfected lines derived from laboratory-induced leukemias or from leukemic patients produce reliable data and are a preferred option. An example is the GM-CSF dependent human cell-line, TF-1 (derived from an erythroleukaemic patient). Such assays require refining for optimal detection of neutralization activity. In some cases, cell lines that proliferate in response to multiple factors/cytokines are useful in determining whether the neutralizing effect is due to specific antibody or interfering factors in the sample matrix. For example, the TF-1 cell-line proliferates in response to several cytokines including GM-CSF, IL-3, erythropoietin (Epo) and IL-5 (Kitamura et al., 1989). In a TF-1 cell-based assay, therefore, one can easily compare the effect of the sample (e.g., serum/plasma) on the proliferation of the TF-1 cells in the presence or absence of the therapeutic product (e.g., GM-CSF) and an irrelevant factor (IL-3). For some biologicals, alternative bioassays using different cell-lines or receptor transfected cell-lines may need to be considered for selection of a more sensitive, suitable assay with good discrimination between signal to noise in the assay matrix. The 32D cell-line transfected with the human Epo receptor is an excellent example of an assay for detection of Epo antibodies; confirmatory response is assessed in the presence of murine IL-3 (Wei et al., 2004). It should be realized that different types of bioassay procedures can be used as the basis of neutralization assays for different biologicals (McKay et al., 2006).

CONSIDERATIONS DURING THE DEVELOPMENT AND DESIGN OF ANTIBODY ASSAYS

The detection, measurement and characterization of antibodies is a significant task and deriving valid, useful results involves more than simply ensuring that appropriate tests are performed. The purpose of the assay, selection of an appropriate assay platform, complex interactions between the critical components of the assay (i.e., antigen, antibody, assay matrix, etc.) are important issues that need to be considered during development and design of antibody assays. Some of these issues are briefly discussed next.

Purpose of the Assay

It is necessary to define the aim of the assay at the outset: that is, whether it is solely a “screening” assay for providing a positive or negative signal or a “quantitative” assay depending on the extent of information needed. While the latter are usually more tedious and labor intensive, they provide data that is generally more useful for comparative purposes. An initial screening assay followed by a “quantitative” assay is usually most logical for assessment of serum samples. Obviously, approaches for assay validation will differ depending on the purpose and the assay design used.

Selection of an Appropriate Assay Format and Design

It is difficult to recommend an assay format for all screening purposes; assay formats tend to vary between different proteins and are often dependent upon the therapeutic protein class. When determining antibodies against a non-immunoglobulin recombinant protein, all of the platforms and reporters listed above (Table 2) are viable options. However, if the protein is a humanized or human immunoglobulin therapeutic, the choices for detection of primate anti-human antibodies (PAHAs) or human anti-human antibodies (HAHAs) are limited, usually due to secondary antibody cross-reactivity.

While selecting a format, consideration should be given to the nature of the product (e.g., a therapeutic protein or a monoclonal antibody), potential interference in the assay from co-medications, dosing regimen (e.g., potential for product to be present in the sample) and/or disease-specific issues (e.g., rheumatoid factor). Other considerations include ability to detect antibodies with desired specificities (IgM, IgG subclasses, etc.) and susceptibility to assay interference from circulating product. The potential for assay interference from the biological itself, particularly with monoclonal antibody therapeutics that have longer half-lives than other recombinant proteins, is significant and strategies to minimize this problem are needed (Patton et al., 2005). During assay development for anti-therapeutic proteins, assay specificity is usually demonstrated by addition of free antigen to a serum sample spiked with known amounts of antibody and looking for inhibition of the expected signal. However, it should be noted that if an assay is tolerant of the therapeutic product, it may be more challenging to demonstrate specificity.

It is important to ensure that the selection of assay parameters and platform are satisfactory for the particular product. The assay should fulfill certain criteria (i.e., adequate sensitivity, the ability to detect all induced antibodies irrespective of their dissociation rates and isotype). In a binding assay, one must consider whether the selected assay format allows for appropriate epitope exposure and detection of antibodies with different on-rates and off-rates. If certain assay formats are incapable of recognizing antibodies with relatively fast off-rates, alternative approaches such as SPR and ECL technologies should be considered for antibody detection and characterization of the immune response.

It should be realized that each assay design is unique and dependent on the type of assay, antigen, treatment schedule, and disease indication. Therefore, adoption of an assay design suitable for the intended purpose of the assay is essential.

Inclusion of Positive and Negative Antibodies as Assay Controls

Inclusion of positive and negative antibodies as controls is vital for monitoring assay performance and for valid interpretation of results. Initially, during product and assay development, a human antibody positive sample is seldom available. Positive control antibodies are generally affinity-purified antisera (from

hyperimmunized animals), which have been spiked into a suitable matrix for use as a control in the assay. Monoclonal antibodies can also be used for positive control, either individually or as a cocktail. As with any assay reagent, such antibodies need to be well characterized and tested in the selected assay matrix. It should be realized that the selection of a reference material may significantly alter the assigned sensitivity value of an antibody assay. Therefore, although an affinity purified polyclonal preparation of antigen reactive antibodies, which contains a mixture of antibodies of variable affinities, is usually the most relevant standard for these assays, a higher sensitivity may be observed if using a high-affinity monoclonal antibody preparation.

As a negative control for demonstrating appropriate assay performance, an irrelevant antibody from the same animal species that has been affinity purified in the same way as the positive control antibody can be used. Sera from normal healthy individuals can also be used as a negative control for assessment of assay performance.

In studies where patient sera are being tested for the presence of antibodies to a biotherapeutic, the patient baseline sample (taken prior to therapy) is the patient specific negative control. This sample should be considered when determining whether the patient samples taken subsequent to therapy are negative or positive for antibodies. If the data for these subsequent samples are the same as the baseline, the patient serum is clearly negative for antibodies.

Interpretation and Expression of Results

Different approaches can be used for interpretation of results. Prior to evaluation of samples in antibody assays, it is necessary to clearly define the criteria for interpretation of results, i.e., antibody-positive or antibody-negative by determining the cutoff values for each assay and devising appropriate methods (titre value, etc.) for expression of results. The positive control antibody can be used as an antibody standard or reference preparation for expressing the levels of antibodies in the test samples relative to the amounts of the antibodies in the reference antibody preparation. It may also be possible to express antibody levels using arbitrary units providing that these have been well defined for the reference material. Although this approach is not ideal (the heterogeneous nature of polyclonal antibodies is particularly problematical), the use of this strategy may provide relatively precise estimates of antibody levels in the test samples and can reduce variability. This situation is likely to occur when a number of sequential samples from the same animal or patient are available and it is difficult to include all samples from all patients in the same assays for establishing a valid comparison of antibody levels between different samples/patients.

VALIDATION AND STANDARDIZATION OF ASSAYS

Validation of antibody assays is an essential prerequisite for obtaining results that are reproducible, accurate and meaning-

ful. It is necessary to develop validation criteria for the assays within various laboratories. Recommendations for design and optimization of immunoassays used in the detection of host antibodies against biotechnology products have been published following a concerted effort by an AAPS group in association with the USFDA; those on neutralization assays are expected soon. Implementation of these recommendations may prevent some of the problems associated with antibody assays.

Standardization of assays is generally not possible as each manufacturer may have unique products and hence there is no possibility of a shared reference preparation. In most cases, standardization of assays is not possible either due to the uniqueness of each biological therapeutic. There are exceptions, however, as some proteins are licensed by multiple manufacturers, e.g., Epo and IFN- β . In the case of these proteins, therefore, standardization of antibody assays is urgently needed for obtaining meaningful and reproducible results. However, it should be realized that it is impossible to draw conclusions on immunogenicity of different products by comparing results from different laboratories using different methods due to different assay formats, sensitivities, subtle differences in drug products, etc.

To summarize, the assay design, specificity, inclusion of appropriate controls and performance of the assay, data analysis and interpretation, validation are all important considerations if reproducible, accurate and meaningful data are to be obtained. It is very important to devise a strategy that includes multiple methods for a detailed understanding of the quantity and type(s) of antibodies generated against a therapeutic product (Figure 1). Correlation of data from antibody assays with clinical observations is also necessary and critical to provide an understanding of the consequences of unwanted immunogenicity.

Strategy for Antibody Detection and Characterization

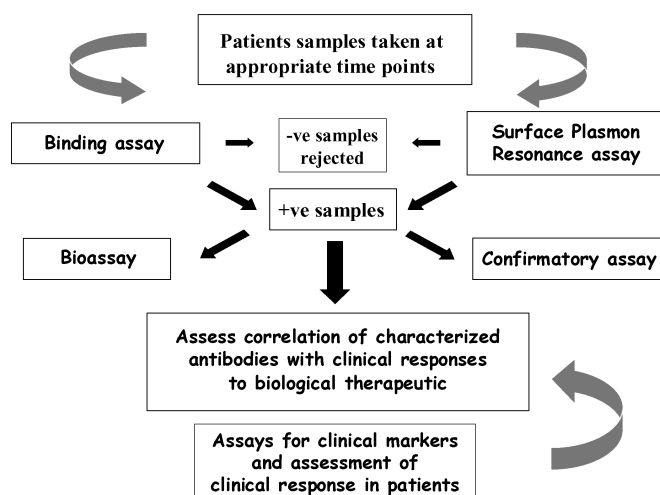


FIG. 1. A simple strategy for detection of antibodies in patients receiving biological products.

FUTURE CHALLENGES

The unexpected and unpredictable aspects of the immunogenicity associated with biological products continue to be of great concern and are clearly very significant for both “novel” and “follow-on” biologicals. The Committee for Medicinal Products for Human Use (EMA) is currently drafting a “Guideline on Immunogenicity Assessment of Therapeutic Proteins” that will provide detailed guidance on appropriate strategies for assessment of immunogenicity in recipients of therapeutic products.

REFERENCES

- Antonelli, G., Currenti, M., Turriziani, O., and Dianzani, F. 1991. Neutralizing antibodies to interferon-alpha: Relative frequency in patients treated with different interferon preparations. *J. Infect. Dis.* 163:882–885.
- Baert, F., Noman, M., Vermiere, S., Assche, G. V., D’Haens, G., Carbonez, A., and Rutgeerts, P. 2003. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn’s disease. *N. Engl. J. Med.* 348:601–608.
- Bertolotto, A. 2004. Neutralizing antibodies to interferon beta: Implications for the management of multiple sclerosis. *Curr. Opin. Neurol.* 17:241–246.
- Bertolotto, A., Deisenhammer, F., Gallo, P., and Solberg Sorensen, P. 2004. Immunogenicity of interferon beta: Differences among products. *J. Neurol.* 251 Suppl 2:II15–II24.
- Casadevall, N., Nataf, J., Viron, B., Kolta, A., Kiladjian, J. J., Martin-Dupont, P., Michaud, P., Papo, T., Ugo, V., Teyssandier, I., Varet, B., and Mayeux, P. 2002. Pure red cell aplasia and anti-erythropoietin antibodies against human erythropoietin in patients treated with recombinant erythropoietin. *N. Engl. J. Med.* 346:469–475.
- CPMP (Committee for Proprietary Medicinal Products). 2004. *Guideline on Comparability of Medicinal Products Containing Biotechnology-derived Proteins as Active Substance. Non-clinical and Clinical Issues.* EMA/CPMP/3097/02.
- Francis, G. S., Rice, G. P., and Alsop, J. C. 2005. PRISMS Study Group. Interferon beta-1a in MS: Results following development of neutralizing antibodies in PRISMS. *Neurology* 65:48–55.
- Hjelm Skog, A. L., Wadhwa, M., Hassan, M., Gharizadeh, B., Bird, C., Ragnhammar, P., Thorpe, R., and Mellstedt, H. 2001. Alteration of IL-2 pharmacokinetics and function by IL-2 antibodies induced following treatment of colorectal carcinoma patients with a combination of monoclonal antibody 17-1A, GM-CSF, and IL-2. *Clin. Cancer Res.* 7:1163–1170.
- International Conference on Harmonization. 1997. *Guidance for Industry. S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals.* Available at (<http://www.fda.gov/cder/guidance/1859fn1.pdf>).
- Kitamura, T., Tange, T., Terasawa, T., Chiba, A., Kuwaki, T., Miyagawa, K., Piao, Y. F., Miyazono, K., Urabe, A., and Takaku, F. 1989. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J. Cell. Physiol.* 140:323–334.
- Koren, E., Zuckerman, L. A., and Mire-Sluis, A. R. 2002. Immune responses to therapeutic proteins in humans—Clinical significance, assessment and prediction. *Curr. Pharm. Biotechnol.* 3:349–360.
- Li, J., Yang, C., Xia, Y., Bertino, A., Glaspy, J., Roberts, M., and Kuter, D. J. 2001. Thrombocytopenia caused by the development of antibodies to thrombopoietin. *Blood* 98:3241–3248.
- McKay, F., Schibeci, S., Heard, R., Stewart, G., and Booth, D. 2006. Analysis of neutralizing antibodies to therapeutic interferon-beta in multiple sclerosis patients: A comparison of three methods in a large Australasian cohort. *J. Immunol. Meth.* 310:20–29.
- Mire-Sluis, A. R., Barrett, Y. C., Devanarayan, V., Koren E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S. J., Taniguchi, G., Wierda, D., and Zuckerman, L. A. 2004. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J. Immunol. Meth.* 289:1–16.
- Patton, A., Mullenix, M. C., Swanson, S. J., and Koren, E. 2005. An acid dissociation bridging ELISA for detection of antibodies directed against therapeutic proteins in the presence of antigen. *J. Immunol. Meth.* 304:189–195.
- Porter, S. 2001. Human immune response to recombinant human proteins. *J. Pharm. Sci.* 90:1–11.
- Rosenberg, M., Kingma, W., Fitzpatrick, M. A., and Richards, S. M. 1999. Immunosurveillance of alglucerase enzyme therapy for Gaucher patients: Induction of humoral tolerance in seroconverted patients after repeat administration. *Blood* 93:2081–2088.
- Saint-Remy, J. M. 2002. Immunology of factor VIII inhibitors. *Semin Thromb. Hemost.* 28: 265–268.
- Stein, K. E. 2002. Immunogenicity: Concepts/issues/concerns. *Devel. Biol.* 109:15–23.
- Steis, R., Smith, J. W., Urba, W. J., Clark, J. W., Itri, L. M., Evans, L. M., Schoenberger, C., and Longo, D. 1988. Resistance to recombinant interferon alpha-2a in hairy-cell leukaemia associated with neutralizing anti-interferon antibodies. *N. Engl. J. Med.* 318:1409–1413.
- Swanson, S. J., Mytych, D., and Ferbas, J. 2002. Use of biosensors to monitor the immune response. *Devel. Biol.* 109:71–78.
- Thorpe, R., and Swanson, S. J. 2005. Current methods for detecting antibodies against erythropoietin and other recombinant proteins. *Clin. Diag. Lab. Immunol.* 12:28–39.
- Wadhwa, M., Bird, C., Fagerberg, J., Gaines-Das, R., Ragnhammar, P., Mellstedt, H., and Thorpe, R. 1996. Production of neutralizing GM-CSF antibodies in carcinoma patients following GM-CSF combination therapy. *Clin. Exp. Immunol.* 104:351–358.
- Wadhwa, M., Skog, A. H., Bird, C., Ragnhammar, P., Lilljefors, M., Gaines-Das, R., Mellstedt, H., and Thorpe, R. 1999. Immunogenicity of granulocyte-macrophage colony stimulating factor (GM-CSF) products in patients undergoing combination therapy with GM-CSF. *Clin. Cancer Res.* 5:1353–1361.
- Wadhwa, M., Bird, C., Dilger, P., Gaines-Das, R., and Thorpe, R. 2003. Strategies for detection, measurement and characterization of unwanted antibodies induced by therapeutic biologicals. *J. Immunol. Meth.* 278:1–17.
- Wei, X., Swanson, S. J., and Gupta, S. 2004. Development and validation of a cell-based bioassay for the detection of neutralizing antibodies against recombinant human erythropoietin in clinical studies. *J. Immunol. Meth.* 293:115–126.