

How to predict and prevent the immunogenicity of therapeutic proteins

Huub Schellekens*

*Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences,
P.O. Box 80.082, 3508 TB Utrecht, The Netherlands*

Abstract. Therapeutic proteins in general induce an immune response, especially when administered as multiple doses over prolonged periods. Non-human therapeutic proteins such as asparaginase and streptokinase induce antibodies by the classical immune reaction and their primary immunogenic factor is the degree of non-self. Human therapeutic proteins such as the interferons and GM-CSF breakdown immune tolerance and protein aggregation is their main factor inducing antibodies. Many other factors influence the level of immunogenicity of proteins, such as storage conditions, contaminants or impurities in the preparation, downstream processing, dose and length of treatment, as well as route of administration, appropriate formulation and disease status and concomitant treatment of patients. Clinical manifestations of antibodies directed against the protein include loss of efficacy, cross neutralization of endogenous proteins and general immune system effects, such as anaphylaxis or serum sickness.

Keywords: immunogenicity, therapeutic proteins, prevention, prediction.

Introduction

The use of therapeutic proteins such as the interferons, growth factors, hormones and monoclonal antibodies has increased dramatically over the past two decades [1]. However, the first use of therapeutic proteins dates back to more than a century ago, when animal antiserum was introduced for the treatment and prevention of infections. This antiserum treatment was associated with a high degree of immunogenicity. As foreign proteins they induced high levels of antibodies in the majority of patients after a single or a few injections sometimes with fatal anaphylactic reactions.

The next generation of therapeutic were of human origin such as the plasma-derived clotting factors and growth hormone isolated from the pituitary glands of cadavers. Also, these proteins induced antibodies in many patients [2,3]. Most of the patients were treated with these products because of an innate deficiency and therefore they lacked immune tolerance for these proteins.

Most of the therapeutic proteins introduced in recent decades are homologues of human proteins. However, in contrast to expectations, these proteins also appear to induce antibodies, and in some cases, in the majority of patients [4,5]. Proteins which have not been reported to induce antibodies are extremely rare. Given that these antibodies are directed against

*Tel.: +3130-2536973. Fax: +3130-2517839.
E-mail: h.schellekens@uu.nl (H. Schellekens).

self-antigens and used in patients with a normal immune tolerance, the immunological reaction involves breaking B-cell tolerance. In general, patients need to be exposed to therapeutic proteins for prolonged periods to activate autoreactive B-cells and the antibody response is relatively limited and usually disappears shortly after stopping treatment.

So when discussing the immunogenicity of proteins it is important to realize that foreign proteins and human homologues in patients lacking immune tolerance induce antibodies by the classical immunological pathways. Human homologues in patients with immune tolerance induce antibodies by breaking B-cell tolerance. There are groups of patients in which both types of antibody induction may occur, *e.g.*, in patients with protein deficiency. Some gene defects lead to complete silencing and lack of immune tolerance. In some cases non-functional but still immune-reactive protein is produced and the patients are immune tolerant, while suffering from a functional deficiency.

Antibody assays

Another important issue is the assays which are used to evaluate the immune response. It is important to realize that the assays are not standardized and there are no international reference preparations or standards. This makes it difficult and in most cases impossible to compare antibody incidences from different trials and different products unless the antibody assays were performed in the same laboratory. The lack of standardization explains why the reported incidence of the same product administered to comparable group of patients differs widely. For example, the reported incidence of an antibody response to interferon alpha 2 in patients with viral infections varied between 0 and >80% [6].

It is also essential to discriminate between the assay performance characteristics and the clinical relevance of the assay results when evaluating the immunogenicity of therapeutic proteins. So a positive signal in an antibody assay does not imply automatically that there are any biological or clinical consequences.

There are two principles for the testing of antibodies: assays that monitor binding of antibody to the drug (EIA, RIA, SPR) and (neutralizing) bioassays. These assays are used in combination: a sensitive binding assay to identify all antibody-containing samples is often more practical as bioassays are usually more difficult and time-consuming. If a native molecule is modified (*e.g.*, pegylated or truncated) to obtain a new product with different pharmacological characteristics, antibodies to both the “parent” and the new molecule should be determined.

A definition for the negative cut-off must be included in the validation process of this assay and is often based on the 5% false positive rate. Such an analytical cut-off *per se* does not predict a biological or clinical effect but is

dependent on the technical limitations of the assay. And because the cut-off is set to include a relative high number of false positives, all initial positive sera should be confirmed by either another binding assays or displacement assay.

The confirmed positive samples should be tested in a bioassay for neutralizing antibodies, which correlate with potential clinical effects in patients because these antibodies interfere with receptor binding. A confirmatory step for neutralizing antibodies is not necessary because these antibodies are a subset of binding antibodies. In some cases it may be important to show the neutralization to be caused by antibodies, if the presence of other inhibitory factors such as soluble receptor has to be excluded.

Further characterization of the neutralizing antibody response may follow, such as determining antibody isotype, affinity and specificity. Often SPR (Biacore) is used for this further characterization.

Evaluating the clinical and biological consequences on the basis of a positive/negative answer from the assays may be misleading; development of antibodies is a dynamic process and therefore the kinetics of antibody development must be plotted quantitatively over time. Usually increasing and high titers of neutralizing antibodies are associated with some biological effect. In many cases a therapeutic protein may not be immunogenic enough to achieve such levels.

Also, an analysis of the biological effect based on incidence alone is misleading. Comparing clinical efficacy in patient groups defined on the basis of mean population antibody titers, taking into account both incidence and titers of neutralizing antibodies, is in general more useful than comparing antibody negative vs. antibody positive patients.

For further information I refer to some recently published excellent reviews regarding the development and validation of the different assay formats for antibodies to therapeutic proteins [7–9].

Mechanisms of antibody induction

As discussed, the mechanism of antibody induction will depend on the origin of the product. A non-human protein will induce antibodies by the classical immune response. Such a response will also occur if the patients do not have immune tolerance to the protein. In case of human proteins antibodies will be induced by breaking B-cell tolerance.

The classical immune response

The classical immune response involves antigen-presenting cells, usually macrophages and dendritic cells which activate both B- and helper T-cells which are necessary for a mature antibody reaction and the formation of

memory cells. For details of this response the reader is referred to general immunology textbooks.

Breaking B-cell tolerance

Therapeutic proteins which are homologues of human proteins induce antibodies by breaking of B-cell tolerance. The details of this mechanisms are not completely understood. There are always autoreactive B-cells circulating, which are kept under control by peripheral mechanisms such as elimination by apoptosis when they meet their antigen. Also receptor editing has been described as a mechanism to make these cells harmless. The most likely silencing peripheral mechanism is the induction of functional anergy in these B-cells. Apparently these cells are not stimulated to produce antibodies by the circulating levels of native proteins such as insulin, interferon and EPO.

Such B-cells may start to antibodies by a second signal or “danger” signal. Bacterial endotoxines which react with Toll receptors may provide such danger signal. This explains the production of antibodies to self-antigen associated with LPS. Also microbial CpG motifs present in DNA may be another factor which can trigger an immune reaction to self-antigens. This T-cell dependent activation is weak. When self-antigens are coupled with foreign T-cell epitopes only a weak IgM response is induced, unless multiple high doses of antigen are given together with immune adjuvants.

The most potent way to induce high levels of IgG independent of T-cell help is to present the self-antigen in arrayed on viruses and viral-like particles [10–12]. The pacing of epitopes with a distance of 50–100 Å is unique to microbial and the immune system has apparently learned to react vigorously to this type of antigen presentation.

Aggregates of proteins also present epitopes in an array form and explain why as described later they are the main cause of antibody induction. Aggregates are also described to be internalized by B-cells which then become helper cells explaining the T-cell independence of the isotype switching.

The induction of antibodies to self-antigens may also occur by immunological mimicry. It needs the presence in the product of a modification of the therapeutic protein which makes it non-self enough to induce a classical immune response but still resembles the human homologue enough to allow cross reaction of the with the original protein. The mirror image of this situation would be patients with an allele of the gene of the proteins which diverges from the gene used for the production of the therapeutic protein. This possibility has been experimentally suggested in mice with different allotypes for *IL-2*. When immunized the mice produced antibodies which reacted with all forms of *IL-2*. Whether this situation also may occur with therapeutic proteins in patients needs to be confirmed.

Factors influencing the immunogenicity of therapeutic proteins

As discussed, the primary factors influencing the immunogenicity of therapeutic proteins are aggregates in the case of human homologues and the degree of non-self and the presence of T-cell epitopes in the case of non-human proteins.

In addition, there are a number of secondary factors which influence the level and intensity of the antibody response. One of these factors is the length of treatment.

Foreign proteins like streptokinase and asparaginase induce antibodies often after a single injection. Breaking B-cell tolerance by human protein takes in general more than six months of chronic treatment.

The route of administration influences the likelihood of an antibody response independent of the mechanism of induction. The probability of an immune response is the highest with subcutaneous administration, less probable after intramuscular administration and intravenous administration is the least immunogenic route. There are no studies comparing parenteral and non-parenteral routes of administration. However, it should be realized that also mucosal tissues are immune competent making intranasal and pulmonary administration of therapeutic proteins potential immunogenic.

The biological activities of the product are influencing the immune response. An immune-stimulating therapeutic protein is more likely to induce antibodies than an immune-suppressive protein. Monoclonal antibodies targeted to cell-bound epitopes are more likely to induce an immune response than monoclonal antibodies with a target in solution. Also the Fc-bound activities of monoclonal antibodies have an influence.

Impurities may influence immunogenicity. I have discussed the presence of modified protein and contaminants such as host-cell derived endotoxins as potential inducers of an immune response. The immunogenicity of products such as human growth hormone, insulin and interferon alpha-2 have declined over the years due to improved downstream processing and formulation, reducing the level of impurities. The probability of an immune response therefore increases with the level of impurities. Impurities may also induce an immune response to themselves and may be the cause of skin reactions, allergies and other side effects.

Products modification which are intended to enhance half-life potentially also increase the exposition of the protein to the immune system and may increase immunogenicity. In addition, the modification may reduce biological activity necessitating more protein for the same biological effect.

Pegylation is claimed to reduce the immunogenicity of therapeutic proteins by shielding [13]. There is evidence that pegylation reduces the immunogenicity of non-human proteins like bovine adenosine deamidase and asparaginase. Whether pegylation also reduces the capacity of human proteins to break B-cell tolerance is less clear. There are reports of high immunogenicity

of pegylated human proteins such as megakaryocyte growth and differentiation factor (MGDF), but the immunogenicity of unpegylated MGDF products is unknown [14].

Gender, age and ethnic background have all been reported to influence the incidence of antibody response to specific therapeutic proteins. However, the only patient characteristic which consistently has been identified for a number of different products is the disease the patients suffer from. Cancer patients are less likely to produce antibodies to therapeutic protein than other patients. The most widely accepted explanation for this difference is the immune compromised state of cancer patients, both by the disease and by anticancer treatment. Also the median survival of patients on treatment by therapeutic proteins may be too short to develop an antibody response. In any case, cancer reduces the probability of an antibody response to a protein considerably.

As the experience in cancer patients shows immune-suppressive therapy reduces the probability to develop an immune response to proteins. In addition, immune-suppressive drugs such as methotrexate are used in conjunction with monoclonal antibodies and other protein drugs in order to reduce the immune reactions.

Prediction on the basis of physicochemical characterization

Non-human proteins

The degree of non-self is an important factor in the potency of a protein to induce antibodies and especially where exactly in the protein the divergence is located. This prediction on the number of amino acid differences with a native sequence alone is misleading. There are examples of proteins in which a single amino acid difference induced strong immunogenicity, while in other proteins more than 10% of changes had no effect [15]. If the proteins differs so substantially that epitopes are formed to which the patients has no innate immunity, then a rapid and intense immune reaction is induced. On the basis of sequence analysis, the immunogenicity of these proteins can be reduced, however, the current algorithms that are designed to predict epitopes are at an early stage of development.

Also, the extreme heterogeneity of HLA responses in humans poses a major hurdle for precise prediction. Therefore epitope prediction is not certain as patients can make antibodies to unpredicted epitopes or fail to mount an immune response to predicted epitopes. On the basis of sequence it is at present impossible to fully predict immunogenicity of biopharmaceuticals. Considering that other factors than sequence are important for immunogenicity and immune responses are genetically determined and therefore highly individual, sequence will probably never be enough to predict and avoid the induction of antibodies.

Human homologues

For biopharmaceuticals with a high degree of homology to native proteins, the main factors in immunogenicity have been reported to be impurities, heterogeneity, aggregate formation and protein degradation such as oxidation and deamidation, which can be monitored by physicochemical characterization during production and storage [16–18]. However, there are factors influencing immunogenicity which cannot yet be identified by the current methods to characterize biopharmaceuticals.

The considerable reduction in immunogenicity seen over the years in products like insulin and growth hormone has been explained by the increased purity. However, which impurities should be avoided is not known. The relative high immunogenicity of first recombinant DNA-derived growth hormone has been blamed by the presence of a high level of host-cell derived endotoxin. However this explanation has been disputed.

Less controversy concerns the role of aggregates in the induction of antibodies by human homologues. They present epitopes in a repeated fashion known to be able to activate B-cells. In transgenic models aggregates have been shown the most decisive product modification able to break tolerance. And reduction of aggregates has also been shown in clinical trials to reduce the induction of antibodies. A number of chromatographic and electroforetic methods can be used to identify some types of aggregates, although the experimental conditions may also dissolve aggregates which have immunogenic potential. Analytical ultracentrifugation is considered the least-destructive method of analysis and capable of identifying all aggregate forms involved in induction of antibodies.

Prediction with animal studies

In principle all proteins intended for therapeutic use in humans are foreign for animals and cause the induction of antibodies. For biopharmaceuticals of microbial, plant or other non-human origins this immunogenic response is similar as in humans as they are comparably foreign for all mammalian species. Therefore, animal studies in which the reduction of immunogenicity is evaluated have a high degree of predictability for immunogenicity in humans. Especially useful are transgenic animals expressing the human MHC-complex, so processing the peptide as humans.

For human homologues standard animals are less predictive because the induction of antibodies in humans is based on breaking B-cell tolerance which differs fundamentally from the vaccine-like reaction seen in conventional animals. Still, animal studies with these type of products in conventional animals may be useful because it may help to identify the relative immunogenicity of different preparations.

If a biological effect develops, preclinical testing may help to identify the possible sequelae of immunogenicity, as has been shown with EPO in dogs. In the canine model human EPO is immunogenic and it induces antibodies which neutralize the native canine EPO leading to pure red-cell aplasia. This severe complication of antibodies to EPO was later confirmed in humans [19]. In addition, antibody positive animals provide sera for the development and validation of antibody assays.

Non-human primates and transgenic animals may be the best models to study immunogenicity of biopharmaceuticals for human use. In human primates there will be a high sequence homology between the product and the homologous molecule to which the animal is immune tolerant. However, one should be aware of strain differences in different monkey species and differences in immunogenicity between monkeys and humans. And there are reports about proteins immunogenic in non-human primates which proved non-immunogenic in human patients and the other way around.

The best models to evaluate the immunogenicity of human proteins are transgenic animals, immune tolerant for the human protein [20–22]. In these animals breaking of immune tolerance can be studied as has been shown with insulin, htPA and growth hormone. Transgenic mice were used some years ago to show that aggregates in Hu IFN alpha 2a produced during storage at room temperature of a freeze dried preparations were responsible for enhanced immunogenicity.

Recently this model was extensively used to study the effect of protein modifications on immunogenicity. These studies not only confirmed the potential of this model but also the central role of aggregates in breaking B-cell tolerance. Results obtained in mice expressing human IFN beta confirmed these observations. Although transgenic immune-tolerant mice are the best choice to study breaking B-cell tolerance, the *in vivo* analysis of the immunogenicity may be complicated, if the therapeutic protein has immune-modulatory effects which may interfere with antibody production or if the transgenesis modulates the immune response.

Consequences of antibodies to therapeutic proteins

In many cases the presence of antibodies is not associated with biological or clinical consequences. The effects which antibodies may induce depend on their level and affinity and can be the result of antigen–antibody reaction in general or of the specific interaction (Table 1). Severe general immune

Table 1. Consequences of antibodies to therapeutic proteins.

General immune effects: anaphylactoid reactions, serum sickness, etc.
Effects on pharmacokinetics
Reduction of efficacy
Cross neutralization of endogenous protein

reactions as anaphylaxis associated with the use of animal antiserum have become rare because the purity of the products increased substantially. Delayed-type infusion-like reactions resembling serum sickness are more common, especially with monoclonal antibodies and other proteins administered in relative large amounts and the formation of immune complexes. Patients with a slow but steadily increasing antibody titer are reported to show more infusion-like reactions than patients with a short temporary response.

The consequences of the specific interaction with protein drugs are dependent on the affinity of the antibody translating in binding and/or neutralizing capacity. Binding antibodies may influence the pharmacokinetic behavior of the product and both increase and reduction of half-life have been reported resulting in enhancement or reduction in activity.

Persisting levels of neutralizing antibodies in general result in loss of activity of the protein drug. Because by definition neutralizing antibodies interact with ligand–receptor interaction, they will inhibit the efficacy of all products in the same class with serious consequences for patients if there is no alternative treatment.

The most dramatic side effects occur if the neutralizing antibodies cross react with an endogenous factor with an essential biological function. This had been described for antibodies induced by epoetin alpha and MGDF which led to life-threatening anemia, respectively, thrombocytopenia sometimes lasting for more than a year.

Reduction of immunogenicity

In [Table 2](#), there are many reports on the reduction of immunogenicity of human growth hormone, factor VIII, insulin and interferon alpha 2a through improvements in manufacturing, specifically purification and formulation. So optimizing downstream processing and the avoidance of aggregates in particular is probably the best possible approach to minimize immunogenicity especially of human homologues.

Chemical modification may be helpful if the therapeutic protein is intrinsically immunogenic as is the case with non-human proteins. Covalent binding with polymers, such as PEG and Dextran, and protection of positive charges with succinic anhydride may reduce the immunogenicity of proteins.

Table 2. Strategies to reduce immunogenicity.

Removal of non-human sequences
Pegylation
Optimizing production and purification
Optimizing formulation

Whether pegylation of products that are more homologous to human proteins generally reduces immunogenicity is much less clear. There are examples of serious immunogenicity of pegylated human homologues such as MGDF and TNF binding protein.

Sequence modification has been reported to reduce the immunogenicity of products like staphylokinase and streptokinase, which are bacterial products carrying neo-antigens. The benefits from sequence modification must be carefully weighed against the risk that these products will cause the breakdown of immune tolerance by introducing novel T- or B-cell epitopes.

The best example of reducing the immunogenicity by sequence modifications are the different generations of monoclonal antibodies [23,24]. The first generation of monoclonal antibodies were of murine origin and therefore highly immunogenic. Several technical advances have been made over the years in both the development as well as the production of monoclonal antibodies. Recombinant DNA technology has made it possible to exchange the murine constant parts of the immune globulin chains with the human counterparts (chimeric mAb) and later to graft murine complementary regions (CDRs) which determine specificity into a human immune globulin backbone creating humanized mAbs. Both the chimeric as well as the humanized monoclonal antibodies have a reduced immunogenicity. At present, transgenic animals, phage display technologies and other developments allow the production of completely human mAb. These human antibodies still have some immunogenicity based on the other factors discussed earlier.

Route of administration is one of the factors influencing the induction of antibodies by proteins. It is possible that local inflammatory reaction, which is occasionally seen after the local injection of biopharmaceuticals, may contribute to the immunogenicity. Alternative forms of delivery may well help to reduce immune reactions. However, the use of pumps delivering insulin intraperitoneally was associated with a higher immunogenicity in about 50% of patients. Sustained release of human growth hormone by encapsulation in microspheres did not lead to different immunogenicity in rhesus monkeys nor transgenic mice which were immune tolerant for rhGH. The use of liposomes to target protein pharmaceuticals has been associated with higher immunogenicity. While it is reasonable to expect that advances in delivery technology will allow one to reduce the immunogenicity, this has not yet been fully established.

References

1. Walsh G. Pharmaceutical biotechnology products approved within the European Union. *Eur J Pharm Biopharm* 2003;55:3–10.

2. Moore WV and Leppert P. Role of aggregated human growth hormone (hGH) in development of antibodies to hGH. *J Clin Endocrinol Metab* 1980;51:691–697.
3. White GC, 2nd, Kempton CL, Grimsley A, Nielsen B and Roberts HR. Cellular immune responses in hemophilia: why do inhibitors develop in some, but not all hemophiliacs? *J Thromb Haemost* 2005;3(8):1676–1681. Review.
4. Schellekens H. Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clin Ther* 2002;24(11):1720–1740.
5. Schellekens H. Bioequivalence and the immunogenicity of biopharmaceuticals. *Nat Rev Drug Discov* 2002;1(6):457–462.
6. Schellekens H, Ryff JC and van der Meide PH. Assays for antibodies to human interferon-alpha: the need for standardization. *J Interferon Cytokine Res* 1997; 17(Suppl 1):S5–S8.
7. Neyer L, Hiller J, Gish K, Keller S and Caras I. Confirming human antibody responses to a therapeutic monoclonal antibody using a statistical approach. *J Immunol Methods* 2006;315(1–2):80–87. Epub Aug 7.
8. Geng D, Shankar G, Schantz A, Rajadhyaksha M, Davis H and Wagner C. Validation of immunoassays used to assess immunogenicity to therapeutic monoclonal antibodies. *J Pharm Biomed Anal* 2005;39(3–4):364–375.
9. Mire-Sluis AR, Barrett YC, Devanarayan V, Koren E, Liu H, Maia M, Parish T, Scott G, Shankar G, Shores E, Swanson SJ, Taniguchi G, Wierda D and Zuckerman LA. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods* 2004;289: 1–16.
10. Bachmann MF, Rohrer UH, Kundig TM, Burki K, Hengartner H and Zinkernagel RM. The influence of antigen organization on B cell responsiveness. *Science* 1993;262:1448–1451.
11. Chakerian B, Lenz P, Lowy DR and Schiller JT. Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. *J Immunol* 2002;169:6120–6126.
12. Fehr T, Bachmann MF, Bucher E, Kalinke U, Di Padova FE, Lang AB, Hengartner H and Zinkernagel RM. Role of repetitive antigen patterns for induction of antibodies against antibodies. *J Exp Med* 1997;185:1785–1792.
13. Veronese FM and Pasut G. PEGylation, successful approach to drug delivery. *Drug Discov Today* 2005;10(21):1451–1458.
14. Vadhan-Raj S. Clinical experience with recombinant human thrombopoietin in chemotherapy-induced thrombocytopenia. *Semin Hematol* 2000;37(2 Suppl 4):28–34.
15. Ottesen JL, Nilsson P, Jami J, Weilguny D, Duhrkop M, Bucchini D, Havelund S and Fogh JM. The potential immunogenicity of human insulin and insulin analogues evaluated in a transgenic mouse model. *Diabetologia* 1994;37:1178–1185.
16. Hermeling S, Schellekens H, Maas C, Gebbink MF, Crommelin DJ and Jiskoot W. Antibody response to aggregated human interferon alpha2b in wild-type and transgenic immune tolerant mice depends on type and level of aggregation. *J Pharm Sci* 2006; 95(5):1084–1096.
17. Hochuli E. Interferon immunogenicity: technical evaluation of interferon-alpha 2a. *J Interferon Cytokine Res* 1997;17(Suppl 1):S15–S21.
18. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. *AAPS J* 2006;8(3):E501–E507.
19. Casadevall N, Nataf J, Viron B, Kolta A, Kiladjian JJ, Martin-Dupont P, Michaud PT, Ugo V, Teyssandier I, Varet B and Mayeux P. Pure red cell aplasia and antierythropoietin antibodies in patients treated with recombinant erythropoietin. *New Engl J Med* 2002; 346:469–475.

20. Braun KL, Labow MA and Alsenz J. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. *Pharm Res* 1997;14:1472–1478.
21. Hermeling S, Jiskoot W, Crommelin D, Bornaes C and Schellekens H. Development of a transgenic mouse model immune tolerant for human interferon Beta. *Pharm Res* 2005;22(6):847–851. Epub Jun 8.
22. Stewart TA, Hollingshead PG, Pitts SL, Chang R, Martin LE and Oakley H. Transgenic mice as a model to test the immunogenicity of proteins altered by site-specific mutagenesis. *Mol Biol Med* 1989;6:275–281.
23. Pendley C, Schantz A and Wagner C. Immunogenicity of therapeutic monoclonal antibodies. *Curr Opin Mol Ther* 2003;5:172–179.
24. Presta LG. Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. *Adv Drug Deliv Rev* 2006;58(5–6):640–656. Epub May 23.