

Factors influencing the immunogenicity of therapeutic proteins

Huub Schellekens

Central Laboratory Animal Institute, Utrecht University, Utrecht, The Netherlands

Abstract

Several diseases and disorders are treatable with therapeutic proteins, but some of these products may induce an immune response, especially when administered as multiple doses over prolonged periods. Antibodies are created by classical immune reactions or by the breakdown of immune tolerance; the latter is characteristic of human homologue products. Many factors influence the immunogenicity of proteins, including structural features (sequence variation and glycosylation), storage conditions (denaturation, or aggregation caused by oxidation), contaminants or impurities in the preparation, dose and length of treatment, as well as the route of administration, appropriate formulation and the genetic characteristics of patients. The clinical manifestations of antibodies directed against a given protein may include loss of efficacy, neutralization of the natural counterpart and general immune system effects (including allergy, anaphylaxis or serum sickness). An upsurge in the incidence of antibody-mediated pure red cell aplasia (PRCA) among patients taking one particular formulation of recombinant human erythropoietin (epoetin- α , marketed as Eprex[®]/Erypo[®]; Johnson & Johnson) in Europe caused widespread concern. The PRCA upsurge coincided with removal of human serum albumin from epoetin- α in 1998 and its replacement with glycine and polysorbate 80. Although the immunogenic potential of this particular product may have been enhanced by the way the product was stored, handled and administered, it should be noted that the subcutaneous route of administration does not confer immunogenicity *per se*. The possible role of micelle (polysorbate 80 plus epoetin- α) formation in the PRCA upsurge with Eprex is currently being investigated.

Keywords: antibodies; epoetin; immunogenicity; loss of efficacy; micelles; pure red cell aplasia; route of administration; therapeutic proteins

Introduction

The introduction of recombinant human proteins, such as recombinant human erythropoietin (rhEPO; epoetin), insulin proteins, growth hormones and cytokines, has revolutionized the treatment of many diseases [1–3]. The recent identification of all 30 000 genes in the human genome [4,5] ensures that many more therapeutic proteins will soon become available. However, most therapeutic proteins have been shown to induce immune responses, in particular when administered as multiple doses over prolonged periods [6,7].

Previously, there has been concern over the development of neutralizing antibodies and the upsurge of cases of pure red cell aplasia (PRCA) among patients with chronic renal failure treated with epoetin- α (Eprex[®]/Erypo[®]; Johnson & Johnson) [8–10], following the change in its formulation in Europe. Although PRCA is normally a rare side effect (20 cases in 100 000 patient years [9]), it is serious and requires patients to be treated with multiple blood transfusions. The mechanisms by which PRCA is generated are therefore significant and the various factors influencing the immunogenicity of therapeutic proteins are discussed here, with particular reference to epoetin.

Immune reactions against biopharmaceuticals

Different types of biopharmaceutical products produce two markedly different immune reactions (Table 1), namely classical immune reactions to neo-antigens and a breakdown of immune tolerance.

Correspondence and offprint requests to: Dr Huub Schellekens, Department of Innovation Studies, Central Laboratory Animal Institute, Utrecht University, PO Box 80.190, 3508 TD Utrecht, The Netherlands. Email: h.schellekens@gdl.uu.nl

Table 1. Types of immune reaction against biopharmaceutical products

	Classical reaction to neo-antigens	Breakdown of immune tolerance
Properties of product	Microbial or plant origin	Human homologue
Characteristics of antibody formation	Fast; often single injection; high incidence; neutralizing antibodies; long duration	Slow; after prolonged treatment; mainly binding antibodies; low incidence; disappear after stopping treatment, sometimes during treatment
Cause of immunogenicity	Presence of non-self-antigens	Impurities and presence of aggregates
Consequences	Loss of efficacy in majority of cases	In majority of patients no consequences

Classical immune reactions

Classical immune reactions were first seen in association with the use of proteins of animal origin, such as antisera from horses and insulin from pigs and cattle. These animal proteins are foreign antigens to humans and result in immunization when they are administered. This classical reaction to neo-antigens also characterizes products of microbial [11] or plant origin [12], such as streptokinase, staphylokinase and asparaginase. It tends to be a rapid reaction, often occurring after a single injection. This type of immune response has a high incidence, while the antibodies are usually neutralizing and persist for a long time. The cause of this immunogenicity is easily explained (i.e. the presence of non-self-antigens) and can be modelled in laboratory animals. The clinical consequence, in most cases, is a loss of product efficacy.

Similar reactions have been seen with human-derived proteins such as growth hormone derived from the pituitary, and factor VIII from serum, primarily used to treat children lacking the native protein [6]. As a result, these patients were immune-naïve to the particular protein product—which was effectively a foreign antigen—and so they had a reaction equivalent to the classical immune response.

Breakdown in immune tolerance

Reactions to recombinant human proteins such as insulin, interferon (IFN) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are different from those discussed above in that these products are not foreign antigens so the patients do not undergo immunization. The reaction in these patients is due to a breakdown in immune tolerance [9], which is a slow process and can take years to become clinically apparent. The incidence of this type of reaction is generally low [13–15], although it can be very high with IFN- β [16,17]. The antibodies may disappear if treatment is stopped, and there are also situations where they disappear in the long term if treatment is continued [18].

Breakdown of immune tolerance is characteristic of human homologue products, including epoetin- α , which was associated with an upsurge in cases of PRCA during the last few years. The main cause of breaking tolerance is the presence of impurities or aggregates in the product [9].

Factors influencing immunogenicity

Factors affecting the immunogenicity of a protein include structural properties (e.g. sequence variation and glycosylation) [19,20], but the lack of standard assays for antibodies makes it difficult to compare results between laboratories and between published studies [21].

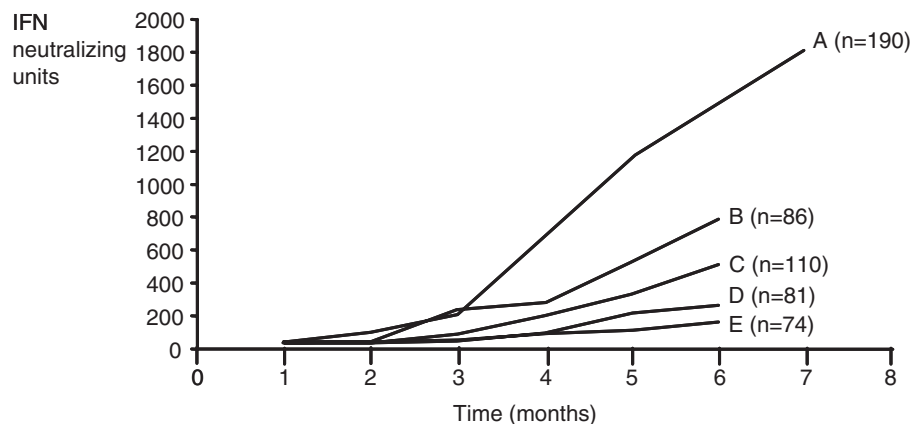
Downstream processing of a product can also influence its immunogenicity. For example, in Belgium and The Netherlands in the late 1990s, antibodies to factor VIII were found to be associated with the introduction of a new pasteurization stage in the manufacturing process. Impurities and contaminants associated with antibody development have also been found in studies on insulin and growth hormone products [22,23]; however, this problem is decreasing as the purity of products improves. There are also likely to be unknown factors that influence immunogenicity without producing any detectable differences in physicochemical characterization of the compound [9].

The frequency and duration of treatment may be of importance in immunogenesis, since it can take many months to break down immune tolerance. An illustration of this is the different amounts of time it takes for antibodies to be produced against the various IFN- β products used to treat multiple sclerosis [17,24].

The genetic background of patients can sometimes influence immunogenicity. There have been conflicting results from studies into the influence of the major histocompatibility complex (MHC) on responses to products—such as growth hormone and insulin—indicating that MHC has no real effect. Another well-established example is with haemophilia, whereby the genetic defect determines whether an individual will or will not produce antibodies [25].

The route of administration can influence the immunogenicity of the protein. Evidence suggests that the subcutaneous (s.c.) environment is relatively hostile to an already immunogenic protein but s.c. administration by itself cannot render a non-immunogenic protein immunogenic. Intramuscular (i.m.) administration is also likely to elicit an immune response, but to a lesser degree than intravenous (i.v.) administration, which in turn is more immunogenic than local (topical) administration [17]. No matter what route of

(a)



(b)

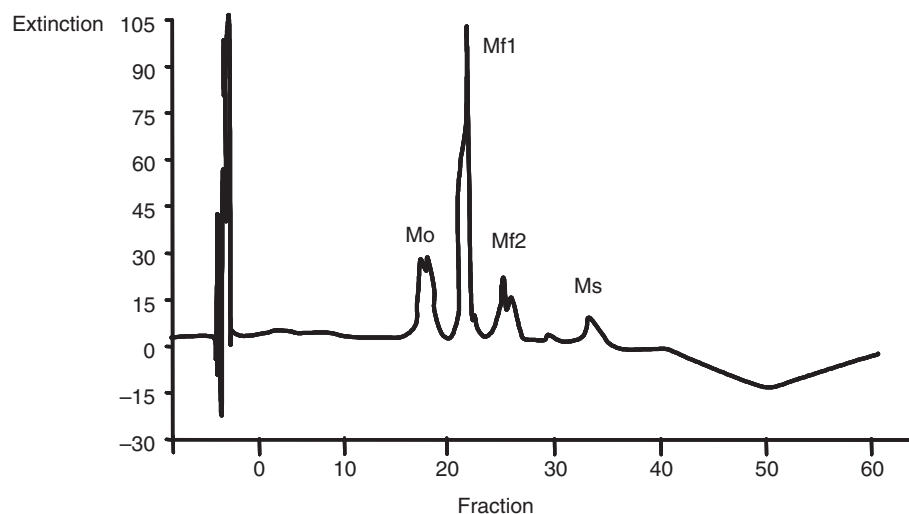


Fig. 1. Variability of IFN- α 2a formulations demonstrated by (a) antigenicity (Ryff [28] with permission) and (b) reverse-phase high-performance liquid chromatography (RP-HPLC) analysis, showing contamination with an oxidized monomer (Mo) (Hochuli [29] with permission).

administration is chosen, it does not confer immunogenicity *per se*: a substance or a product is either immunogenic or it is not. There have been no published cases whereby a change in administration route completely negated immunogenicity [10].

Appropriate formulation of a protein product is highly important, particularly with respect to stabilization, because if this is inadequate the protein may aggregate or denature, which increases its immunogenic potential [26]. Formulation becomes even more crucial for products that may not be optimally stored or handled [27]. For example, in a study of IFN- α 2a formulations, the most immunogenic (A) was a freeze-dried, human serum albumin (HSA)-containing formulation, which was kept at room temperature in accordance with the product's handling and storage instructions [28] (Figure 1a). Other HSA-free formulations (B, C, D and E) were less immunogenic. In another study [29], examination of the components of the IFN- α 2a formulation showed that the molecule became oxidized at room temperature

(Figure 1b). The contents of this peak are more immunogenic than normal IFN- α 2a and react with HSA to form aggregates, which in turn induce an immune response. Changing to a liquid, HSA-free formulation, and recommending storage at 4°C have reduced the immunogenicity of this product [28].

Consequences of antibody formation

Antibodies usually have no adverse clinical consequences except a loss of product efficacy, an effect observed with many different products, e.g. insulin, streptokinase and IFN- α 2a. In one study, patients with hepatitis C virus (HCV) infection were treated with IFN- α 2a. Sustained response rates could be correlated with antibody status; for example, patients with the highest antibody titres had the lowest response rates to IFN- α 2a treatment (Figure 2 [28]).

In some cases, the immunogenicity can be overcome to a certain extent by increasing the dose to reverse the

loss of efficacy, while in a small number of instances, efficacy can be enhanced; e.g. it has been shown that antibody development to growth hormone in children results in enhanced hormone efficacy [30]. This is because binding antibodies stabilize the growth hormone.

Antibody formation can also have general immune effects, such as allergy, anaphylaxis and serum sickness [31], but these are becoming increasingly rare because of the purity of the proteins now in use. Neutralization of the native protein by antibodies can have more serious consequences for the patient. This has been seen with megakaryocyte-derived growth factor (MDGF [32]), where administration to volunteers and patients with cancer produced MDGF antibodies, which neutralized their own thromboopoietin. This resulted in severe thrombocytopenia and the patients needed platelet treatment to survive. The second known example of native protein neutralization is

with epoetin- α [8,33], which led to an upsurge in cases of PRCA.

Relationship between epoetin- α and the epidemic of PRCA

The reports of PRCA associated with epoetin treatment [8] appeared to be primarily associated with the epoetin- α formulation available in Europe (Eprex/Erypo). Only a few sporadic cases have involved patients treated with other epoetin- α products (Procrit[®]; Ortho Biotech, Epogen[®]; Amgen) or epoetin- β (NeoRecormon[®]; Roche Pharmaceuticals), and the increased number of PRCA cases was only observed in countries where Eprex was marketed. Notably, the upsurge corresponded with the removal of HSA from the Eprex formulation and its replacement with glycine and polysorbate 80 (Figure 3) [33,34].

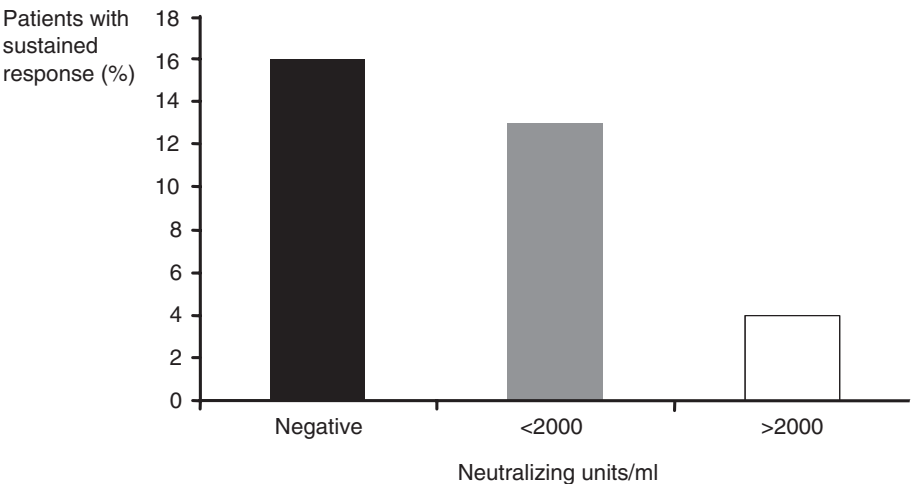


Fig. 2. Relationship between sustained response and antibody level in IFN- α 2a-treated patients with HCV infection.

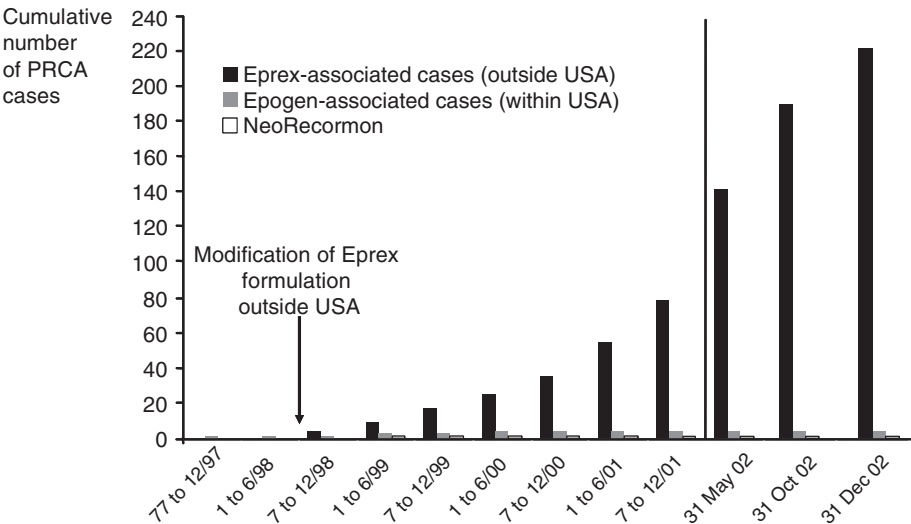


Fig. 3. Cumulative PRCA cases over a 5 year period (1997–2002) (adapted from Gershon *et al.* [33] with permission).

The main stabilizers used in currently available epoetin formulations are summarized in Table 2.

The Eprex formulation in Europe was changed in 1998 to comply with new European recommendations for the removal of human-derived products (in this case HSA) from pharmaceutical products; this stabilizer was replaced by polysorbate 80 and glycine. There was no such recommendation in the USA so the epoetin- α formulation (Procrit, Epogen) still contains HSA. No increase in the annual number of PRCA cases in the USA has been observed since 1998 (see Figure 3), in contrast to the situation in Europe. NeoRecormon (epoetin- β) has retained an HSA-free formulation since its launch in 1990 and instead of polysorbate 80, glycine and polysorbate 20 have been used as stabilizers (Table 2).

Hermeling *et al.* [35] used size exclusion analysis (SEC) to compare the formulations of NeoRecormon and Eprex (Figure 4a) and demonstrated that there was an extra peak (peak 1) with Eprex, compared with NeoRecormon. The extra peak is a result of micelle formation, and further analysis with enzyme-linked immunosorbent assay (ELISA) and Western blotting techniques demonstrated that the micelles contained epoetin- α (Figure 4b). This raises the question of

whether the micelles have a role in the breakdown of immune tolerance.

The role of micelles

An important determinant of protein immunogenicity is the tendency of the protein to form aggregates or micelles. Micelles may simulate a viral structure, and the human immune system has evolved to react vigorously to viruses. Unlike other epoetin formulations, the currently available European formulation of Eprex may have a tendency to form micelles, possibly due to its high concentration of polysorbate 80. During encapsulation of a protein in micelle formation, antigenic hydrophilic sites may be left exposed in an array form. Work is currently under way to investigate whether micelle formation is the cause of the problem with Eprex. (Since the presentation of this paper in 2003, additional data have been published. Kerwin and co-workers have also found a direct interaction between polysorbate 80 and epoetin- α , and hypothesized that it may lead to changes in the molecule [36]. Also, the suggestion has been raised that the presence of leachates from the rubber stoppers used in the pre-filled syringes of Eprex may be implicated in its immunogenicity [37].)

Influence of route of administration

The majority of PRCA cases occurred with s.c. administration of Eprex [8,38]. As a result, European regulatory authorities restricted the use of Eprex to the i.v. route [39]. Although s.c. administration of Eprex is contraindicated in renal patients in Europe, no restrictions have been placed on s.c. epoetin- β . A group of experts organized by the Canadian Society of Nephrology concluded that there is no evidence to support a general switch to i.v. administration of

Table 2. Main stabilizers used in epoetin formulations

Epogen/Procrit (USA)	Eprex (pre-1998)	Eprex (post-1998)	NeoRecormon (1990 launch)
HAS	HSA	Polysorbate 80 Glycine	Polysorbate 20 Glycine Complex of five other amino acids Calcium chloride Urea

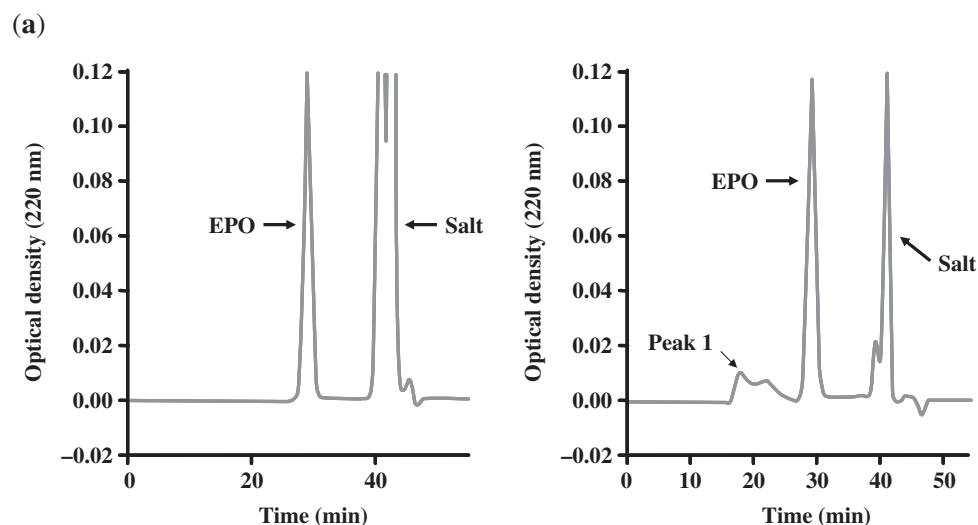


Fig. 4. Formation of micelles (Hermeling *et al.* [35] with permission). (a) Size exclusion analysis (SEC) of Eprex (Erypo) and NeoRecormon demonstrates an extra peak (peak 1) in Eprex, compared with NeoRecormon. (b) Enzyme-linked immunosorbent assay (ELISA) and western blotting (lower right) analyses of peak 1 demonstrate that the micelles contain epoetin- α .

(b)

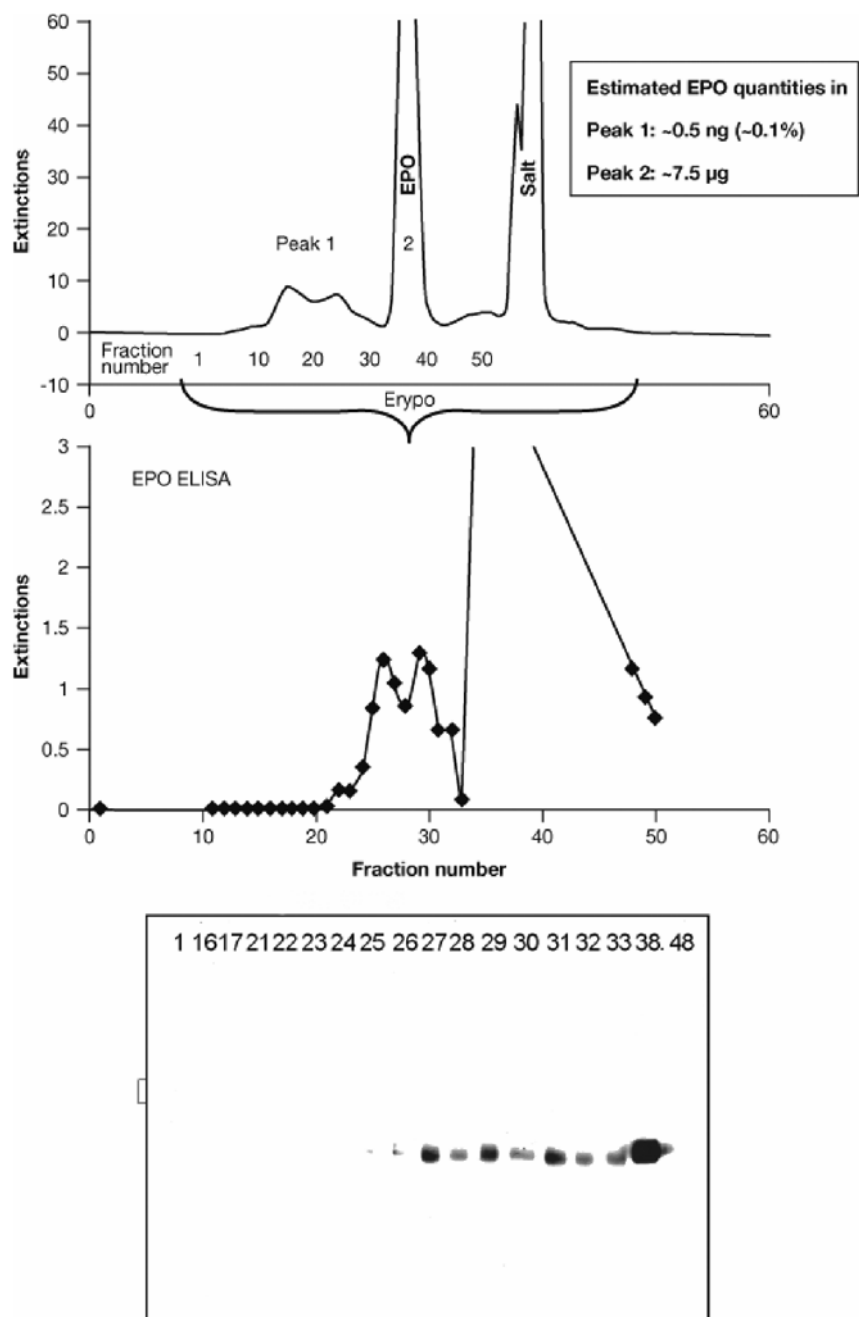


Fig. 4. Continued.

epoetin [40] because the route of administration cannot render a protein immunogenic, although it can increase the likelihood of an immune reaction to a protein that is already immunogenic.

Conclusions

Most biopharmaceutical products have immunogenic potential. The breakdown of immune tolerance seen

with some human homologues may be related to the way epitopes are presented, e.g. aggregates or micelles. Appropriate formulation of a protein product is of great importance with respect to stabilization because, if it is inadequate, the protein may aggregate or denature, which increases its immunogenic potential. Biogenerics, as well as modified proteins, should be carefully evaluated as therapeutic options and their benefits weighed against successful, time-tested formulations.

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