BIOEQUIVALENCE AND THE IMMUNOGENICITY OF BIOPHARMACEUTICALS

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The expiry of the first patents for recombinant-DNA-derived biopharmaceuticals will open the possibility of marketing generics, if they can be shown to be essentially similar to the innovator product. However, as shown by the problem of immunogenicity, the properties of biopharmaceuticals are dependent on many factors, including downstream processing and formulation. Products from different sources cannot be assumed to be bioequivalent, even if identical genes are expressed in the same host cells and similar production methods are used. Some of the influencing factors are still unknown, which makes it impossible to completely predict biological behaviour, such as immunogenicity, which can sometimes lead to serious side effects.

RECOMBINANT DNA
The combination of DNA from different species; for example, the introduction of human genes into microorganisms.

GENERIC
A copy of a drug that is introduced after the patent expires.

BIOEQUIVALENCE
Similarity of biological properties.

BIOPHARMACEUTICAL
A pharmaceutical product that consists of protein and/or nucleic acid.

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In the near future, the patents of some RECOMBINANT-DNA-derived pharmaceuticals will expire. In the case of classical drugs, patent expiration opens the possibility of introducing GENERIC products. For classical generics, limited documentation to show chemical similarity and BIOEQUIVALENCE in a small volunteer study is, in general, sufficient to obtain marketing authorization. However, it is unlikely that the concept of developing generics for small-molecule therapeutics can be extrapolated to most BIOPHARMACEUTICALS, which tend to be large proteins. We lack the technology to establish whether the structures of two biopharmaceuticals are completely identical. Moreover, the properties of biotechnology products are highly dependent on the production process, and their biological behaviour remains partly unpredictable.

To highlight the difficulties in comparing biotechnology products, this review discusses the IMMUNOGENICITY of biopharmaceuticals. It also shows that the clinical consequences of insufficient biological characterization could be severe. The term 'biopharmaceutical', as used here, excludes antibodies — the immunogenicity of which raises separate questions — but includes therapeutic proteins from natural sources and those produced by recombinant-DNA technology.

Immunogenicity

Immunogenicity has always been associated with the medical use of proteins, derived from both human and non-human sources. As immunogenicity can have severe clinical consequences, such as loss of efficacy of the product or even life-threatening complications, it is important to establish the factors that contribute to the immunogenicity of biopharmaceuticals. Initially, when proteins of animal origin, such as bovine and porcine insulin, were used as therapeutics[1], the foreign origin of these proteins was considered to be the main cause of immunogenicity. Later, it was found that products purified from human tissue or sera, such as growth hormone and factor VIII, also seemed to induce an immunological response[2,3]. These products were given to patients with innate deficiencies, which were considered to be the cause of the lack of self-tolerance.

The introduction of recombinant technology made it possible to develop products that were identical or nearly identical to native human proteins. In the past two decades, an increasing number of biopharmaceuticals produced by genetically modified cells have entered clinical practice. The first generation of products comprised mainly copies of naturally occurring growth factors, cytokines or hormones, sometimes with minor

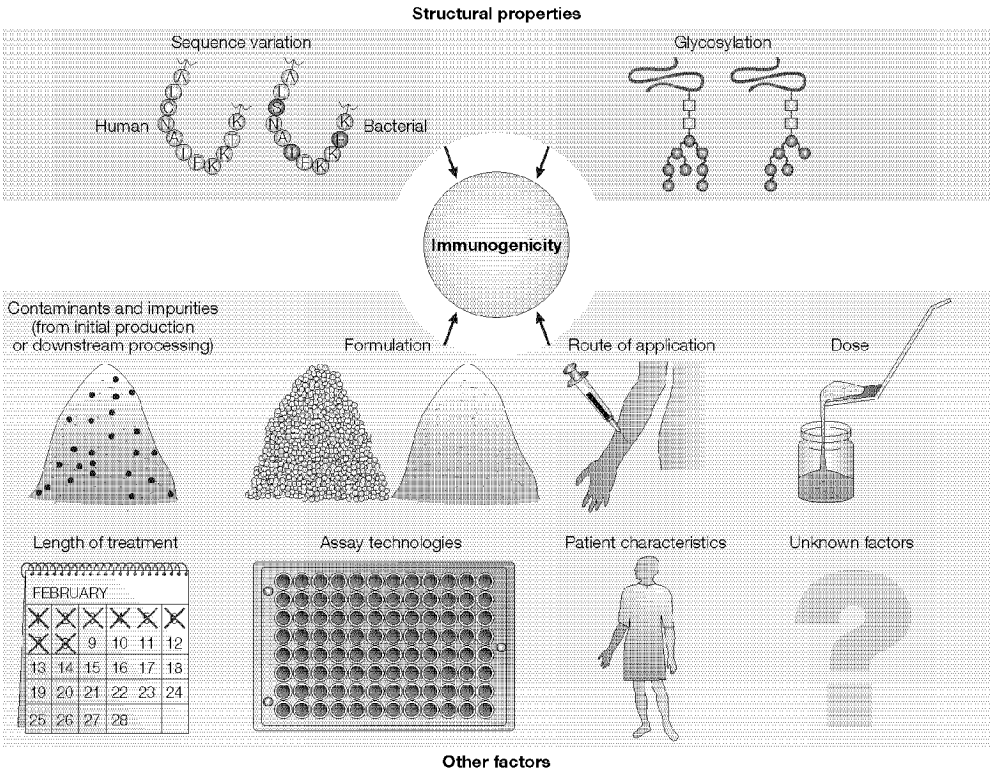

Figure 1 | **Some of the many factors that influence the immunogenicity of biopharmaceuticals.** The immunogenic potential of therapeutic proteins can be reduced by the design of the production system and downstream processing. However, some of the factors involved are still unknown.

modifications to enhance stability or allow expression in bacterial hosts. However, in cases in which proteins were replaced with recombinant products, the problem of immunogenicity — although reduced —still persisted[4,5].

Factors that influence immunogenicity

FIGURE 1 lists the main factors that are thought to influence the immunogenicity of biopharmaceuticals.

Sequence variation. Predictably, proteins that are derived from non-human sources, such as streptokinase[6], staphylokinase[7], bovine adenosine deaminase[8] and salmon calcitonin[9], have shown considerable immunogenicity in patients. Their degree of divergence from the human protein sequence fully explains their immunogenicity. However, similarity to the human sequence does not necessarily imply a lack of immunogenicity. There have been cases of high and/or clinically relevant levels of antibody production after the administration of natural biopharmaceuticals derived from human organs and cells; for example, factor VIII (REF 10), growth hormone[11] and human interferon-β (IFN-β)[12]. Furthermore, recombinant-DNA-derived pharmaceuticals produced on the basis of a sequence that was completely identical to the human gene have shown immunogenicity for example, erythropoietin (EPO)[13], IFN-α2B[14] and recombinant IFN-β[15].

Conversely, there are many examples of biopharmaceuticals that differ from the natural sequence in which the variation from the naturally occurring human sequence has not led to increased immunogenicity. Examples include IFN-α2A[16], consensus IFN-α[17] and methionyl human growth hormone[18].

Glycosylation. The immunogenicity of several biopharmaceuticals, including IFN-β and granulocyte–macrophage colony-stimulating factor (GM-CSF), has been related to de-glycosylation. For GM-CSF, the increased immunogenicity of the non-glycosylated, bacteria-derived protein is thought to be caused by the exposure of antigenic sites[19].

In the case of IFN-β, the higher immunogenicity of the *Escherichia coli*-derived product compared with the mammalian-cell product has been explained by the reduced solubility of the non-glycosylated bacterial product[20]. The carbohydrate side chains are thought to shield hydrophobic sites of mammalian IFN-β. So far, hyperglycosylation has not been correlated with increased immunogenicity[21].

Host cells. The particular host cell that is used for recombinant-protein production is a crucial determinant of the immunogenicity of biopharmaceuticals, although the relative contribution of the host depends on the product.

IMMUNOGENICITY
The capacity to elicit an immune response, such as the production of specific antibodies.

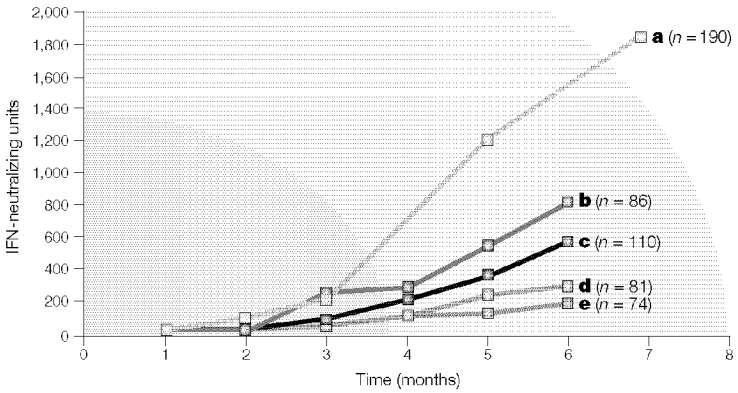

Figure 2 | **Immunogenicity of different human IFN-α2A preparations.** The immunogenicity of human interferon-α2A (IFN-α2A) is highly dependent on the formulation and storage conditions, as shown here by the mean-population antibody titre in patients treated with different IFN preparations: **a** | lyophilized powder stored at room temperature; **b** | lyophilized powder stored under refrigeration; **c** | human serum albumin (HSA)-containing liquid stored under refrigeration; **d** | ultrapure liquid formulation (HSA-free) stored under refrigeration; and **e** | ultrapure lyophilized powder stored under refrigeration. IFN-neutralizing units; arbitrary unit of neutralizing activity; *n*, number of patients. Reproduced with permission from REF. 26 © (1997) Mary Ann Liebert, Inc.

Natural IFN-α2 (REF. 22) and interleukin-2 (IL-2)[23] were reported to be less antigenic than products made by *E. coli*. However, for growth hormone[24] and IFN-β[25], the opposite correlation was found.

Contaminants and process-related impurities. There are many examples in which contaminants or process-related and product-related impurities were associated with immunogenicity, and these factors can be considered to be the main cause of immunogenicity over the years[26,27]. Host-cell contaminants (such as lipopolysaccharides (LPS)), protein modifications (such as oxidation and deamidation) and aggregate formation have been implicated in the immunogenicity of many biopharmaceuticals, including insulin, growth hormone, IFN-α2A and factor VIII. Sometimes, these impurities and contaminants were present during the initial production process, and improving the purification procedure[28] reduced the problem. In other cases, however, the problem of immunogenicity was introduced by changes in production or downstream processing[29].

Formulation and storage. Formulations that allow protein oxidation or aggregation, such as freeze-drying preparations, can enhance immunogenicity[30]. Storage of freeze-dried materials at room temperature could enhance the problem. Human serum albumin (HSA) is commonly added to proteins as a stabilizing agent, as it is believed to reduce protein aggregation. However, in the case of IFN-α2A, the presence of HSA was actually correlated with the formation of aggregates and the problem of immunogenicity[31] (FIG. 2).

Route of administration. In studies in which routes of administration were analysed, the intramuscular and subcutaneous routes were shown to be the most immunogenic[32,33]. Intravenous application is comparatively less immunogenic, with local treatment being the route of administration that is the least associated with immunogenicity. However, mucosal application of products such as calcitonin and deoxyribonuclease can still be associated with considerable antibody production[34,35].

Dose and length of treatment. In general, the dose and total amount of a drug that are received are related to immunogenicity. However, the effect of treatment length on immunogenicity seems to be a factor that is independent of the amounts applied. Both *E. coli*- and Chinese hamster ovary (CHO)-derived IFN-β products induce antibodies after 6–12 months of treatment, although the amounts injected show a tenfold difference in protein content[36]. In general, acute administration of biopharmaceuticals is less likely than chronic administration to be associated with immunogenicity.

Patient characteristics. Patients with impaired immune systems, such as cancer patients, are less likely to develop antibodies than patients with intact immune systems[37]. Individuals who are deficient for a functioning gene might lack the normal immune tolerance to certain products, such as factor VIII and growth hormone; this deficiency renders such patients more prone to the production of antibodies compared with those who express the protein[38]. Such immunogenicity is sometimes dependent on the type of gene defect that causes the deficiency[39]. In some cases, immunogenicity has been related to certain human leukocyte antigen (HLA) types[40]. In other cases, this relationship could not be established[41].

Assays and types of antibody. Much of the blame for the highly variable and sometimes conflicting results reported concerning immunogenicity can be attributed to the assay technology that is used. The lack of international standardization of the assays and international reference preparations makes it virtually impossible to compare test results from different test laboratories. Even in the case of an international, endorsed assay format, results differ widely[42].

Unknown factors. Although important factors that contribute to immunogenicity have been identified over the years, there are still several unknowns. A good example of such uncertainty is CHO-cell-derived IFN-β, for which a significant reduction in immunogenicity was noted when the production site for this biopharmaceutical was changed. Although it was extensively analysed, the cause of the reduced immunogenicity could not be pinpointed by the manufacturer (S. Goelz (Biogen), personal communication).

In addition, the recent problem with recombinant EPO shows how unpredictable the problem of immunogenicity can be[43]. Recombinant EPO has an excellent safety record, and it has been prescribed to millions of patients without major problems. Recently, however, an epidemic of pure red-cell aplasia has been identified that is linked to antibodies that are induced by a specific recombinant-EPO product. The first 13 cases were published recently[43], and since the submission of this report,

Table 1 | **Clinical consequences of antibodies**

Consequence of antibody	Biopharmaceutical	References
Loss of efficacy	Insulin	5
	Streptokinase	6
	Staphylokinase	7
	ADA	63
	Salmon calcitonin	9
	Factor VIII	3
	IFN-α2	14,26
	IFN-β	15
	IL-2	23
	GnRH	64
	Denileukin diftitox	65
	HCG	66
	GM-CSF/IL-3	67
Enhancement of efficacy	Growth hormone	2
Neutralization of native protein	MDGF	45
	EPO	13,43

ADA, adenosine deamidase; EPO, erythropoietin; GM-CSF, granulocyte–macrophage colony-stimulating factor; GnRH, gonadotropin-releasing hormone; HCG, human chorionic gonadotropin; IFN-α2, interferon-α2; IL-2, interleukin-2; MDGF, megakaryocyte-derived growth factor.

the number of cases has increased to more than 40. The antibodies that are induced by the recombinant EPO crossreact with the endogenous protein, which leads to a complete blockade of the differentiation of red blood cells. The patients survive only by frequent blood transfusions, and although antibody levels decrease when the treatment is stopped, about 50% of the patients remain transfusion dependent. Although the exact cause has not yet been identified, the type and duration of treatment, geographical distribution of the cases and timing indicate that a change in the production process might be the cause.

Clinical and biological consequences

The clinical effects of the antibodies that are induced by the different biological products are summarized in TABLE 1. For most biopharmaceuticals, the presence of antibodies has no consequences. Occasionally, antibodies are even related to enhanced efficacy of the product[44]. In the case of negative consequences, the main effect is loss of efficacy of the treatment. Only in cases in which the biopharmaceutical induces antibodies that neutralize proteins that have an important physiological function will severe side effects develop[45].

Predictability of immunogenicity

Protein sequence. The degree of homology with the native protein is an important factor in determining the potency of a protein to induce antibodies. Note that protein-sequence differences can be distributed more or less evenly across the sequences that are being compared, or, alternatively, they can be concentrated in sections in which the sequences are very different. The degree of homology described as an overall per-cent identity is, therefore, a crude measure of differences. The distribution of differences might be expected to have profound effects on the probability of generating new T- or B-cell EPITOPES. If the proteins differ so substantially that epitopes are formed to which the patient has no innate immunity, then a rapid and intense immune reaction is induced. The best examples of this situation are products of microbial or plant origin, such as streptokinase and trichosanthin[46], which have low homology to human proteins. On the basis of sequence analysis, the immunogenicity of these proteins can be reduced; however, the algorithms that are designed to predict epitopes are still only at an early stage of development[47,48].

Considering that factors other than sequence are important for immunogenicity, and that immune responses are genetically determined and, therefore, highly individual, sequence analysis alone will probably never be sufficient to predict and avoid antibody induction.

Physicochemical characterization. For biopharmaceuticals that have a high degree of homology to native proteins, the chief factors to cause immunogenicity have proven to be impurities, heterogeneity, aggregate formation and protein degradation — for example, by oxidation or deamidation (FIG. 3). Each of these factors can be monitored by physicochemical characterization during production and storage. However, other factors influence immunogenicity that cannot be identified by the methods that are used at present to characterize biopharmaceuticals. Highly sophisticated methods, such as real-time affinity assays based on SURFACE PLASMON RESONANCE (SPR) and three-dimensional computer modelling, are now available to study antibody–antigen interactions[49]. These techniques are important both as diagnostic tools for identifying immunogenic reactions to proteins, and as methods for discovering the factors that determine immunogenicity.

Animal models. The immune reaction in animals to biopharmaceuticals of microbial or plant origin is similar to that 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[50,51].

The development of antibodies has been observed regularly in preclinical studies in animals of biopharmaceuticals that are homologous to human proteins[52]. As such a response is considered to be a normal reaction to a foreign protein, it has led to the generally held assumption that immunogenicity testing, and, in some cases, any preclinical testing at all, in animals is irrelevant. However, not all antibodies interfere with the biological activity of a biopharmaceutical or induce other effects. If a biological or clinical effect develops, preclinical testing can also help to identify the possible sequelae of immunogenicity, as has been shown with EPO in dogs[53]. In the canine model, human EPO is immunogenic, and it induces antibodies that neutralize the native canine EPO and lead to pure red-cell aplasia. This severe complication of EPO was later confirmed in humans. In addition, antibody-positive animals could provide sera for the development and validation of antibody assays. Furthermore, preclinical testing in animals might help to evaluate the relative immunogenicity of different variants of biopharmaceuticals[54].

Non-human primates and transgenic animals might be the best models for studying the immunogenicity of biopharmaceuticals for human use. In non-human

EPITOPE
Part of a protein that is recognized by an antibody.

SURFACE PLASMON RESONANCE (SPR.) This occurs when surface plasmon waves are excited by light deflection at a metal–liquid interface. SPR can be used to investigate protein–protein interactions, such as antibody–antigen interactions.

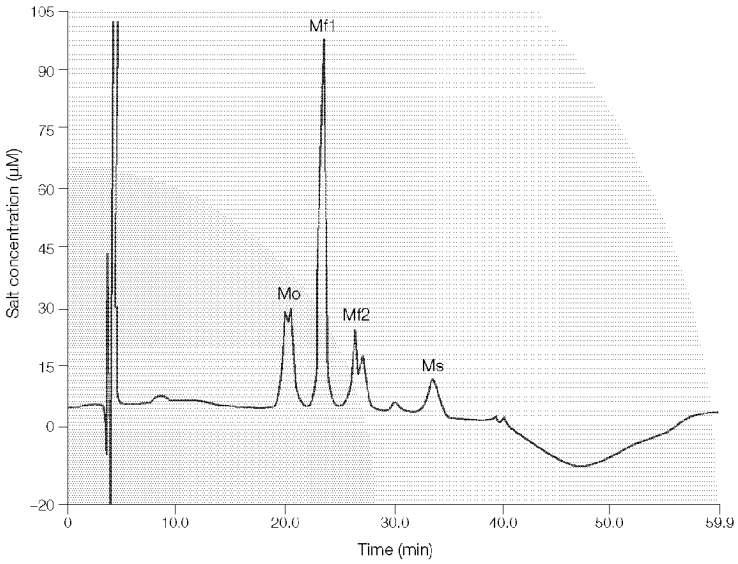

Figure 3 | **RP-HPLC of a highly immunogenic batch of interferon (IFN)-α2A.** The chart shows that this sample contains high levels of the oxidized form (Mo) of IFN-α2A. This oxidized form is more immunogenic than the non-oxidized form (Mf1), and it also contributes to the formation of aggregates, which greatly enhance immunogenicity. Mf2 is the acetylated form, and Ms is the form with only a single disulphide bridge. RP-HPLC, reversed-phase high-performance liquid chromatography. Reproduced with permission from REF. 28 © (1997) Mary Ann Liebert, Inc.

primates, there will generally be a high sequence homology between the product and the native monkey molecule to which the animal is immune tolerant. A recent review[55] suggested using a hyperimmunization protocol — including the use of an ADJUVANT — to determine a worst-case scenario of immunogenicity.

Theoretically, transgenic animals are highly attractive models for monitoring the immunogenicity of biopharmaceuticals. These animals are immune tolerant for the human protein that they express. The caveats are that the wild-type mouse strain that is used for transgenesis should be able to produce antibodies to the protein, and the transgenic mice should have immune tolerance to the native molecule. Studies in mice that are transgenic for human insulin showed that immunogenicity to variant insulin molecules was dependent on the number of amino-acid substitutions[56]. Variants of tissue plasminogen activator (tPA) with a single amino-acid substitution were shown to be antigenic in mice that were transgenic for human tPA, which highlights the discriminatory potential of the model[57].

The transgenic approach also proved useful when mice were made transgenic for IFN-α2 (REF. 58). In this case, the presence of aggregates and oxidized proteins proved to be the main cause of immunogenicity, both in transgenic animals and in human patients. On the basis of this limited data, transgenic animals seem to be the best animal model for predicting immunogenicity, especially when assessing the contribution of aggregate formation or LPS contamination. However, the model needs further evaluation, as there might be differences in the processing of antigens and epitope recognition between mice and men.

ADJUVANT
A substance that can boost the immune response.

NEO-ANTIGEN
A non-self antigen that is new for an individual.

Reactivity with positive sera. The reactivity of patient sera that is positive for antibodies to the unmodified product has been used as an approach to screen for reduced immunogenicity of protein variants. Although this approach will show whether the original epitopes have (partly) disappeared, it will not reveal whether new epitopes have been introduced[59].

Summary

In general, biopharmaceuticals are homologous to, or have a high degree of similarity with, human native proteins. These products act as foreign antigens only in patients who lack immune tolerance because of an innate insufficiency. In these patients, the only consequence of antibody induction is the loss of efficacy of the product. In other patients, immunogenicity is based on the loss of immune tolerance, which is caused by an unnatural presentation of the self-antigens; for example, in the form of aggregates. This is a form of autoimmune response that is normally weak, occurs after long-term treatment, usually has no clinical consequences and sometimes disappears after prolonged treatment[60]. In some cases, the antibodies might be associated with loss of efficacy, which can possibly be overcome by increasing the dose[61]. In rare cases, the antibodies neutralize the native protein, an effect that potentially has biological consequences. Because this type of immune reaction is mainly caused by secondary factors, such as protein modification, impurities and aggregation, it can be avoided by improvements in production and downstream processing. It is still unclear whether chemical modifications, such as polyethyleneglycol (PEG)ylation, reduce this type of immune reaction.

There are also examples of mixed types of immune reaction that are caused by a product carrying NEO-ANTIGENS that induce antibodies that crossreact with the native protein. The degree of variation from the native protein that is needed to trigger this type of reaction is generally uncertain, and some products with a relatively high degree of amino-acid variation failed to induce antibodies.

Sequence analysis, epitope identification and studies of immunogenicity in conventional animals are important tools in helping to understand the factors that are involved in immunogenicity. So far, the best predictor of immunogenicity has been the physicochemical characterization of the product to identify protein modifications such as impurities and, especially, the presence of aggregates — and the use of immune-tolerant transgenic mice.

Conclusion — the possibility for biogenerics

The many factors that influence immunogenicity — some of which have not yet been defined — show that it is inconceivable at present to manufacture a biopharmaceutical that can be shown to be therapeutically equivalent to another product, other than by extensive clinical comparisons. The current analytical methods do not allow a full prediction of the biological and clinical properties of a protein. So, the concept of generics in the case of biopharmaceuticals that consist of large proteins is misleading. We suggested recently the term 'off-patent

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