Pharmaceutical Research, Vol. 21, No. 6, June 2004 (© 2004)

Review Article

Structure-Immunogenicity Relationships of Therapeutic Proteins

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Received August 31, 2003; accepted February 16, 2004

As more recombinant human proteins become available on the market, the incidence of immunogenicity problems is rising. The antibodies formed against a therapeutic protein can result in serious clinical effects, such as loss of efficacy and neutralization of the endogenous protein with essential biological functions. Here we review the literature on the relations between the immunogenicity of the therapeutic proteins and their structural properties. The mechanisms by which protein therapeutics can induce antibodies as well as the models used to study immunogenicity are discussed. Examples of how the chemical structure (including amino acid sequence, glycosylation, and pegylation) can influence the incidence and level of antibody formation are given. Moreover, it is shown that physical degradation (especially aggregation) of the proteins as well as chemical decomposition (e.g., oxidation) may enhance the immune response. To what extent the presence of degradation products in protein formulations influences their immunogenicity still needs further investigation. Immunization of transgenic animals, tolerant for the human protein, with well-defined, artificially prepared degradation products of therapeutic proteins may shed more light on the structure-immunogenicity relationships of recombinant human proteins.

KEY WORDS: aggregation; animal models; immunogenicity; protein structure; therapeutic proteins.

INTRODUCTION

Nowadays, proteins are widely used as therapeutic agents. When the first proteins became available for therapeutic use [in the 1920s, insulin was the first therapeutic protein introduced (1)], concerns were raised about their immunogenicity. Initially, proteins from animal origin (e.g., bovine and porcine insulin) were used. The non-human origin of these proteins was believed to be the reason for their immunogenicity. Later on, proteins purified from human tissue or sera were introduced. Surprisingly, these products also proved immunogenic. These products, such as factor VIII and growth hormone, were given mainly to children with an innate deficiency. The immunogenicity was explained by their lack of immune tolerance. Also, the impurities in these products were considered to be an important reason for the immunogenicity.

The introduction of recombinant DNA techniques enabled the large-scale production of highly purified proteins identical or nearly identical to the endogenous proteins. It was hoped that this would reduce the immune responses to the proteins. Currently, more than 60 recombinant human proteins [including recombinant human(ized) monoclonal antibodies] are available for therapeutic purposes in the European Union (2). Although, in general, the level and incidence of immune responses against recombinant human proteins are low and variable, most of these products have been shown to be immunogenic. In some cases, the immunogenicity of therapeutic proteins leads to serious problems.

Immunogenicity of recombinant human proteins can have a number of clinical consequences. Binding antibodies may influence the pharmacokinetics of the proteins (1,3). High neutralizing antibody levels may result in more serious consequences, such as inhibition of the therapeutic effect or the neutralization of essential endogenous proteins. An example is the antibody formation reported after the long-term subcutaneous administration of recombinant human erythropoietin (epoetin) in patients with chronic renal failure: the antibodies also neutralize endogenous erythropoietin, which results in pure red cell aplasia (4–6).

Table I shows that several factors can influence the immunogenicity of therapeutic proteins in man. This review

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ABBREVIATIONS: CHO-rhIFNβ, recombinant human interferon beta expressed in CHO-cells; *E. coli*-rhIFNβ, recombinant human interferon beta expressed in *E. coli*; epoetin, recombinant human erythropoietin; hGH, human growth hormone; hIFNβ, interferon beta obtained from human fibroblasts; HSA, human serum albumin; humatrope, natural sequence recombinant human growth hormone; met-rhGH, recombinant human growth hormone with an extra methionine; MSA, murine serum albumin; PEG, polyethylene glycol; pit-hGH, human growth hormone purified out of human pituitaries; rhGM-CSF, recombinant human granulocyte macrophage colony stimulating factor; rhIFNα2, recombinant human interferon alpha2; rhtPA, recombinant human-tissue plasminogen activator; rmIFNα2, recombinant murine interferon alpha2; VLP, viral like particles.

Table I. Factors Influencing the Immunogenicity of Therapeutic Proteins in Man

Factors directly affecting the immune response	Factors affecting the measured immune response
Protein structure	Timing and frequency of sampling
Immune modulatory effect of the protein	Assay methods
Formulation	Expression of titers
Contaminants and impurities	
Route, dose, frequency, and duration of administration	
Major histocompatibility complex (MHC) genotype of the patient	
Associated diseases and concomitant therapy	

Adapted from (Refs. 35 and 42).

only focuses on the formation of antibodies after administration of recombinant human proteins to man in relation to the structural properties of the protein. Monoclonal antibodies will not be included in this review, because they are at best humanized and thus not completely human (2). First, the probable mechanisms of the immune responses are discussed, followed by the factors influencing the observed antibody response. The models used for the prediction of immunogenicity will be evaluated. Next, the protein structure in relation to immunogenicity will be dealt with. In the final section, formulation aspects are considered.

MECHANISMS OF IMMUNOGENICITY

There are two basic immunological mechanisms by which therapeutic proteins induce antibodies in man. Proteins carrying foreign epitopes like the proteins from microbial origin (e.g., streptokinase) induce a classical immune reaction that involves the presentation of the epitope by antigen-presenting cells. These cells activate B-cells and T-helper cells resulting in antibody formation and in the induction of memory cells, leading to an enhanced reaction upon rechallenge.

The other mechanism by which recombinant human proteins induce antibodies is based on the breaking of immune tolerance to self-antigens (7). This tolerance is based on the elimination of the immune cells reacting with self-antigen in the thymus during early development of the individual. This central mechanism of tolerance induction only concerns self-antigens that are present in sufficient amounts in the thymus. Some B-cells may escape elimination in the thymus. Peripheral mechanisms, however, keep the B-cells directed to self-antigens under control. These B-cells may be eliminated by apoptosis when they meet their antigen. Also, receptor editing has been described as a mechanism to make these cells harmless. The most likely 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 endogenous proteins such as insulin, interferon, and erythropoietin. In mice transgenic for human insulin, low levels of insulin silence the B-cells, although a few cells may escape silencing and produce antibodies. This silencing mechanism may act through antigen-antibody complexes, which react with the low affinity IgG receptor Fcγ2b on B-cells comparable with the mechanism by which antirhesus prophylaxis induces tolerance to RhD (8).

Anergic B-cells only start to produce significant amounts of antibodies after receiving a second signal or "danger" signal from T-helper cells. Bacterial endotoxins that react with Toll receptors may provide such a danger signal. This explains the production of antibodies to self-antigen associated with lipopolysaccharide (LPS). Also, oligonucleotides containing unmethylated cytosin-guanosin dinucleotides (CpG) motifs present in DNA can trigger an immune reaction to self-antigens. This T-cell–dependent activation may be weak. When self-antigens are coupled with foreign Th 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 arrayed on viruses and viral-like particles (VLP). The spacing of epitopes with a distance of 5–10 nm is unique to microbial antigens, and the immune system has apparently learned to react vigorously to this type of antigen presentation. Self-antigens conjugated with papilloma VLP evoked a strong antibody response to the self-antigens (9). It is the density of the exposed self-antigens on the VLPs that determines the level of the effect. VLP pentamers had the same effect as complete particles.

Apart from the self-antigens in viral-like arrays, other mechanisms exist that lead to the formation of antibodies to self-antigens:

1. Modification of the molecules, which gives antibodies cross-reacting with the unmodified self-antigens (T-cell responses are in general specific for the modified antigens).
2. Different allotypes for the gene coding for the product. This has been shown in mice with different allotypes for IL-2. When immunized, the mice produced antibodies that reacted with all forms of IL-2. However, adjuvant was used to immunize the mice (10).
3. Binding to a non–self-antigen. This has been shown for DNA bound to T-antigen from polyomavirus (11).

PREDICTIVE MODELS FOR IMMUNOGENICITY

As we will see later, physicochemical characterization of the therapeutic proteins will not completely predict their immunogenicity.

In vivo studies on immunogenicity of recombinant human proteins in conventional animals have limited value because these proteins, in general, are foreign in animals and will induce a classical immune response. These animals only provide the opportunity to study the relative immunogenicity of different protein formulations. The best models are transgenic animals, immune tolerant for the human protein, in which breaking of immune tolerance can be studied. This has been done by Ottesen *et al.* (12), Palleroni *et al.* (13) and Stewart *et al.* (14) for recombinant human insulin, recombinant human interferon alpha2 (rhIFNα2), and recombinant human-tissue plasminogen activator (rhtPA), respectively.

Stewart *et al.* studied the effect of the addition of an adjuvant on the immunogenicity of (mutated) rhtPA in transgenic mice (14). They immunized transgenic mice with unaltered rhtPA and with a mutant containing one amino acid substitution [rhtPA(E275)]. Mice were immunized with these proteins with or without adjuvant. The results, summarized in Table II, clearly show that the adjuvant did not increase the reaction to the ′self-protein′ but increased the immune response against the altered protein in the transgenic mice. Adjuvant only stimulated the classical immune response in this model. Most of the *in vivo* immunogenicity studies of therapeutic proteins have been done with mice, either wild type or transgenic. Zwickl *et al.* (15) performed studies in rhesus monkeys. They claim that the relative immunogenicity of various forms of human growth hormone (hGH) as predicted by the monkey model was confirmed in extensive clinical trials. However, according to the authors, the monkey model might not be suitable for every protein.

The *in vivo* analysis of the immunogenicity may be complicated, if the therapeutic protein has immune modulatory effects that may interfere with antibody production (e.g., interferons). Braun *et al.* studied the immunogenicity of rhIFNα2 in transgenic mice tolerant for human IFNα2. Because of the species specificity of IFNα2, they injected recombinant murine IFNα2 (rmIFNα2) or polyIC (an interferon inducer) together with rhIFNα2 to mimic its immune modulatory effects (16). In transgenic mice, the concurrent treatment did not break the tolerance toward rhIFNα2 monomers. In wild-type mice, antibody titers to rhIFNα2 increased due to the co-injection of rmIFNα2 or polyIC (16).

PROTEIN STRUCTURE AND IMMUNOGENICITY

Factors Influencing Immunogenicity

Comparison of immune responses induced by different formulations can become complex due to the issues stated in Table I. Besides factors affecting the intrinsic response, assay variations can largely influence the measured immune response. Not only do the methods used to determine antibody levels vary, but also the ways the results are reported. For instance, in enzyme-linked immuno-sorbent assays (ELISAs) the procedure used to coat the plate with antigens is of crucial importance. Depending on the chosen procedure, antibodies directed against either native or non-native epitopes will be measured (17). In patient studies, some groups report average antibody levels but no percentages of patients with antibodies, whereas other groups only mention percentages of patients with antibodies. So, standardization and validation of the assays and data presentation (e.g., level of antibodies and number of responders) is crucial.

Protein structure is one of the factors that can affect the immune reponse in man (see Table I). Proteins are complex molecules, so that a small change at a particular site may result in a major change in the overall properties. For instance, oxidation of a few amino acids of rhIFNα2 may lead to aggregation of the molecule (18). But, in general, it is very difficult to relate a particular change in protein structure to a change in immunogenicity, as will be discussed in the sections below.

Amino Acid Sequence

Divergence of the primary structure from the human counterpart explains why therapeutic proteins of animal sources are immunogenic. An example is insulin. The differences in primary structure between human, bovine, and porcine insulin are depicted in Fig. 1. Porcine insulin induced slightly higher antibody levels in patients than human insulin (1,12,19,20). Even trace amounts of bovine insulin in porcine insulin preparations increased the immune response (20). Remarkably, deletion of A19Tyr from recombinant human insulin resulted in a decrease of antibody titers to 2.2% as compared to the native form, whereas the molecule lost one third of its receptor binding activity (21). This deletion results in a small conformational change and an increase in hydrophobicity, as measured by reversed-phase high-pressure liquid chromatography (RP-HPLC). Changes in the A-chain of insulin have more effect on the immunogenicity than changes in the B-chain (12), which is not surprising as the A-chain is located at the outside of the insulin hexamers.

RhIFNα2 exists in different subtypes (Table III). RhIFNα2a and rhIFNα2c both differ in one amino acid from rhIFNα2b. The majority of the human population expresses the gene encoding rhIFNα2b (13). Differences in immunogenicity seen in patients could not be related to differences in primary structure. Antibodies from patients treated with either rhIFNα2 showed complete cross-reactivity (13).

Human growth hormone was available in three different forms: purified out of human pituitaries (pit-hGH), recombinant human growth hormone with an extra methionine (met-rhGH), and natural sequence recombinant human growth

Table II. Effect of Adjuvant on Immunogenicity of rhtPA and Mutant

	Immunogen			
	rhtPA		rhtPA(E275)	
	Without adjuvant	With adjuvant	Without adjuvant	With adjuvant
Control mice	40/42	12/12	79/80	12/12
Transgenic mice	0/21	0/8	8/44	4/8

Mice were immunized with rhtPA or rhtPA(E275) (with or without Freund's adjuvant). The numbers refer to the number of mice making antibodies against the immunogen. The sera were analyzed using a standard radioimmunoprecipitation assay using polyethylene glycol to precipitate the antibodies. After Ref. 14.

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A-chain 1          11          21
Human   giveqcctsi  cslyqlenyc  n
Bovine  giveqcc***as*** cslyqlenyc  n
Porcine giveqcctsi  cslyqlenyc  n

B-chain 1          11          21
Human   fvnqhlcgsh  lvealylvcg  ergffytpkt
Bovine  fvnqhlcgsh  lvealylvcg  ergffytpk***a***
Porcine fvnqhlcgsh  lvealylvcg  ergffytpk***a***
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Fig. 1. The amino acid sequences of human, bovine, and porcine insulin. Differences with the human sequence are depicted in bold and italic (after Ref. 12). Disulfide bridges between A7Cys-B7Cys and A20Cys-B19Cys connect the A- and B-chain (43).

Table III. Amino Acid Differences Between Different rhIFNα Variants

Variant	AA 23	AA 34
rhIFNα2a	Lys	His
rhIFNα2b	Arg	His
rhIFNα2c	Arg	Arg

RhIFNα2a and rhIFNα2c both differ from rhIFNα2b by one amino acid (13,26).

hormone (humatrope). Pit-hGH and humatrope had the same primary structure, whereas met-hGH had one extra N-terminal methionine residue. Differences in immunogenicity were shown but could not be related to the methionine residue (15,20). More detailed studies are needed to explain the differences in immunogenicity.

RhtPA was mutated by one amino acid (R275 → E275). Transgenic mice that were tolerant for htPA were immunized with the mutant and with the nonaltered form. The altered protein elicited antibodies in a significantly higher percentage of transgenic mice than the nonaltered protein (see Table II) (14).

The impact of a mutation on the immunogenicity is dependent on the position and the type of amino acid involved. For rhtPA, a positively charged amino acid was changed into a negatively charged one. The question arises if a more subtle change (e.g., R275 → K275, both positively charged amino acids) also would give a different immune response.

As the changes in the primary structure reported are diverse and different test systems have been used (patients, monkeys, transgenic mice, and non-transgenic mice), it is very difficult to draw any general conclusions about the influences of primary structure on the immunogenicity.

Glycosylation

Several therapeutic proteins are glycosylated. Glycosylation is one of the most common posttranslational modifications. Glycosylation may differ by sequence, chain length, and position of linkage to the polypeptide chain and of branching sites (22). Glycosylation is species- and cell-specific. Moreover, the culture conditions of production cells have direct effects on N-linked glycosylation patterns (23). Thus, recombinant human glycoproteins will never have exactly the same glycosylation patterns as their endogenous counterparts.

The sugars of glycoproteins have various functions (listed in Table IV). Below we will give some examples to illustrate the effect of the glycosylation profile on the immunogenicity of glycoproteins. Other posttranslational modifications (e.g., phosphorylation, acetylation, methylation) also may influence the immunogenicity, but published data are lacking.

Four out of 13 patients who received yeast-derived recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) formed antibodies to this product. The cross-reactivity of the antibodies with other forms of rhGM-CSF was tested (Fig. 2). Because the antibodies had the same reactivity with *Escherichia coli*–derived rhGM-CSF (no glycosylation), the authors concluded that the antibodies could not be directed against the sugars. The antibodies showed no cross-reactivity with CHO-cell–derived rhGM-CSF (O-linked and N-linked sugars). When the CHO-cell–derived rhGM-CSF was N-cleaved, cross-reactivity remained absent. However, when the CHO-cell–derived rhGM-CSF was O-cleaved, the cross-reactivity was completely regained, irrespective of the presence of N-linked sugars. It was concluded that the antibodies were directed against a part of the peptide backbone that is protected by the O-linked glycosylation in the endogenous protein. Only three patients received unglycosylated rhGM-CSF (produced in *E. coli*), and although none of them developed antibodies, definite conclusions cannot be drawn about the immunogenicity of *E. coli*–derived rhGM-CSF (24).

Naturally occurring human IFNα2 is O-glycosylated at Thr106 (25). No significant antigenic differences between glycosylated natural and nonglycosylated rhIFNα2 proteins have

Table IV. Functions of Glycoprotein Glycans

Type	Function
Physicochemical	Modify solubility, electrical charge, mass, size, and viscosity in solution
	Control protein folding
	Stabilize protein conformation
	Confer thermal stability and protection against proteolysis
Biological	Regulate intracellular trafficking and localization
	Determine circulation half-life
	Modify immunological properties
	Modulate activity
	Act as cell surface receptors for lectins, antibodies, toxins, and so forth
	Participate in cell-cell interactions

After Ref. 22.

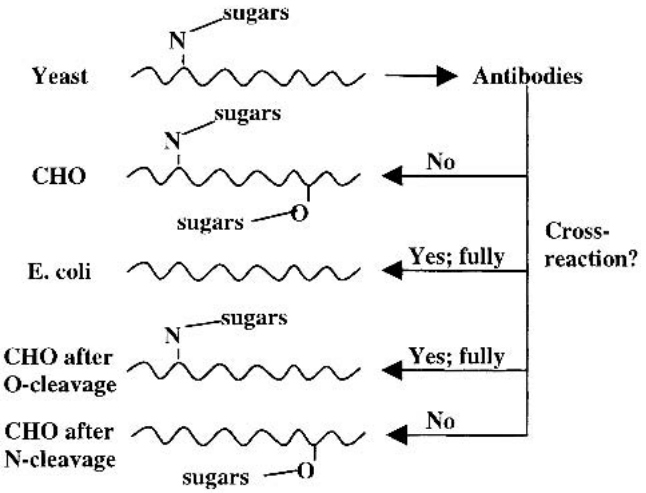

Fig. 2. Schematic representation of the cross-reactivity between antibodies against differently glycosylated forms of rhGM-CSF. RhGM-CSF produced in yeast-induced antibodies in 4 out of 13 patients. The antibodies were fully cross-reactive with rhGM-CSF produced in *E. coli*. The antibodies did not recognize the mammalian rhGM-CSF (produced in CHO-cells). When the mammalian form was O-cleaved, the antibodies did fully recognize the rhGM-CSF. This means that the antibodies are directed against a portion of the amino acid backbone protected by O-glycosylation in the native product and the product derived from CHO-cells (24).

been identified (26). So, glycosylation probably does not affect the immunogenicity of human IFNα2.

Recombinant human interferon beta (nonglycosylated) expressed in *E. coli* (*E. coli*-rhIFNβ), and CHO-cells (CHO-rhIFNβ, glycosylated) as well as (nonrecombinant human, glycosylated) interferon beta obtained from human fibroblasts (hIFNβ) were compared for their immunogenicity. The three forms possess equal antiviral activities and a mouse monoclonal anti-hIFNβ antibody had the same affinity for the three variants. However, the antiviral activity of CHO-rhIFNβ was neutralized more effectively by mouse monoclonal anti-hIFNβ than were hIFNβ or *E. coli*-rhIFNβ, which may be explained by glycosylation differences. Furthermore, during the production process of *E. coli*-rhIFNβ, the protein is extracted from the bacteria with a strong detergent (whereas CHO-rhIFNβ and hIFNβ are secreted in the medium). This could result in a conformational change that might explain the different neutralization activity of the mouse monoclonal anti-hIFNβ (27).

In the above examples, only cross-reactivity of an antibody with glycosylation variants was tested. Clearly, sugar chains are able to mask antigenic sites *in vitro*. Whether this has implications for the immunogenicity *in vivo* remains to be seen. A complicating factor when comparing the cross-reactivity of antibodies against recombinant proteins produced in different species is that the glycans of species differ. So, when the antibodies are directed against the glycans, cross-reactivity may never be seen.

Pegylation

Chemical conjugation of proteins with polyethylene glycol (PEG, a nontoxic water-soluble polymer) reduces their immunogenicity (28). As pegylated proteins have a prolonged circulating time (29), pegylation has the potential to reduce the immune response as a result of less frequent administration schemes. In addition, it may shield immunogenic sites, like glycosylation does. In most cases, pegylation does not lead to conformational changes of the polypeptide chain (28). Important issues regarding protein activity and immunogenicity are the choice of the PEG chain length, the conjugation method, the number of PEG chains per protein molecule, and the pegylation sites (30).

Conjugates of rhIFNα2a with PEG of various molecular weights and structures (branched or linear) decreased the immunogenicity in comparison to nonpegylated rhIFNα2a. In addition, one of the pegylated forms showed no immunogenicity in mice (31,32).

The immunogenicity of monopegylated recombinant human insulin with distinct sites of substitution and varying molecular weights of the PEG was studied in mice (33). All forms, irrespective of molecular weight or site of conjugation, reduced the level of circulating insulin-specific IgG antibodies 10- to 1000-fold.

The heterogeneity in the length of the PEG chain and the site where the PEG is attached make it difficult to establish structure-immunogenicity relationships. In our opinion, the best place to attach the PEG chain would be around possible antigenic epitopes, if this does not result in decreased activity of the protein.

Physical Degradation

Proteins have complex three-dimensional structures. Physical degradation processes such as unfolding, misfolding, and aggregation of the protein can result in an immune response. Physical degradation of the protein can occur during the production and purification but can also result from improper formulation or storage or handling conditions. The choice of the formulation and the dosage form requires extensive research to guarantee the physical stability of the protein and thus minimize immunogenicity.

Especially aggregates have been shown to increase the immunogenicity of various therapeutic proteins, which might be explained by their multiple-epitope character (cf. the viral-like arrays described earlier) and/or to conformational changes of the individual aggregated protein molecules.

For instance, aggregates of insulin can lead to antibody formation (1). Evidence exists that insulin aggregates possess antigenic sites that are absent in monomers. Further research is needed to determine whether these aggregates promote the formation of antibodies against insulin monomers (34).

Aggregates were present in rhIFNα2a formulations containing human serum albumin (HSA) stored for a prolonged period at 25°C. Not only rhIFNα2-rhIFNα2 aggregates, but also HSA-rhIFNα2 aggregates were present, which might have contributed to the observed immunogenicity (13,18,35). The effect of different rhIFNα2 aggregates on the immunogenicity was studied systematically in mice. Not only rhIFNα2-rhIFNα2 aggregates isolated from an expired bulk solution were injected intraperitoneally (i.p.) in wild-type and transgenic mice tolerant for human IFNα2, but also rhIFNα2-rhIFNα2, rhIFNα-HSA, and rhIFNα2-MSA (murine serum albumin) aggregates, made with glutaraldehyde. As controls, rhIFNα2 monomers or a mixture of either HSA/rhIFNα2 or MSA/rhIFNα2 were injected. The aggregates (either one) induced antibody formation in both conventional and transgenic mice. In the transgenic mice, the rhIFNα2-rhIFNα2 aggregates and albumin (human and murine)-rhIFNα2 aggregates could break an existing tolerance toward rhIFNα2 monomers, in contrast with nonaggregated rhIFNα2 formulations with or without albumin (16).

The presence of protein aggregates in formulations of (nonrecombinant) hGH has been correlated with an increased frequency of immune responses. Three distinct patterns of antibody formation in patients were observed when different preparations of hGH were used. The patients were divided in three groups according to antibody formation (Table V). The first group of patients developed antibodies that were persistent irrespective of the length and type of hGH therapy. The second group of patients developed anti-

Table V. Role of Aggregated hGH and Patient Features in Immunogenicity of (Nonrecombinant) hGH Formulations

Group	Antibodies	Number of patients	Formulation
1	Persistent	11	50–70% aggregated
2	Transient	18	<5% aggregated
3	No antibodies	33	Different types

Patients were divided in groups according to antibody formation. See text for details. Adapted from Ref. 36. hGH, human growth hormone.

bodies that disappeared after switching to a different preparation of hGH or when the therapy was discontinued. The third group developed no antibodies. The first group had received a preparation of hGH that contained 50–70% aggregates, the second group had received a preparation with less than 5% aggregated hGH, and the third group had received different types of hGH (either the 50–70% aggregated, less than 5% aggregated, or monomeric), as determined after the study. It was concluded that the formation of antibodies was dependent on both the level of aggregates present and patient characteristics (36).

Aggregates are a decisive factor for an antibody response. Aggregates not only increase the classical immune response in conventional animals but also break immune tolerance in transgenic animals made tolerant for the therapeutic protein.

Chemical Degradation

Oxidation and deamidation, sometimes followed by isomerization, are the major causes of chemical degradation of proteins (e.g., during storage or handling). One of the impurities found in rhIFNα2a formulations because of improper storage was an oxidized form (18). The oxidation sites were not determined. The oxidation product was isolated and compared with nonoxidized rhIFNα2a for its immunogenicity in wild-type mice. The oxidized form was more immunogenic than the nonoxidized rhIFNα2a (18,35).

Deamidation mainly occurs at asparagine residues. In this reaction, the asparagine residue is, via a succinimide intermediate, converted into an aspartate or an iso-aspartate residue (37–39). Also, glutamine residues can undergo deamidation resulting in glutamate residues. The rate of deamidation is dependent on the type of nearby amino acids and on the conformation (37,40). Not much is known about the immunogenicity of deamidated proteins. Chen *et al.* showed that antibodies can be raised specifically against the succinimide derivative of peptides, showing weak cross-reactivity with the parent peptide (41).

CONCLUDING REMARKS AND DISCUSSION

Structural changes in proteins may affect their immunogenicity. The presence of grafts (sugar or PEG chains) on the polypeptide chain may decrease their immunogenicity. There are no reports about the introduction of antigenic sites due to these additions. The most important recognized structural change known to increase the immune response is aggregation. Even small amounts of aggregates may be sufficient to elicit an immune response. The aggregation either reveals new epitopes recognized as non-self or leads to the spacing of the epitopes known to break self-tolerance. Aggregates occur in different sizes, and the proteins in the aggregates can have a native or non-native conformation. Folding probably has a great influence on immunogenicity because the misfolded protein may present different epitopes than the native protein. The effect of non-native conformers present in protein formulations on the immunogenicity is still unclear and needs to be investigated further.

To what extent do the antibodies induced by structural variants of a protein cross-react with the native protein? If the immune response is directed toward an epitope specific for the variant, the only clinical consequence may be the loss of effect of the therapeutic protein involved. However, when the immune response is directed toward an epitope present in the endogenous protein, the antibodies induced may also neutralize this endogenous protein, and serious clinical consequences may be anticipated.

The formulation of proteins plays an important role in the occurrence of immunogenicity. Excipients in the formulation added to stabilize the protein may affect its immunogenicity. The excipients could change the presentation of the protein to the immune system (i.e., in a spacing of epitopes known to be a strong stimulus for the immune system). Lyophilization of HSA-containing rhIFNα2a formulations and storage at room temperature induced aggregates (HSA-rhIFNα2a and rhIFNα2a aggregates). Removal of the HSA and storage in a refrigerator led to less aggregation and diminished immunogenicity (35).

Still, little is known about the relationship between changes in the structure of therapeutic proteins and their immunogenicity. The development of new and improvement of current analytical techniques will help in identifying impurities and non-native conformers in protein formulations. With the advent of transgenic animals tolerant for human proteins, systematic studies on the correlation between conformational and chemical changes and immunogenicity can be conducted. Well-defined degradation products can be prepared, and their immunogenicity can be compared to that of native proteins. It is unlikely that one can completely predict the immunogenicity in patients by using transgenic animal models. However, such models will at least be useful to screen formulations or structural variants for their immunogenicity and can therefore be valuable during the development of new therapeutic protein formulations as well as for the establishment of proper storage and handling conditions.

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