Factors that contribute to the immunogenicity of therapeutic recombinant human proteins

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Summary
Use of recombinant human proteins has revolutionized medicine by providing over 200 highly purified hormones and proteins that effectively treat many inherited and acquired peptide hormone and protein deficiencies. With the exception of therapeutic monoclonal antibodies, these biological medicines are synthesized by cultured cells using DNA sequences that would yield proteins with identical amino acid sequences as endogenous human proteins. Therefore, there was the broad expectation that recombinant human biological medicines would be non-immunogenic in patients capable of synthesizing even sub-optimal levels of these therapeutic proteins to which they are innately tolerant. However, the widespread clinical use of recombinant human proteins has demonstrated that nearly all of them are immunogenic. This observation suggests that factors additional to differences in amino acid sequences of endogenous and biotherapeutic proteins contribute to the immunogenicity of therapeutic proteins. The main aim of this review is to summarize some of the factors that are known to contribute to the immunogenicity of recombinant therapeutic proteins.

Keywords
Therapeutic proteins, immunogenicity, recombinant human proteins

Introduction
Severe inherited or acquired protein deficiencies may have severe and chronic clinical consequences if left untreated. Replacement of missing proteins is the most effective method for treating protein deficiencies as conventional synthetic low-molecular-weight drugs generally cannot effectively mimic the many biological activities of proteins (1). The availability of recombinant human proteins now makes effective treatments of many diseases possible, and over 200 biopharmaceutical proteins had been approved for clinical use by 2007. Over 75% of the approved therapeutic proteins are recombinant human proteins (2, 3). The immunogenicity therapeutic proteins derived from human plasmas is well known. The birth of recombinant DNA technology led to the creation of proteins initially thought to be structurally and biologically identical to their counterparts found in man (4, 5).

The immunogenicity of animal insulins and that of recombinant human insulin soon became apparent after their widespread clinical use (6). Clinical uses of human growth hormone and human plasma-derived or recombinant human FVIII also had immunological consequences (7–9). It is reasonable to expect that individuals congenitally unable to synthesize specific proteins would mount immune responses when treated with therapeutic proteins that provide the missing ones, since they lack innate immune tolerance to the therapeutic proteins. Widespread use of biopharmaceuticals has, however, amply demonstrated that nearly all approved biologicals can elicit antibody responses, even in some patients who should be innately tolerant to them. Induction of antibody responses to therapeutic proteins created from the genetic sequences of human peptide hormones and proteins (4, 5, 10) clearly indicates that factors additional to their amino acid sequences contribute to the immunogenicity of human therapeutic proteins. The incidence of the antibody responses, a major concern for regulatory authorities, varies among biopharmaceuticals, and the severity of the antibody-mediated clinical consequences also markedly varies (11). The purpose of this review is to consider some of the mechanisms known to govern some immune responses to haemostatic and other therapeutic proteins.
**Antibody formation and its clinical consequences**

In the context of this review, immunogenicity of therapeutic proteins refers to their ability to elicit two classes of humoral immune responses. Non-neutralizing antibodies (NNAbs) generated in response to therapeutic proteins bind to antigenic sites in the proteins in ways that do not disrupt the normal functions of the therapeutic proteins. In most cases, NNAbs have no impact on the efficacy of the therapeutic proteins, e.g., NNAbs generated against a tumour necrosis factor receptor fusion protein or recombinant human growth hormone (12, 13). In some cases, however, NNAbs can accelerate the clearance of therapeutic proteins, e.g., yeast-derived granulocyte macrophage colony-stimulating factor (GM-CSF) and recombinant IFN-α (14–16). In other cases, NNAbs can unexpectedly enhance the efficacy of biopharmaceutical proteins, e.g., anti-recombinant human methylionyl-growth hormone (8). NNAbs can, however, be a cause for concern when immune complexes of NNAbs and therapeutic proteins trigger anaphylaxis, serum sickness and other clinical complications (6, 17–21).

Neutralizing antibodies (NAbs) bind to sites in therapeutic proteins that directly impair or abrogate the biologic functions of the therapeutic proteins. NAb responses have the potential to cause negative clinical consequences by neutralizing the therapeutic product and therefore reducing the efficacy, e.g., factor VIII or streptokinase (9, 22). However, the situation may be worsened if the NAbs neutralize not only the therapeutic proteins but also their endogenously produced forms resulting in severe adverse consequences for the patients. Examples of anti-drug antibodies that also activate autologous proteins (i.e., by the generation of autoantibodies) include autoantibodies against recombinant human thrombopoietin (23), erythropoietin (EPO) (24–27), GM-CSF (28), and many interferons (17, 18, 21, 29–33). The most devastating consequence of neutralizing antibody development is complete neutralization of endogenous proteins by autoantibodies generated. Healthy human platelet donors treated with pegylated recombinant human megakaryocyte growth and differentiation factor containing the first 163 amino acids of thrombopoietin also developed neutralizing antibodies to native thrombopoietin that caused severe long-term thrombocytopenia (23). Similarly, patients with chronic renal failure treated with rhEPO for anemia formed NAbs to their own erythropoietin and developed pure red cell aplasia (24–27). It is noteworthy that relative to the number of patients treated with biopharmaceutical proteins, the number of patients affected by the undesired immunological consequences attributed to NAbs is rather small (26, 27).

**T cell-dependent mechanism of antibody formation**

The immune system can generate antibodies to therapeutic proteins by two general mechanisms. One relies on T-cell co-stimulation of B cells while the other can proceed independently of T-cell help. In order to elicit a T-cell-dependent antibody response, a therapeutic protein must first interact with antigen presenting cells (APC), e.g., dendritic cells (DCs), B cells, or macrophages. Immature and mature DCs and macrophages take up large amounts of antigens by non-specific macropinocytosis. In contrast, naïve B cells bind and internalize specific antigens via their B-cell antigen receptors (BCRs) by receptor-mediated endocytosis. Protein antigens internalized by APC are proteolyzed into peptide fragments that bind MHC class II molecules in intracellular vesicles and are then targeted for display as peptide:MHC class II complexes on the B-cell surface. Antigen presentation by B cells is highly efficient, since specific antigens are presented on the B-cell surface at a high density. This is because B-cells constitutively express high levels of MHC class II molecules that, upon antigen uptake, present high levels of peptide:MHC class II complexes on their cell surfaces (34).

Following antigen uptake and presentation, APCs must activate naïve CD4+ T-cells by two distinct signals based on recognition of peptide:MHC class II complexes on the APC surface by T-cell receptors (TCRs) present on the antigen-specific naïve CD4+ T-cell, and by ligation of CD28 on the T-cell surface by a B7 co-stimulatory molecule on the APCs. Expression of the B7 co-stimulatory molecules B7.1 and B7.2 on APCs can be induced by various microbial agents, by cytokines secreted by nearby cells during infection or inflammation. Recognition of the peptide:MHC class II complex on APC surfaces by TCRs on the naïve CD4+ T cells has two possible outcomes that depend on the B7 co-stimulatory molecules. In the absence of co-stimulation, the peptide:MHC class II complexes cannot activate naïve CD4+ T cells, causing them to become anergic. In the presence of co-stimulatory molecules on the B-cell surface, peptide-specific naïve CD4+ T cells are activated to differentiate into armed CD4+ T-helper (Th) cells (34).

Naïve B cells require two signals for their proliferation and differentiation into antibody-secreting plasma cells. The first signal is generated by the direct binding of the antigenic protein to the BCRs on naïve B-cell surfaces. The second signal is delivered by the armed Th cells, which recognize the same antigen, via the binding of the TCRs to the peptide:MHC class II complexes on the surfaces of the naïve B cells. Direct contact between the B cells and the armed Th cells leads to the expression of the B-cell co-stimulatory molecule CD154 (CD40L) on the Th-cell surface, and to the secretion of B-cell stimulatory cytokines IL-4, IL-5, IL-6 by the Th cells. B-cell activation occurs through the interaction of the peptide:MHC class II complex on the B-cell surface with the TCRs on the Th cells, and by the binding of B-cell surface CD40 (a co-stimulatory molecule present on APCs), to CD154 (CD40L) expressed on activated Th cells. The second signal for B-cell activation requires Th cells that respond to the same antigen, but not the same epitope, a concept known as linked recognition (34).

Memory B cells generate the characteristically rapid secondary immune response following antigen re-exposure, and this strictly depends on B-cell interactions with activated Th cells for their re-stimulation (34). In the case of FVIII specific memory B-cell re-stimulation, activated Th cells can be specific for either FVIII or a third-party antigen. Re-stimulation by a third-party antigen requires the presence of FVIII (35), indicating that both BCR activation and interactions with activated Th cells are required. In addition, blockade of the T-cell co-stimulatory signal, B7-CD28 or CD40-CD40L interactions, was sufficient to...
prevent the re-stimulation of the FVIII specific memory B-cells in mouse models of haemophilia A (36–38).

**T cell-independent mechanism of antibody generation**

Eliciting antibody production directed against therapeutic proteins may also proceed by direct B-cell activation, and without Th-cell co-stimulation. This ability to bypass T-cell activation produces a more rapid and efficient antibody response. This type of B-cell response is typically evoked by particulate antigens, and by sequences of microbial and viral origin (39, 40). Changes to the structure of a therapeutic protein may alter its miscibility in ways that enhance its aggregation, or cause it to resemble a pathogen, therefore greatly increasing its antigenicity. Blood-borne peripheral DCs (CD11c<sup>+</sup> Mac1<sup>+</sup>) engulf particulate protein antigens and migrate to the spleen where they activate and enhance the survival of marginal zone and B1 B cells, inducing a T cell-independent antibody response. Particulate aggregates of therapeutic proteins may meet a similar fate (40).

The immune system has evolved to recognize repetitive displays of proteins, polysaccharides, nucleic acids, or lipids (39). T cell-independent immune responses have been demonstrated for polyvalent antigens of bacterial and viral origin (41–43). Naïve B cells may recognize epitopes on the aggregated protein and bind to them. Successful B-cell stimulatory signals are only delivered when a minimum number of BCRs simultaneously bind to aggregated proteins and connect in a spatially contiguous cluster resulting in BCR cross-linking (44). Antibodies produced by direct B-cell stimulation are primarily low-affinity, high-avidity IgM and some low-affinity IgG isotypes, specific for the epitope initially recognized by the BCR. While this activation signal is sufficient to stimulate the proliferation of B cells, the delivery of a second signal by Th cells or by other pathways, such as those mediated by Toll-like receptors may be required. Th-cell co-stimulation allows for affinity maturation and class switching, creating a more efficient antibody response (41–44).

**Factors influencing the immunogenicity of biopharmaceutical proteins**

The factors detailed in the sections below are known to contribute to immunogenicity of therapeutic proteins.

**Patient characteristics**

Patient characteristics strongly influence the immunogenicity of biopharmaceuticals. For example, patients with impaired immune systems (e.g. some cancer patients receiving chemotherapy) may be less likely to develop antibodies to therapeutic proteins than immunocompetent individuals (45, 46). As could be expected, exposure of patients to human therapeutic proteins that the patients cannot synthesize frequently leads to the generation of neutralizing antibodies after single or repeated exposures. Development of neutralizing antibodies frequently occur in severe congenital factor (F) XI-, FIX-, and FVIII-deficient patients treated with the missing therapeutic proteins (9, 47–50). It should be noted that there may be many polymorphic versions of human proteins with different amino acid sequences.

Haemophilia A is useful as a prototypic monogenic disease for analyzing how specific factor VIII mutations influence the risk of neutralizing anti-factor VIII antibody development. Generally, haemophilia A patients fall into one of two groups. The first includes patients with the types of molecular defects such as large deletions, nonsense mutations, and intron-22 inversions that result in the non-detection of factor VIII protein in the plasmas of affected individuals. This group of patients that would be expected to lack innate immunity to factor VIII exhibits an anti-FVIII antibody prevalence of more than 30%. The second group includes patients with small deletions/insertions, missense, and splice site mutations who synthesize some defective and non-functional FVIII proteins and may therefore be innately tolerant to therapeutic FVIII administration. The latter group of patients has an anti-FVIII antibody prevalence of less than 10% (51).

According to data obtained from the HAMSteRS haemophilia mutation registry when mutation types are further subdivided based on their risk of antibody formation, patients with large deletions have the highest risk (~75%), followed by patients with nonsense mutations and those with inversions of intron-22 (52). Since small deletion/insertion mutations cause a frameshift, resulting in a subsequent stop codon and truncated protein, a risk similar to that of a major genetic defect is expected. Surprisingly, patients with small deletion/insertion mutations had a low risk of developing antibodies (7.4%) (53). Polymerase errors during DNA replication/RNA transcription apparently allowed for the endogenous restoration of the reading frame for small deletion/insertion mutations located near stretches of adenines (52–54). The small amount of endogenous FVIII thus produced is apparently sufficient to prevent antibody development. If the small deletion/insertion mutation does not occur in a poly A region, the risk of antibody development becomes substantially higher (20.5%). Patients with missense mutations synthesize some non-functional FVIII which in most patients is sufficient to induce immune tolerance, and thus an antibody risk of only 4.3% is observed. Splice site mutations bear almost no risk for antibody development. This is likely because some FVIII mRNA molecules are spliced normally, leading to the biosynthesis of FVIII at levels sufficient to induce immune tolerance (55).

However, specific FVIII gene mutations do not necessarily explain all the differences in immune responses seen in severe FVIII-deficient patients. An interesting observation arises from analyzing the risk of antibody formation in patients with the same mutation, such as the intron-22 inversion which results in failure of FVIII protein expression. Only one third of the patients with this mutation develop anti-FVIII antibodies. Speculations explaining this finding include presentation of maternal FVIII to the fetal immune system to induce immune tolerance. Another explanation offered for the reduced penetrance is polymorphism in the immune systems of the patients, which either hinders or synergizes anti-FVIII antibody formation (47).

A meta-analysis conducted by Scharrer et al. elucidated the influence of patient race on the risk of antibody formation (55). The incidence of antibody formation in African-Americans was double that of Caucasians (51.9%, 14 of 27 vs. 25.8%, 51 of 191). This finding provided indirect evidence that genetic poly-
morphism in immune response genes, such as the MHC classes and various cytokines, influences the risk of anti-FVIII antibody development. Furthermore, the incidence of antibody formation in haemophilia A siblings (50%) is significantly higher than in extended haemophilia A relatives (9%) (56). Since the genetic defects in the FVIII gene are assumed to be similar in siblings and extended relatives, polymorphisms in immune response genes may influence the risk of anti-FVIII antibody formation. Although various studies have attempted to address the influence of MHC classes on the risk of antibody formation, inconsistent results have been obtained due to the influence of the FVIII gene defect, as this variable was not controlled for (57–61). A recent study of patients with only the intron-22 mutation attempted to clarify the influence of MHC class I/II genes on the risk of antibody formation. Different alleles in the MHC classes were assigned as risk or protective alleles based on whether they occurred more often in patients who formed antibodies or not. MHC class I/II alleles A3, B7, C7, DQA0102, DQB0602, and DR15 were assigned as risk alleles (relative risk 1.9–4.0). MHC class I/II alleles C2, DQA0103, DQB0603, and DR13 were assigned as protective alleles (relative risk 0.1–0.2), since they occurred more often in patients who did not develop antibodies than in those who did. Definitive conclusions cannot be drawn, however, since the sample size in the above study was too small to achieve a clear statistical significance. In another study, the presence of MHC class II haplotype DRB1*0701 in patients with multiple sclerosis was strongly associated with the development of anti-IFN-β antibodies (62).

Product characteristics

Animal-derived therapeutic proteins

Animal-derived proteins and recombinant counterparts based on animal amino acid sequences are often immunogenic in humans because the amino acid sequences of animal and analogous human proteins often differ. For example, recombinant bovine and porcine insulin are more immunogenic in insulin-dependent diabetic patients than recombinant human insulin. Porcine insulin only differs from human insulin in the concelade B-chain (63). This may explain why porcine insulin is only slightly more immunogenic than human insulin, while bovine insulin is more immunogenic than either recombinant porcine or human insulin (64, 65).

Human therapeutic proteins

Differences between therapeutic and patient protein polymorphisms could be potential sources of immunogenicity. However, such differences may not always predict immunogenicity (66, 67). The types of changes in amino acid sequences, and their positions in the primary sequences, will have varying impacts on the immunogenicity of a protein. For example, deletion of chain A19 Tyr from recombinant human insulin decreased the anti-insulin antibody titers in patients. However, this mutation also led to the loss of insulin receptor binding activity (68). On the other hand, single amino acid mutants of recombinant human tissue plasminogen activator elicited neutralizing antibody production in a significantly higher percentage of patients than wild-type tissue plasminogen activator (69).

Another class of therapeutic proteins that show variable degrees of immunogenicity is engineered monoclonal antibodies. As would be expected, 38 of 44 murine monoclonal antibodies used to treat several diseases were immunogenic in nearly 100% of patients. Further, 80% of these murine therapeutic proteins elicited marked anti-monoclonal antibody responses, and most of them ultimately failed as therapeutic proteins because of their poor safety profiles (70). Hwang and Foote report that chimeric monoclonal antibodies, the next generation of engineered antibodies are generally less immunogenic in patients than murine monoclonal antibodies. They classified 40% of 15 chimeric monoclonal antibodies as causing marked anti-antibody responses (70). Some of these chimeric antibodies induced no human anti-chimeric antibody production, e.g. Ritusimal (targeting CD20 and used in non-Hodgkin’s lymphoma [71]) and cMOv18 (used in ovarian carcinoma [72]) were non-immunogenic. Other chimeric monoclonal antibodies, however, were immunogenic, e.g. 5% of patients treated with Abciximab (a platelet glycoprotein IIb/IIIa antagonist [73]) and 60% of patients treated with Infliximab/Remicade (targeting tumor necrosis factor-α and used in Crohn’s disease and rheumatoid arthritis [74]). The least immunogenic of therapeutic monoclonal antibodies are humanized monoclonal antibodies (70). For example, no immunogenic response was mounted against Hu5c8 targeting CD154 (CD40 ligand) and used to induce immune tolerance in severe haemophilia A patients who have made factor VIII-neutralizing antibodies (75). This very low immunogenicity of humanized monoclonal antibodies reprises that ofIVIG preparations used to treat a variety of conditions including idiopathic thrombocytopenic purpura (76).

Impact of expression systems used on immunogenicity of therapeutic proteins

Biosynthesis of recombinant therapeutic proteins requires different expression systems because cell lines differ in their abilities to carry out post-translational modifications. Most therapeutic proteins are expressed in mammalian cell lines, such as Chinese hamster ovary (CHO) cells and baby hamster kidney (BHK) cells. Therapeutic proteins not requiring post-translational modifications can be successfully produced in yeast or Escherichia coli (E. coli) (77–79). The prokaryotic E. coli protein-expression system lacks the chaperones, specialized post-translational modifications, the ability to form complexes with stabilizing binding proteins, and to be targeted to subcellular localizations found in eukaryotic cells. The absence of these functional entities has the potential to cause misfolding and/or aggregation when some eukaryotic-derived proteins are expressed in E. coli (78, 79). Expression of recombinant human FVIII and FIX in mammalian cells can have measurable impact on the function or survival of the recombinant proteins. Significant fractions of the FVIII proteins in approved recombinant FVIII products do not bind von Willebrand factor, the carrier protein of FVIII in vivo. In contrast, virtually all the FVIII proteins in plasma-derived FVIII concentrates bind von Willebrand factor (80). The contribution of the non-von Willebrand factor binding fraction of recombinant FVIII products to their immunogenicity is controversial (81–83). The half-life of recombinant human FIX is approximately 50% of that of plasma-derived FIX (84). How-
ever, this difference has no impact on the immunogenicity of recombinant and plasma-derived FIX (84).

The influence of the expressing cells on immunogenicity is also highlighted by the different immune responses to recombinant FIX obtained using different expressing cells in the same mouse model of severe FIX deficiency. In a cellular therapy for haemophilia B in mice, recombinant transformed C2C12 murine myoblasts genetically engineered to produce human FIX were transplanted into FIX-deficient mice (85). While partial phenotypic correction was obtained, all the treated mice also developed antibodies to the recombinant human FIX thus delivered in vivo (86, 87). Replacement of C2C12 cells with fetal murine G8 myoblasts similarly engineered to produce recombinant human FIX also resulted in partial phenotypic correction of FIX deficiency (87). In contrast to haemophilia mice with C2C12 implants, however, mice transplanted with G8 cells did not generate anti-human FIX antibodies. Thus, C2C12 myoblasts apparently produced a more immunogenic form of human FIX than G8 myoblasts in mice. Although similar studies have not been performed with other biopharmaceutical proteins in other animal models or in man, the above results highlight the potential role that the cells used to produce a therapeutic protein can have on the immunogenicity of the product.

Impact of post-translational modifications on immunogenicity

Mammalian cells are used to produce therapeutic glycoproteins requiring post-translational modifications to function effectively (88). The most common post-translational protein modifications are glycosylation reactions. The enzymes that add glycan structures to proteins can generate distinct glycoform profiles. For example, 44 different glycoforms of recombinant human erythropoietin (rhEPO) have been identified (89). rhEPO and endogenous human EPO can be distinguished by differences in their glycan structures and their isoelectric points. The latter are largely determined by their sialic acid content (90, 91). Creating protein biopharmaceuticals in CHO cells and BHK cells with glycoform profiles identical to the same proteins made in humans is virtually impossible. Humans do not synthesize the Galα1–3Gal structure at the non-reducing ends of glycan chains, while the mammalian cell line cells routinely used to express recombinant therapeutic human proteins synthesize this highly immunogenic disaccharide (92). Since it is important to ensure that the glycosylation profiles of production batches of recombinant glycoprotein remain consistent (93), factors that influence glycoform profiles must be controlled, especially when some glycoforms of therapeutic proteins have greater therapeutic efficacy than other glycoforms. Many pharmaceutical proteins are synthesized as mixtures of glycoforms with distinct glycan structures attached to invariant peptide backbones. Human glycoproteins have N- and O-linked glycans added by glycosyl transferases during Golgi modifications, creating characteristic glycoform profiles (94). The glycosylation enzyme repertoire of the recombinant protein production cells, product transit times in the Golgi, and availability of sugar-nucleotide donors all affect the final glycoform profiles. Variations in glycan profiles of recombinant human erythropoietin can affect their pharmacokinetic properties, biologic activities, solubility, receptor recognition, and antigenicity (95–98). The host cell line (99–102), methods of culture (102–106) and extracellular environmental variables can all affect the resulting glycoform profiles of therapeutic proteins (107).

Variations in glycosylation can influence the immunogenic potential of therapeutic proteins. Four of 13 patients receiving yeast-derived rhGM-CSF developed antibodies that cross-reacted with E. coli-derived rhGM-CSF. These antibodies did not, however, cross-react with rhGM-CSF produced in CHO cells. Yeast cells only attach N-linked sugars, while E. coli does not attach sugar residues to proteins. CHO cells, however, attach both O-linked and N-linked sugars. When O-linked sugars were cleaved from rhGM-CSF produced in CHO cells, the antibodies against yeast derived rhGM-CSF then reacted with this isoform. However, the antibodies produced against yeast-derived rhGM-CSF did not react with rhGM-CSF made by CHO cells from which only N-linked sugars were removed. This suggests that O-linked sugars were blocking antigenic sites in CHO-derived rhGM-CSF (7, 8).

Pegylation

Biopharmaceutical proteins have several intrinsic features that can limit their clinical effectiveness. These include short circulatory half-life, short shelf-life, low solubility, rapid kidney clearance, and susceptibility to destruction by proteolytic enzymes (108–110). The covalent attachment of water soluble polyethylene glycol (PEG) moieties to proteins in vitro, PEGylation, may improve some pharmacokinetic properties of therapeutic proteins (108, 111–115). Specifically, pegylation may reduce renal clearance of small proteins and reduce degradation of therapeutic proteins by proteolytic enzymes. PEGylation may mask antigenic sites in a manner similar to glycosylation (116–120). However, there may be a trade-off between biological activity and shielding of antigenic sites for some therapeutic proteins modified by pegylation. This may be compensated for by the longer circulatory residence time of the PEGylated than the non-conjugated therapeutic protein. A PEGylated rhIFN-α, Pegasys, retains only 7% of the antiviral activity of native rhIFN-α in vitro, but still has greater efficacy over unmodified rhIFN-α because of its improved pharmacokinetics (119). Ongoing clinical trials of pegylated factor VIII are expected to determine the immunogenicity of this new product.

Contaminants arising during the production of therapeutic proteins

The choice of expression system influences the presence of contaminants that may be immunogenic in themselves or enhance the immunogenicity of biopharmaceutical proteins (121). Before highly purified recombinant human insulin became available, the less purified porcine and bovine insulin preparations used to treat insulin-dependent diabetics were highly immunogenic. Subsequent purification techniques have resulted in the removal of nearly all contaminants (122, 123). Impurities have been correlated with the development of antibodies against recombinant human insulin and rhGH (123, 124). Contaminating proteins secreted by the biosynthetic cells that make rhIL-2, rhGM-CSF, and rhIFNs can also elicit immune responses (125, 126).
Formulation
Biopharmaceutical proteins are usually stored in solution or after freeze drying at 4°C to maintain acceptable half-lives (127). Stabilizers can be added to biopharmaceutical formulation to prevent protein degradation. The formulation, however, can increase the immunogenic potential and or modify the biological activities of therapeutic proteins. For instance, polysorbate 80 accelerated the formation of the main degradation product and decreased the biological activity of rhIL-2 (128, 129). Formulations of rhIFN-α2a containing human albumin were found to have aggregates of rhIFN-α2a or complexes of rhIFN-α2a with albumin. The latest human albumin-free formulations of rhIFN-α2a are less immunogenic (14).

Storage and handling
Even in sealed containers, biopharmaceutical proteins remain susceptible to significant modification by external factors, including shaking and shearing forces, changing temperatures, light, reactive gases, and water vapor that can impact their immunogenicity (46, 130). Many proteins including rhfactor XIII, rhGH, and recombinant human insulin, easily aggregate by shaking or shearing (131–139). Surfactants are commonly used to inhibit protein aggregation caused by shaking/shearing forces (138, 140–142). Specifically, Pluronic F88 and Tween 20 are used to inhibit shear/shaking-induced aggregation of rhGH (137–143). Aggregation of PEG-GM-CSF and insulin were also successfully inhibited with the surfactants Tween 20 and glycerol monoleate respectively (144, 145).

Excessive heat and/or light, and exposure to air during the storage of therapeutic proteins can increase the rates of formation of degradation products. rhIFN-α2a formulations containing human albumin that are meant to be stored at 4°C generated aggregates on improper storage at room temperature and storage between 2°C and 8°C is now recommended (146). Peroxides of polysorbate 80 can be generated in the presence of light and air at elevated temperatures to oxidize a rhIL-2 mutein (147). Oxidized forms of proteins generated in polysorbate 80 can be more immunogenic than the non-oxidized forms (146). Antioxidants such as methionine and thiolsulfate can be used in the formulation of biopharmaceuticals to prevent oxidation and thereby reduce immunogenicity (148).

Container closure
The container closures meant to protect biopharmaceutical proteins from the external environment can interact with therapeutic proteins to affect their biological activity and immunogenicity (15). Contaminants in glass, the glass and air interfaces, lubricants, and the materials used in syringe hubs, can all influence antibody development to biopharmaceutical proteins. Arsenic, a potential contaminant from the manufacturing of glass containers, can increase the immunogenicity of biopharmaceuticals (131). Limits on the levels of arsenic and similar contaminants are thus regulated to ensure that containers are of proper quality. The choice of container can also impact the quality of the stored product. Vials introduce less risk to immunogenicity than syringes since they only consist of a glass surface and a coated vial stopper. Syringes on the other hand may enhance protein adsorption. Adsorption to syringes is a rapid process that can change the secondary structure of a protein, and destabilizing it or inhibiting its biological activity (149). Hydrophobic surfaces and lubricants may mediate protein adsorption that may lead to the denaturation and degradation of therapeutic proteins (144, 150, 151).

Organic compounds from vulcanization or plasticization can leach from rubber stoppers into biopharmaceutical protein formulations (152). Use of Eprex, a rhEPO formulation, was associated with a sharp increase in the incidence of autoantibodies formed against endogenous erythropoietin to cause pure red cell aplasia. Subsequent investigation established that phenolic derivatives from uncoated rubber syringe stoppers were extracted by polysorbate 80, an excipient in the Eprex formulations. Replacement of uncoated stoppers with Fluro-Tec®-coated rubber stoppers decreased the incidence of PRCA (153).

Chemical degradation
Oxidation, deamidation, and aggregation are the three most common degradation pathways for proteins in vitro (154). Protein oxidation can be induced by contaminant oxidants, catalyzed by light and transition metal ions. Methionine, cysteine, histidine, tryptophan, and tyrosine residues are most susceptible to oxidation due to their high reactivity with reactive oxygen species. Oxidation of a protein therapeutic can result in a loss of biological activity or have other undesirable clinical consequences (154). For example, oxidized versions of rhIFN-α2a formed during improper storage were more immunogenic in mice than the non-oxidized counterparts (146). These oxidative modifications can be influenced by pH, temperature, and buffer composition, and can occur both in solution and during freeze-drying (155, 156).

Protein denaturation can arise from oxidation, deamidation, extreme temperatures, surface interactions, changes occurring during freeze-drying, or with time (154). Denaturation may favor protein aggregate formation arising from either native proteins assembling into polymeric structures, polymers of denatured protein aggregating by hydrophobic interactions, or covalent linking of proteins in their native or denatured state. Aggregate-induced immunogenicity has been reported for rhIFN-α2a, rhFVIII, rhGH and IFN-β and rhGH (46, 132, 156, 157).  

Frequency, duration, and routes of administration
The frequency and duration of administration may play an important role in the immunogenicity of a therapeutic protein since it can take many months to break immune tolerance. An example of this is illustrated by the lengths of time it takes to elicit antibody production against various IFN-β products used to treat multiple sclerosis (16, 157) or chronic hepatitis C with IFN-α (16). Although most chemically synthesized drugs are administered orally, this route is ineffective for therapeutic proteins (1). Their high molecular mass and polar surfaces render these molecules essentially impermeable across epithelial and endothelial cell lining. These physical barriers, along with various chemical and enzymatic barriers encountered by proteins prior to entering the bloodstream, require parenteral administration of therapeutic proteins for their efficient systemic delivery. Different routes of parenteral administration have varying levels of immunogenicity. For example, in mice that received rhIFN-α intraperitoneally,
intronavely, subcutaneously, or by intramuscular injection, the highest antibody titers were elicited against intraperitoneal and subcutaneous injections, but the subcutaneous route evoked a much faster and stronger response. Intramuscular administration elicited few antibody responses, and intravenous injection elicited none. Intravenous injection is thus the favored route of administration (157). Exuera, the inhaled form of insulin is more immunogenic than injected insulin albeit with no apparent impact on the clinical response (158). Despite its remarkable efficiency, parenteral administration has a number of disadvantages including unpredictable patient compliance, complications associated with use in non-clinical settings, and safety issues with reuse of needles.

Conclusions

Clinical use of recombinant human proteins to treat diseases has revolutionized modern medicine. However, nearly all biopharmaceutical proteins can elicit antibody responses in treated patients. The human immune system generates antibody responses to biopharmaceuticals by both T cell-dependent and T cell-independent mechanisms. The consequences of antibody generation against therapeutic proteins include no effects, reduced efficacy, inhibition of the biological activity of the therapeutic and/or homologous endogenous proteins. The immunogenicity of recombinant biopharmaceutical proteins arises from factors including patient characteristics, the doses, frequency and routes of administration, the amino acid sequences selected, post-translational modifications carried out by the expression systems used, purification, formulation, storage and handling, container closure and chemical degradations. The interplay of these factors makes predicting and identifying a single cause of the immunogenicity of any therapeutic protein nearly impossible. Harmonization of the methodologies for antibody identification and quantification may facilitate meta-analysis of studies aimed at establishing the safety of a given therapeutic protein and for comparing similar recombinant products. Finally, it would be ideal if each step in the development of a biopharmaceutical were subject to great scrutiny, and if widespread pharmacovigilance was employed to monitor the safety of biopharmaceutical proteins after product approval. These steps are even more crucial whenever changes, however minor, are made to the production, processing, or administration of a biopharmaceutical protein.

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