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ScienceDirect
Biotechnology Advances 25 (2007) 325–331
BIOTECHNOLOGY ADVANCES
www.elsevier.com/locate/biotechadv

Research review paper

Immunogenicity of therapeutic proteins. Part 3: Impact of manufacturing changes

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Available online 30 January 2007

Abstract

Immunogenicity of biopharmaceuticals relates to the intrinsic complexity of proteins as well as the complexities of the manufacturing process. The manufacture of biopharmaceuticals involves a number of complex processing steps designed to create a highly pure, stable, safe, and effective product. The process often lasts many months and can be divided into seven stages — host cell development, master cell bank establishment, protein production, purification, analysis, formulation, and storage and handling. Even minor variations at any of these stages can lead to clinically relevant changes in efficacy and/or safety of the end product. Due to the complexity of the process and the inherently unstable nature of proteins outside the body, compositional changes can occur, leading to decreased biological activity, alteration of molecular structure, and possible increased risk of host immune responses following administration. Examples are discussed whereby immunogenicity associated with some of these changes has occurred with potentially serious clinical consequences.

Keywords: Biopharmaceutical; Therapeutic protein; Biosimilar; Immunogenicity; Manufacturing changes; Epoetin; PRCA

Contents

1. Introduction

Immunogenicity is an important aspect of the safety of biopharmaceuticals. All exogenous proteins have the potential to induce antibody production, with clinical consequences ranging from allergic reactions to loss of efficacy and neutralization of the native protein (Koren et al., 2002; Schellekens, 2002). The immunogenicity of therapeutic proteins is influenced by extrinsic factors relating to administration of the dosage form as well as the introduction of impurities into the product, while

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0734-9750/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.biotechadv.2007.01.007

intrinsic factors relating to the protein itself may also trigger immune responses (Koren et al., 2002). In addition, patient-specific factors such as genetic background, nature of the disease, immune status, and concomitant medications contribute to immunogenicity (Schellekens, 2002).

Most pharmaceuticals elicit an immune response, but the incidence of detectable antibodies is highly variable, ranging from less than 1% to over 70% for some products (Koren et al., 2002). Although reduced efficacy has been reported for non-neutralizing antibody responses, the induction of a neutralizing antibody response has the greatest potential clinical impact, both in terms of reduction of efficacy and effect on endogenous protein production. However, with most products, the number of patients affected by deleterious antibody responses is small relative to the number of patients treated (Schellekens, 2002).

The issue of immunogenicity is receiving increasing levels of scrutiny, particularly as the patents of many innovator biopharmaceuticals have expired or will soon expire, and biosimilar versions of these therapeutic proteins are expected to enter markets in the USA and Europe. Market admission of biosimilar products is likely to require more than the limited documentation required for conventional off-patent pharmaceuticals. In the absence of suitable predictors of immunogenicity other than clinical trials and active pharmacovigilance, the evaluation of biosimilar products based only on physicochemical characterization, preclinical studies, and demonstration of pharmacokinetic bioequivalence would compromise patient safety (Schellekens, 2004a). The issues for biopharmaceuticals not only relate to the intrinsic complexity of proteins, but also to the complexities of manufacturing, and establishing standards for processing, packaging, and quality assurance (Schellekens, 2005).

Thus, protein production, purification, formulation (i.e. selection of excipients and container closure system), and storage and handling (i.e. storage temperature and exposure to light) can have an impact on the immunogenicity of a biopharmaceutical product. Examples from the literature indicate that product handling has contributed to the immunogenicity of two monoclonal antibodies, interferon alpha, insulin, and erythropoietin (Gregory et al., 1991; Hochuli, 1997; Kroon et al., 1992; Lam et al., 1997; Sharma et al., 2004). Container closures also have the potential to affect immunogenicity, with glass surfaces, air–liquid interfaces, and lubricants able to mediate protein denaturation and aggregation (Jones et al., 2005; Mizutani, 1980; Treuheit et al., 2002), while extractables/leachates in plastics and latex rubber contribute to product impurities, allergic reactions and enhanced immune responses (Hill et al., 2003; Larsen et al., 2001; Primeau et al., 2001). The impact of product handling and container closure on the immunogenicity of therapeutic proteins is presented in the first and second papers in this series.

Manufacturing changes or differences in manufacturing processes between two related products can also influence immunogenicity and is the focus of this third and final article on immunogenicity of therapeutic proteins.

2. Potential sources of immunogenicity resulting from the manufacturing process

The manufacturing process for biotechnology products must be designed to control the complexity inherent in using living systems to prepare products. In addition, manufacturing facilities for biotechnology products must be specifically designed to prevent contamination of the product with adventitious agents (such as viruses, mycoplasma, and bacteria), and to securely contain microorganisms used for production. Appropriate facility design, construction, validation, and testing, in association with extremely vigilant, well-designed and well-implemented manufacturing practices, have contributed to excellent product quality. Public health protection requires that the current high standards of manufacturing compliance remain uncompromised. Inadvertent contamination and even subtle differences in manufacturing must not occur or, more importantly, go undetected.

The manufacture of biopharmaceuticals is a far more complicated process than the processes used by manufacturers of synthetic pharmaceuticals. The recombinant DNA and hybridoma technologies used in biopharmaceutical manufacture include numerous extraction, purification, and concentration steps that can impact on a protein's three-dimensional structure and, therefore, its stability and biological properties (Patro et al., 2002; Schellekens, 2004a). Proteins that become denatured as a result of an unstable tertiary structure can present novel antigenic epitopes not found in the parent molecule, with a resulting increase in immunogenicity (Koren et al., 2002). As an example of the production cascade from raw materials to end product, the seven major steps in biopharmaceutical manufacture, each with the potential to impact on immunogenicity, are host cell development, master cell bank establishment, protein production, purification, analysis, formulation, and storage and handling.

Host cell development, i.e. the cell line used to produce the recombinant protein, is an important component of manufacturing because cell culture and maintenance of the cell line are complex procedures that require controlled manipulation of many different factors. The choice of cell line or expression system, for example, may determine what impurities are introduced into the product given that host cell proteins are also released into the culture media along with the target recombinant molecule. These host cell impurities can act as adjuvants or be directly immunogenic (Chirino and Mire-Sluis, 2004). Host cell selection is also important in terms of degree of glycosylation, with proteins produced in *Escherichia coli* expression systems not glycosylated and, therefore, potentially exposed as novel antigenic sites (Schellekens, 2002).

Protein production conditions can affect the distribution of glycoforms. In a study on the production of monoclonal antibodies, the carbohydrate glycoforms of monoclonal antibodies produced under various conditions were analyzed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Differences in glycoform profiles of the antibodies were observed with different production conditions. These data confirm that production conditions can affect glycoform distribution in both murine-origin and humanized versions of monoclonal antibodies (Kroon et al., 1995).

Any change in the purification process or the material made by the manufacturer or the vendor, respectively, can have an impact on the outcome of the product. It is even possible for the initial validated process to fail to remove trace amounts of impurities. Therefore, the purification process must be sufficiently robust to provide high-purity material consistently.

As it is well established that cell growth conditions can affect the distribution of glycoforms, following a different purification process can result in a product with a significantly different product profile. As an example, a study comparing epoetins produced by different manufactures reported wide variability in bioactivity that corresponded to a high degree of heterogeneity in the molecular isoforms of epoetin found in each preparation (Fig. 1) (Schellekens, 2004b). Different degrees of glycosylation of epoetin alfa have an impact on its biological activity and highly sialylated glycoforms are more active than less sialylated forms (Fukuda et al., 1989; Gokana et al., 1997). However, it remains to be determined whether there are any differences in safety with long-term use in humans.

Selection of proper analytical methods with correct sensitivity also has an important role in ensuring consistent production. For example, an extra peak was occasionally identified on reverse-phase high-performance liquid chromatography in some lots of purified epoetin alfa that was not evident in the reference standard. Interestingly, this anomaly was not observed by any other analytical test and did not affect the activity. The unknown peak was identified as an anomalous epoetin alfa peptide fragment resulting from an extremely low amount of residual carboxypeptidase B-like activity in these lots. The total loss of the extra peak in the boiling and additional experiments indicated that the impurities were related to metalloproteases. The results, however, demonstrate that the proper selection of analytical methods is important and helps to check the consistent quality of the product (DePaolis and Sharma, 1994).

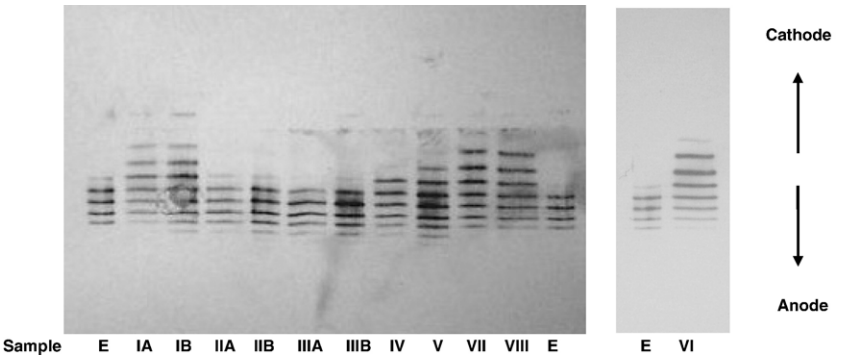

Fig. 1. Molecular heterogeneity among available epoetin alfa products manufactured in Asia, India, and South America. Samples IA, IB, IIA, IIB, IIIA, and IIIB were obtained from Korea; samples IV and V were obtained from Argentina, sample VI was obtained from India, and samples VII and VIII were obtained from China. Sample E is the epoetin alfa control. [Adapted with permission from European Journal of Hospital Pharmacy, 2004, volume 3, published by Pharma Publishing and Media Europe bvba, Belgium (Schellekens (2004b)].

Steps in the production cascade subsequent to protein purification are collectively referred to as formulation and fill-finish operations. These operations include freezing and thawing of the purified protein bulk, product formulation with the addition of excipients, sterile filtration, filling into ampoules, vials, or syringes, freeze-drying, container closure, inspection, and packaging (Patro et al., 2002). Protein molecules are readily destabilized during in vitro processing, with the resulting conformational changes most commonly giving rise not only to aggregation, but also to oxidation and deamidation, which have consequences that vary from reduced potency to enhanced immunogenicity (Cleland et al., 1993; Patro et al., 2002). Efficient development of stable formulations of protein pharmaceuticals requires an intimate knowledge of the protein and its chemical and physical properties. In particular, understanding the mechanisms by which a protein could degrade is critical for designing and testing formulations. The introduction of excipients during formulation of the product is intended to protect the protein against degradation by stabilizing its native conformation. However, the choice of excipient is critical, not only to protect the protein against multiple mechanisms of degradation, but also because excipients themselves may influence protein aggregation. For example, the use of human serum albumin (HSA) as an excipient for interferon alpha-2a has been associated with aggregation and increased immunogenicity, despite being used as an excipient for other products without apparent effect on immunogenicity (Palleroni et al., 1997; Schellekens, 2002).

3. Examples of immunogenicity associated with manufacturing changes

Choice of host cell line or tissue source used in the manufacture of biopharmaceuticals has had an impact on the immunogenicity of human growth hormone (hGH), insulin and interferon beta. In the case of hGH, problems with immunogenicity date back to its first use in GH-deficient patients, as well as subsequent uses involving more pure preparations of human pituitary-derived hormone and those manufactured using recombinant techniques (Pirazzoli et al., 1995). The first preparation of recombinant hGH, methionyl-recombinant hGH (met-rhGH), demonstrated greater immunogenicity than pituitary-derived hGH as a result of contamination and aggregation during product processing. In a study of 46 GH-deficient children treated with met-rhGH for at least 12 months, 15 of 20 patients naïve to hGH treatment, and 3 of 26 patients previously treated with pituitary-derived hGH developed a persistent GH-specific antibody response (Massa et al., 1993). Switching to a non-methionyl rhGH completely ablated detectable antibodies in 9 of 11 patients, indicating that host expression system rather than use of recombinant DNA techniques per se was an important factor in the immunogenicity of rhGH.

Recombinant human interferon beta is a first-line therapy for relapsing-remitting multiple sclerosis where it has been shown to reduce the exacerbation rate, severity of relapse, and progression of neurological dysfunction (Sorensen et al., 2003). A large study of patients with relapsing-remitting multiple sclerosis found there was a higher proportion of patients with neutralizing antibodies in those treated with interferon beta-1b (Avonex® and Rebif®) than in those taking interferon beta-1a (Betaferon®) (Ross et al., 2000). The two recombinant interferons differ in that interferon beta-1a is produced in mammalian cell lines and interferon beta-1b is produced in *E. coli* (Abdul-Ahad et al., 1997). Given that proteins produced in *E. coli* are not glycosylated, thereby leaving potential antigenic sites exposed for antibody binding, it is likely that the choice of expression system is contributory to the increased immunogenicity of interferon beta-1b. The use of glycosylated proteins generated in mammalian cell lines may offer safety advantages due to the glycosylation of proteins expressed in this manner (Schellekens, 2002).

Early forms of insulin were derived from porcine and bovine tissues and demonstrated considerable immunogenicity (Schellekens, 2002). With the development of better purification techniques it became possible to remove many impurities, thereby promoting greater discrimination between products. For example, Chance and colleagues showed that single-component purification of porcine insulin led to a product that was less immunogenic than either a conventional preparation derived from crystalline insulin or a single-peak formulation purified using a less rigorous process (Chance et al., 1976). The introduction of highly purified porcine insulin products and, subsequently, human insulin preparations has reduced the incidence of immune-mediated insulin resistance, while lipoatrophy, reported in a high proportion of patients treated with nonpurified bovine/porcine preparations, has all but disappeared with exclusive human insulin treatment (Schernthaner, 1993).

Changes in formulation are commonly applied in biopharmaceutical manufacture to take advantage of new processing technologies or to improve safety. Recombinant human interferon alpha-2a is approved in the treatment of a range of oncological and viral diseases (Kontsek et al., 1999). The discovery that HSA had a destabilizing effect on interferon alpha-2a that resulted

in increased immunogenicity prompted a change in formulation of the product (Ryff, 1997). The impact of serial process improvements on immunogenicity of the product was subsequently evaluated over five clinical trials in patients with chronic hepatitis C. Retrospective comparison between trials was possible due to similar patient populations, standardized procedures for collection, handling, storing, and shipping of serum samples to a central laboratory, and standardized assays for antibody screening and bioassay (Ryff, 1997). Decreased immunogenicity compared with the starting product was associated with the use of single-peak refrigerated lyophilizate that was formulated without HSA. In the HSA-formulated interferon alpha-2a, immunogenicity was correlated with the presence of both interferon–interferon and interferon–HSA aggregates, with little aggregation found at product storage temperatures of 4 °C (Hochuli, 1997).

Processes such as pasteurization, freeze-drying, and pegylation can alter the three-dimensional conformation of a protein. Pasteurization was introduced into the manufacture of factor VIII to reduce the risk of viral transmission, but resulted in increased immunogenicity as a consequence of a conformational change (Schellekens, 2002). In addition to allowing protein aggregation, freeze-drying may also result in protein oxidation, which not only reduces biological activity but may also increase the immunogenicity of some proteins (Cleland et al., 1993). Although polyethylene glycol can increase stability and potentially even decrease the immunogenicity of therapeutic proteins, pegylation may also contribute to immunogenicity (Li et al., 2001).

Another example of immunogenicity that is related to process changes is considered in the context of pegylation. While pegylation is intended to stabilize proteins, providing increased solubility, extending in vivo circulation and, in some cases, reducing immunogenicity, pegylation has also had undesirable effects. As a potent stimulator of platelet production, recombinant human thrombopoietin has been used to treat patients with thrombocytopenia, particularly in the nonmyeloablative chemotherapy setting. Two recombinant products have undergone clinical testing, a glycosylated molecule identical in amino acid sequence to native thrombopoietin (rHuTPO), and pegylated recombinant megakaryocyte growth and development factor (PEG-rHuMGDF), which is a non-glycosylated molecule sharing the first 163 amino acids of the native protein sequence (Li et al., 2001). In clinical studies, some patients treated with PEG-rHuMGDF, but not the glycosylated molecule, paradoxically developed persistent thrombocytopenia due to the development of neutralizing antibodies against endogenous TPO. Those affected included healthy volunteers as well as a small proportion of oncology patients. While route of administration may have contributed to the immunogenicity of PEG-rHuMGDF, which was administered subcutaneously versus intravenously for rHuTPO, the PEG-rHuMGDF-associated antibody response was targeted towards epitopes in the first 163 amino acids of TPO (Li et al., 2001). Given that this sequence is also found in rHuTPO and this product was not immunogenic, it is likely that the immunogenicity of PEG-rHuMGDF was related either to the absence of glycosylation or an inability of polyethylene glycol to protect the therapeutic protein against conformational instability.

The development of pure red cell aplasia (PRCA) in patients with chronic kidney disease who were treated with epoetin alfa (Eprex®) is an example of the impact of increased immunogenicity of a biopharmaceutical agent, in this case associated with product handling, container closures, and the production process. Prior to 1998, Eprex® was formulated with HSA as an excipient before being replaced with polysorbate 80 in markets outside the USA to prevent possible transmission of virus. Following this formulation change, the incidence of PRCA in patients with subcutaneous exposure to Eprex® began to increase substantially, prompting risk mitigation initiatives by the product manufacturer. Subsequent investigations revealed that the change in formulation coupled with the use of prefilled syringes with uncoated rubber stoppers resulted in leachates with adjuvant activity being released into the product (Boven et al., 2005; Sharma et al., 2004). The solution in this case was to use FluroTec®-coated rubber stoppers in conjunction with improved cold-chain storage and handling of Eprex® (Boven et al., 2005). These measures were accompanied by an immediate decrease in the number of new cases of antibody-mediated PRCA.

The most recent example of a process-related change in immunogenicity is Omnitrope (Sandoz GmbH, Kundl, Austria), the first biosimilar recombinant human growth hormone (GH) approved by the European Medicines Agency (EMEA). The active drug substance manufactured by Covance was initially used during the development program. Improvements in the purification process were implemented at the Kundl site leading to significantly enhanced host cell protein (HCP) clearance, and the active drug substance from the Kundl process (Omnitrope) was used for commercialization (EMEA, 2006). In clinical studies with the Covance active substance, up to 60% of enrolled patients developed non-neutralising anti-GH antibodies and all patients developed anti-HCP antibodies (EMEA, 2006). It is known that these

HCP can enhance the antibody reaction against GH. With the improved HCP clearance, anti-GH antibody formation returned to within the range reported with other GH-containing products (EMEA 2006; Lundin et al., 1991).

4. Conclusion

In this final paper, we have shown that differences in the manufacturing process between preparations of the same protein and changes in the manufacturing process of a single therapeutic product can have an impact on the immunogenicity of a biopharmaceutical agent. Some biopharmaceutical manufacturers now conduct clinical trials for a product with an existing license that has undergone a change in formulation in order to ensure that the manufacturing change or changes do not alter the immunogenicity of the dosage form (e.g. Avonex®). Given the obvious potential for immunogenicity and its unpredictable nature, such measures are prudent and are likely to become a requirement by regulatory authorities.

In the second paper in this series we showed that although container closures are a seemingly minor aspect of the production of biopharmaceuticals, they have an important bearing on the potential immunogenicity of the product within the container. When the contents of the container are able to interact with the container closure system, extractables and leachables may appear as contaminants in the product with resulting deleterious effects. Compounds that have been shown to leach from plastic containers and rubber stoppers include allergens and adjuvants. In the first paper in this series we showed that product handling can affect the immunogenicity of a biopharmaceutical, also with potentially serious clinical sequelae. Although the use of prefilled syringes allows patients to self-administer their medication, physicians, pharmacists, and patients all need to be aware that proper storage is essential to ensure they can be used safely.

Patents of innovator biopharmaceuticals have expired or will soon expire, creating opportunities for biosimilar versions to enter markets in the USA and Europe. Despite new guidelines by the European Medicines Agency, which indicate that biosimilar products will need to undergo a more rigorous evaluation process than conventional generic pharmaceuticals, immunogenicity remains a concern because it cannot be detected outside the clinical trial setting and, in some cases, requires active postmarketing pharmacovigilance. Thus, even small changes in the three-dimensional composition of a protein that may go undetected by physicochemical characterization techniques may invoke a potentially serious immune response in a portion of treated patients. Given the complexity of proteins and their intrinsic instability under in vitro conditions, each step in the biomanufacturing process from selection of host cell line to the container used to dispense the product is a potential juncture for breakdown of the process.

Acknowledgements

Disclosure: The author received editorial/writing support in the preparation of this manuscript, funded by Johnson & Johnson Pharmaceutical Services. The author, however, was fully responsible for contents and editorial decisions for this manuscript.

Amy Ferry provided editorial/writing support for this manuscript.

Carol Keiling provided typing/administrative support for this manuscript.

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