Antibodies to Heterologous Proteins in Hemophilia A Patients Receiving Recombinant Factor VIII (Recombinate™)

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Keywords

Factor VIII; recombinant proteins; CHO cells; antibodies, monoclonal; serum albumin, bovine

Summary

As a consequence of the manufacturing process, trace quantities of Chinese hamster ovary cell protein, bovine serum albumin and murine immunoglobulin G are present in Recombinate™ recombinant human factor VIII (rhFVIII). The development of antibodies (Abs) to these heterologous proteins was evaluated during long-term rhFVIII therapy of hemophilia A in 68 previously treated and 73 previously untreated patients. Ab prevalence was also assessed in 157 non-hemophilic subjects. Abs against heterologous proteins could be detected in varying percentages of patients and non-hemophilic subjects. Abs arose in patients sporadically, and levels were typically low. There were no adverse events associated with development or presence of anti-heterologous protein Abs. These data indicate that sustained immune responses to trace levels of heterologous proteins are very infrequent during long-term rhFVIII therapy.

Introduction

Recombinant human factor VIII (rhFVIII) affords an attractive alternative to plasma-derived factor VIII (pdFVIII) concentrates. The potential for transmission of blood-borne viral pathogens is essentially eliminated with rhFVIII, and availability is not dependent upon human plasma supplies. Inhibitors arising in recipients of rhFVIII are typically of no greater incidence than that for closely monitored patient cohorts infused with pdFVIII concentrates (1, 2). Many inhibitors observed with rhFVIII treatment are low in titer and often transient.

The safety and efficacy of Recombinate™ rhFVIII (Baxter BioScience, Glendale, California, USA) for hospital or home treatment of hemophilia A patients has been extensively documented in clinical studies (1, 3-9). This rhFVIII preparation, introduced in 1992, is highly stable under varied conditions of use including continuous infusion and resistant to heat- or light-induced degradation (10).

Recombinate™ is synthesized in Chinese Hamster Ovary (CHO) cells transfected with the cDNAs for human factor VIII (FVIII) and von Willebrand factor (11). One constituent of the media used during synthesis is bovine serum albumin (BSA). Purification of rhFVIII is accomplished by immunoaffinity chromatography with monoclonal murine immunoglobulin G (MuIgG) directed against human FVIII. CHO protein, BSA and MuIgG are present in the final rhFVIII product at levels of < 1.5, < 1.0 and < 0.1 ng/IU rhFVIII, respectively. These trace levels are generally too low to provoke an immune response. However, the possibility needs to be investigated that patients receiving rhFVIII repeatedly over a protracted time period at high doses might develop antibodies (Abs) against these heterologous proteins. Relatively few data on this issue have been previously reported. In the present study anti-CHO protein, anti-BSA and anti-MuIgG Abs were monitored longitudinally over an extended course of rhFVIII therapy in previously treated patients (PTPs) and previously untreated patients (PUPs) with hemophilia A. In addition, the prevalence of these Abs against heterologous proteins was assessed in adult and pediatric non-hemophilic subjects unexposed to rhFVIII.

Materials and Methods

Subjects

The study population was comprised of 141 hemophilia A patients and 157 non-hemophilic subjects. Of the hemophilia A group, 68 were adult and pediatric PTPs and 73 PUPs. The 68 PTPs were subjects of a prospective, open-label multicenter clinical trial of rhFVIII safety and efficacy for long-term home treatment under a protocol approved by the Institutional Review Boards or Ethics Committees at the respective study centers, as previously described (9). Their baseline endogenous FVIII was ≤ 5% of normal. Patients ≥ 18 years of age and the parents or guardians of patients < 18 years granted informed written consent. Detectable baseline neutralizing anti-FVIII Ab (inhibitor) was absent in all but two cases. Patients received rhFVIII either on demand for management of acute bleeding episodes or for prophylaxis.

The PUPs were participants in a prospective, open-label multicenter clinical trial focused inter alia on inhibitor formation in rhFVIII recipients, as elsewhere reported (1). Approval was obtained from the study center Institutional Review Boards or Ethics Committees. Informed written consent was rendered by the parents or guardians of the PUPs. Their rhFVIII regimen involved on-demand treatment, prophylaxis or both. In cases of inhibitor formation, they were offered the option to undergo immune tolerance induction (ITI) according to a pre-designated regimen designed specifically for this study. After commencement of rhFVIII therapy 5 PUPs who developed high responder inhibitors elected to undergo ITI.

The composition of the non-hemophilic group was 57 normal adult volunteers with no known history of exposure to hamster, bovine or murine antigens and 100 infants and children with no prior exposure to rhFVIII or pdFVIII at University Hospital Skejby, Aarhus, Denmark, under a protocol approved by the Biomedical Ethics Committee of Aarhus County, Denmark. Of the 100 non-hemophilic pediatric subjects, 60 were individuals referred for evaluation of suspected congenital cardiac disorder and 40 healthy child volunteers donating blood in connection with a study of coagulation activation markers.

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Antibodies directed against CHO protein, BSA and MuIgG were measured by enzyme-linked immunosorbent assay either at the laboratories of Baxter BioScience (Duarte, California, USA and Round Lake, Illinois, USA) or University Hospital Skejby (Aarhus, Denmark). The same methodologies, materials and reagents were used at all laboratories. Assays were conducted using 96 well microtiter plates coated with 0.1 M carbonate buffer solutions (pH 9.6) containing 20 mg/mL CHO protein obtained from lysates of cultured CHO cells; 2.5 mg/mL BSA (Sigma Chemical, St. Louis, Missouri, USA); or 5 mg/mL F8/1 monoclonal MuIgG (Baxter BioScience), the same monoclonal Ab (MoAb) used for purification of rhFVIII by immunoaffinity chromatography during the manufacturing process. The wells were washed four times with phosphate buffered saline (PBS) containing 0.5% Tween-20 (pH 7.4) and then incubated 1 h at 37° C in the presence of 1% w/vol ovalbumin blocking solution. After 4 further washing steps, 100 μL of serum sample or control diluted 1:50 in PBS were added to the wells and incubated 2 h at 37° C. Thereupon, the wells were again washed four times and incubated 1 h at 37° C with 100 μL of alkaline phosphatase conjugated to goat anti-human IgG, IgA and IgM (Harlan Sera-Lab Ltd., Loughborough, Leicestershire, UK). After 4 additional washes, alkaline phosphatase activity was determined using p-nitrophenyl phosphate as substrate.

Positive anti-CHO protein and anti-BSA controls were secured by immunoaffinity fractionation of a commercially available pooled intravenous immunglobulin preparation (Gammagard®, Baxter BioScience) on a Sepharose 4B (Pharmacia & Upjohn, Uppsala, Sweden) chromatography column conjugated with CHO protein or BSA. Anti-murine immunglobulin control was purified from the blood of a single individual with high Ab titers. Pool normal human serum (Sigma Chemical) served as negative control. In a previous report (12) anti-MuIgG Ab in this commercial pooled normal human serum preparation, though not undetectable, was found to be low in concentration (1.4 μg/mL). Assay blanks contained buffer only. Six replicate determinations were performed for each sample and control.

Results were expressed as reaction scores, which were calculated as follows. The mean of the 6 replicate blank absorbance values (background absorbance) was subtracted from the means of both the specimen and negative and positive control replicates. Then the net mean negative control values were subtracted from the net specimen mean. Positive reaction scores were considered to signify the presence of Ab against heterologous protein. For assay data to have been judged valid, the ratio of net mean positive to net mean negative control absorbance in the microtiter plate must have exceeded 3.0 for anti-CHO protein and anti-MuIgG Ab determinations and 4.0 for anti-BSA Ab.

In hemophilic patients serum samples were assayed for anti-heterologous protein Abs at baseline and intervals of 3 months thereafter. Patients were classified on the basis of immunoreactivity to heterologous proteins as exhibiting: (1) no immune response based on negative assay results before and during rhFVIII therapy; (2) pre-existing Abs as judged by pretreatment positive assays with or without measurable immunoreactivity during treatment; or (3) new-onset or anamnestic response in cases of one or more positive assay findings during therapy in patients without detectable pretreatment immunoreactivity.

Adverse Events

Patients were examined at 3 month intervals during rhFVIII therapy. At each visit the occurrence of any therapy-related adverse events was documented based on clinical observations and interviews with the patients and, in pediatric cases, their parents or guardians.

Data Analysis

Stata 7.0 (Stata Corp., College Station, Texas, USA) statistical software was used for data analysis. Proportions of patients exhibiting immunoreactivity were calculated with exact binomial 95% confidence limits (95% CI). Between-group differences in proportions of patients with immunoreactivity were evaluated by Fisher exact test. Continuous data are presented as mean with range or mean ± standard deviation. The relationship between de novo development of anti-heterologous protein Abs and duration of rhFVIII therapy was assessed by linear regression analysis.

Results

Of 69 PTPs enrolled in a prospective, open-label multicenter clinical trial, 68 were evaluated up to 57 months for development of anti-heterologous protein Abs. Severe or moderately severe hemophilia A (baseline endogenous FVIII <2% of normal) was present in 67 cases and moderate (2-5% of normal) in 2. This PTP population consisted of 46 adults of mean age 34 years (range, 18-63 years) at the time of first rhFVIII infusion and 23 children of mean age 12 years (range, 3-17 years). The cumulative administered rhFVIII averaged 241 ± 180 exposure days during the course of the study. The median duration of follow-up was 42 months (range, 6-57 months). Additional characteristics of this group appear elsewhere (9).

The PUPs presented with severe or moderately severe severe hemophilia A (baseline endogenous FVIII <2% of normal) and were of mean age 10.3 months (range, 0.1-50.0 months) at time of first rhFVIII infusion. Their average duration of follow-up was 51.5 months (range, 22.2-68.5 months).

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Fig. 1 Percentages of PTPs, PUPs and non-hemophilic subjects with (a) anti-CHO protein, (b) anti-BSA and (c) anti-MuIgG Abs. Error bars indicate 95% CI.
Mean cumulative administered rhFVIII in the PUPs was 102.9 ± 71.8 exposure days, excluding ITI treatments. Further details of this population have been previously reported (1).

**Anti-CHO Ab**

Frequencies of anti-CHO protein Ab are summarized in Fig. 1(a). Prior to rhFVIII treatment anti-CHO protein Ab was detected in 0/68 PTPs (0.0%; 95% CI, 0.0-5.3 %) and 0/73 PUPs (0.0%; 95% CI, 0.0-4.9%). Measurable anti-CHO protein Ab was present in 1/57 non-hemophilic adults (1.8%; 95% CI, 0.0-9.4 %) and 10/100 non-hemophilic pediatric subjects (10.0%; 95% CI, 4.9-17.6%). The difference in pre-existing Ab frequency between adult and pediatric non-hemophilic subjects was of borderline statistical significance (p = 0.058). For the total of 10 positive anti-CHO protein Ab assays in the pediatric non-hemophilic group the median reaction score was 0.185 (range, 0.004-1.461).

In 3/68 PTPs (4.4%; 95% CI, 0.9-12.4%) and 18/73 PUPs (24.7%; 95% CI, 15.3-36.1%) anti-CHO protein Ab appeared de novo after commencement of rhFVIII therapy [Fig. 1(a)]. The frequency of de novo anti-CHO immunoreactivity was significantly higher in PUPs than PTPs (p = 0.001). In one PTP, serologic evidence was present of sensitization to anti-CHO protein, as well as to both BSA and MuIgG.

When present, new-onset or anamnestic anti-CHO protein immunoreactivity was sporadic, and measured Ab fluctuated over time at very low levels, as indicated in Fig. 2. For the 3 PTPs with de novo anti-CHO immunoreactivity (Fig. 2), a total of 4 positive assay results were obtained with a median reaction score of 0.100 (range, 0.026-0.194). Among the 18 PUPs with de novo anti-CHO immunoreactivity, the median reaction score for all 39 positive assays was 0.069 (range, 0.004-0.842). For 64% of the positive assays in the PUPs the reaction score was less than 10% as large as the observed maximum of 0.842. Thus, the preponderance of anti-CHO protein Ab measurements in patients with new-onset or anamnestic Abs were low compared with the highest observed values. Linear regression analysis of pooled data from PTPs and PUPs with positive assays for de novo anti-CHO protein immunoreactivity failed to reveal any significant trend toward increasing reaction scores in relation to duration of rhFVIII therapy [Fig. 3(a)]. In no case did a positive assay temporally coincide with an adverse event. There were no cases of incremental immunoreactivity as a function of increasing cumulative rhFVIII exposure. Among the 5 PUPs undergoing ITI [Fig. 4(a)-(e)], sporadic low-level positive assays were recorded in 2 patients [Fig. 4(a)-(b)], and their cumulative rhFVIII exposure was lower by 2-6 fold than that of the 3 not exhibiting immunoreactivity. Thus, even in these cases of relatively high cumulative rhFVIII exposure there was no evidence of a dose-response relationship with respect to anti-CHO protein immunoreactivity.
In 5/68 PTPs (7.4%; 95% CI, 2.4-16.3%) and 35/65 PUPs (53.8%; 95% CI, 41.0-66.3%). The pre-existing Ab frequency difference between PTPs and PUPs was significant (p < 0.0005). Assays of anti-BSA Ab were not performed prior to treatment in 8 PUPs. In none of the PTPs or PUPs with pre-existing Abs was there evidence of an anamnestic response characterized by incremental immunoreactivity over time proportionate to cumulative rhFVIII exposure. Pre-existing anti-BSA reaction score data of the PTPs are shown in Fig. 5(a)-(e). Sporadic positive assays were recorded in PUPs undergoing ITI [Fig. 4(f)-(j)]; however, no pattern of progressively rising immunoreactivity could be discerned. Anti-BSA Abs were present in 71/100 non-hemophilic pediatric subjects (71.0%; 95% CI, 61.1-79.6 %), a significantly higher proportion (p <0.0005) than the 8/57 (14.0%; 95% CI, 6.3-25.8%) observed for non-hemophilic adults [Fig. 1(b)]. The median reaction score for the 71 positive anti-BSA Ab assays in non-hemophilic pediatric subjects was 0.798 (range, 0.002-2.419).

New-onset or anamnestic anti-BSA Abs [Fig. 1(b)] developed during rhFVIII therapy in 5/63 PTPs (7.9%; 95% CI, 2.6-17.6 %) and 24/65 PUPs (36.9%; 95% CI, 25.3-49.8%), and the between-group difference in frequency of de novo anti-BSA Abs was significant (p <0.0005). New-onset or anamnestic anti-BSA immunoreactivity was generally small in magnitude or sporadic [Fig. 5(f)-(j)]. For the 28 and 196 total positive assays among the 5 PTPs and 24 PUPs with de novo anti-BSA Abs the median reaction scores were 0.080 (0.006-1.504) and 0.725 (range, 0.002-2.419), respectively. Based on the pooled positive assay data for PTPs and PUPs with new-onset or anamnestic anti-BSA Abs there was no evidence of increasing reaction scores associated with longer duration of rhFVIII therapy [Fig. 3(b)]. None of these patients experienced an adverse event related to rhFVIII therapy. De novo formation of both anti-BSA Abs and anti-MuIgG was detected in one PTP. In only one case, that of a PUP observed over a 54 month period, did anti-BSA Ab reaction score consistently increase in relation to higher cumulative rhFVIII exposure. Although there was no detectable anti-CHO protein immunoreactivity in this patient [Fig. 6(a)], both anti-BSA[Fig. 6(b)] and anti-MuIgG [Fig. 6(c)] Ab reaction scores rose progressively during treatment. The history of this patient during rhFVIII therapy was otherwise unremarkable, and no specific basis for the incremental immunoreactivity could be identified.

Anti-MuIgG Ab

In 9/68 PTPs (13.2%; 95% CI, 6.2-23.6 %) and 33/64 PUPs (51.6%; 95% CI, 38.7-64.2 %) measurable anti-MuIgG Ab was present prior to rhFVIII therapy [Fig. 1(c)]. The difference in frequency of pre-existing anti-MuIgG Ab was significant (p <0.0005). Incremental immunoreactivity suggestive of an anamnestic response was not observed in any PTPs. The pattern of pre-existing immunoreactivity in the PTPs is depicted in Fig. 7. In 9 PUPs assays of anti-MuIgG Ab were not performed before treatment. Anti-MuIgG Ab was also detected in 11/57 non-hemophilic adult (19.3%; 95% CI, 10.0-31.9%) and 53/100
Fig. 4  Reactions scores of individual patients undergoing ITI for (a)-(e) anti-CHO protein Abs, (f)-(j) anti-BSA Abs and (k)-(o) anti-MuIgG Abs. Data in vertically aligned panels are derived from the same patient.

Fig. 5  (a)-(e) Pre-existing and (f)-(j) new-onset or anamnestic anti-BSA immunoreactivity in the individual PTPs.
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(53.0%; 95% CI, 42.8-63.1%) non-hemophilic pediatric individuals [Fig. 1(c)]. The difference in Ab frequency between the adult and pediatric non-hemophilic groups was statistically significant (p = 0.006). There were a total of 53 positive anti-MuIgG Ab assays among non-hemophilic pediatric subjects with a median reaction score of 0.524 (range, 0.003-1.840).

Anti-MuIgG Ab [Fig. 1(c)] arose de novo during rhFVIII treatment in 4/59 PTPs (6.8%; 95% CI, 1.9-16.5%) and 26/64 PUPs (40.6%; 95% CI, 28.5-53.6%), and the between-group difference in frequency was significant (p < 0.0005). There were no adverse events contemporaneous with new-onset or anamnestic anti-MuIgG Ab, and immunoreactivity was typically minor and variable (Fig. 8). For the 4 PTPs and 26 PUPs with de novo anti-MuIgG immunoreactivity there were respectively a total of 21 and 212 positive assays with median reaction scores of 0.151 (range, 0.037-0.561) and 0.395 (range, 0.002-1.921). Reactions scores for positive de novo anti-MuIgG assays in PTPs and PUPs did not exhibit any pattern of progressive increase as a function of rhFVIII therapy duration [Fig. 3(c)]. As indicated above, steadily increasing levels of anti-MuIgG Ab as well as anti-BSA were observed in one PUP. Anti-MuIgG data for the 5 PUPs undergoing ITI are shown in Fig. 4(k)-(o). Persistent immunoreactivity was evident in only one of these patients [Fig. 4(m)], but in this case increasing anti-MuIgG Ab levels over time were not apparent.

Discussion

Heterologous proteins are present in an array of foods as well as protein therapeutics in current clinical use. Immunoaffinity purification with MuIgG is employed in the production of several proteins including both pdFVIII (13-15) and rhFVIII (9). Residual MuIgG is present at trace levels in the final products. Similarly, CHO cell expression systems are commonly used for synthesis of recombinant protein therapeutics such as rhFVIII (11), tissue plasminogen activator (16), interferon beta-1a (17), deoxyribonuclease I (18), Trastuzumab anti-HER2 MoAb (19) and Rituximab anti-CD20 MoAb (20). Residual CHO proteins remain in the final products at low levels.

Despite exposure of large numbers of patients to residual heterologous proteins, the potential immunological sequelae have received comparatively little attention, either among hemophilia patients or other patient populations. The available studies have primarily focused on formation of anti-murine Abs in recipients of therapeutic proteins containing low levels of MuIgG as a manufacturing process-related impurity. In 7 patients with severe hemophilia A there was no evidence of de novo anti-MuIgG formation after 6 months of treatment with immunoaffinity-purified pdFVIII (21). In a study of 18 adult and pediatric hemophilia A patients treated chronically with monoclonally purified pdFVIII and followed for 12-36 months, pre-existing anti-muIgG Abs were detected in two infants (22). No seroconversion to mouse MoAb was observed over a median follow-up period of 9 months (range, 3-18 months) in 60 PTPs and 17 PUPs undergoing treatment with immunoaffinity-purified pdFVIII (23). Moderate to high levels of anti-MuIgG were reported in 5/8 healthy volunteers, 3/3 patients with genital warts receiving placebo and 2/3 such patients treated with intralesional human leukocyte interferon alpha-n3 purified by murine MoAb immunoadfinity chromatography (24). Surveillance of 13 severe hemophilia A patients receiving substantial dosages of the same pdFVIII product over an average observation period of 28 months failed to reveal evidence of human anti-murine Abs (14). Pre-existing anti-MuIgG Abs were detectable in 18% of 146 hemophiliacs and 10% of 20 normal donors (25). After initiation of recombinant factor VIIa therapy 10/146 (7%) patients developed anti-MuIgG de novo. In 9 hemophilia A and 11 hemophilia B PUPs monitored for mean periods of 21 months and 3 months, respectively, treatment with immunoaffinity-purified pdFVIII and plasma-derived factor IX concentrate, respectively, did not result in significantly increased levels of either anti-murine IgG or IgM Abs (15). Of 58 PTPs receiving exclusively one rhFVIII product (Kogenate®, Bayer Corp., West Haven, Connecticut, USA) as part of a 5 year multicenter prospective study of home therapy, 8.6% exhibited pre-existing anti-MuIgG (26). An undisclosed percentage developed de novo anti-MuIgG.

Data on immunoreactivity against bovine and hamster proteins in recipients of recombinant therapeutic products are surprisingly limited.
Pre-existing Abs directed against hamster cell protein and bovine serum protein were evident in 16% and 2% of hemophiliacs, respectively, and 5% and 0% of normal blood donors, respectively (25). Among recombinant factor VIIa recipients new-onset or anamnestic Abs to hamster cell and bovine serum proteins were apparent in 6% and 0%, respectively (25). Pre-existing and de novo Abs against hamster protein were demonstrable in 3.4% and 1.7% of PTPs, respectively, undergoing long-term home therapy with Kogenate® (26).

The present report provides the most extensive data thus far on formation of Abs against heterologous proteins in hemophilia A patients receiving rhFVIII, as well as prior pdFVIII in some cases, and in non-hemophilic subjects. Prior to rhFVIII therapy anti-CHO protein Abs were rare in both PTPs and PUPs. New-onset or anamnestic immunoreactivity was significantly more frequent in PUPs than PTPs. Nevertheless, in all patients the temporal pattern of immunoreactivity was sporadic, and measured Ab levels were low. Importantly, the appearance of anti-CHO protein Abs bore no relationship to the occurrence of rhFVIII-related adverse events, which were in any case infrequent.

Anti-BSA and anti-MuIgG Abs were present prior to treatment in substantial percentages of PUPs (53.8% and 51.6%, respectively). This observation may in part reflect the pediatric composition of the PUP population, since such antibodies were also frequently detected in non-hemophilic pediatric subjects (71.0% and 53.0%, respectively). The genesis of these Abs is poorly understood but, since the non-hemophilic subjects were not exposed to rhFVIII, such immunoreactivity presumably can result from environmental exposure. With continuing environmental exposure over time tolerance may develop, so that by adulthood the prevalence of anti-BSA and anti-MuIgG Abs would be lower, as observed in the present study. In any case, the presence of Abs directed against these two heterologous proteins exhibited no direct relationship to cumulative rhFVIII exposure, except in the case of a single patient. Abs levels were generally low and highly variable, and no associated adverse events were encountered.

The present findings provide reassurance that enduring sensitization or anamnestic response to heterologous proteins resulting from long-term rhFVIII treatment is very infrequent. Even in ITI patients receiving high-dose rhFVIII regimens, evidence was lacking of a sustained immune response. The presence of anti-heterologous protein Abs can be demonstrated in both hemophilia A patients and non-hemophilic subjects. However, such Abs do not signal the development of therapy-related complications in patients.

The observed lack of abiding immune response likely reflects the low levels of heterologous proteins in rhFVIII. It is clear that, at least in the case of MuIgG, therapeutic doses can elicit Ab responses in a high proportion of patients. For instance, 86% of patients receiving 5 mg intravenous muromonab-CD3 daily for the treatment of acute renal allograft rejection developed IgG Abs against this murine MoAb. Muromonab-CD3 is also associated with a high incidence of serious adverse events (27).

The introduction of rhFVIII marked a major advance in the clinical management of hemophilia A, affording safe, efficacious therapy with...
negligible risk of viral transmission and without reliance on human plasma supplies. The present findings provide further support for the safety of rhFVIII during long-term administration. Trace amounts of heterologous proteins in rhFVIII appear to entail little risk of sustained immune responses or adverse events.

Appendix

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