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Effect of route of administration of human recombinant Factor VIII on its immunogenicity in Hemophilia A mice

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Abstract

Factor VIII is a multi-domain glycoprotein and is an essential cofactor in the blood coagulation cascade. Its deficiency or dysfunction causes Hemophilia A, a bleeding disorder. Replacement using exogenous recombinant Factor VIII (FVIII) is the first line of therapy for Hemophilia A. Immunogenicity, the development of binding (total) and neutralizing (inhibitory) antibody against administered protein is a clinical complication of the therapy. There are several product related factors such as presence of aggregates, route and frequency of administration and glycosylation have been shown to contribute to immunogenicity. The effect of route of administration of Factor VIII on antibody development in Hemophilia A is not completely understood. Here we investigated the effect of route of administration (sc or iv) on immunogenicity in Hemophilia A mice. The total and inhibitory titers were determined using ELISA and modified Bethesda Assay respectively. The results indicated that sc is more immunogenic compared to iv route in terms of total antibody titer development (binding antibodies) but no significant differences in inhibitory titer levels could be established.

Keywords

Hemophilia A; Inhibitor development; Immunogenicity; Route of administration

Introduction

Factor VIII is a glycoprotein comprised of six domains A1-A2-B-A3-C1-C2. The protein is secreted as a hetero-dimer made of heavy chain (A1-A2-B) and light chain (A3-C1-C2) and are held together by a divalent cation (1) (2,3). Factor VIII plays a central role in the coagulation cascade. Its deficiency or dysfunction causes bleeding disorder Hemophilia A. Replacement using recombinant full length Factor VIII or B-domain deleted Factor VIII is first line therapy for Hemophilia A. Immunogenicity i.e., development of inhibitory anti Factor VIII antibodies that abrogate the activity of the protein is a clinical complication in the management of the disease (4).

Immunogenicity is one of the important concerns that impact protein based therapeutics (3,5). The immune response to protein therapeutics leads to development of anti product antibodies often referred as binding (total) titers. Some responses are neutralizing antibody

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responses that can abrogate the activity of the protein and are considered inhibitory antibody. There are several product and process related factors that contribute to the development of total antibody responses. Presence of aggregates, route and frequency of administration and glycosylation that have been shown to contribute to immunogenicity (6). Recently, we investigated the presence of non-native aggregates of FVIII on eliciting immune response in Hemophilia A mice (7). However the effect of product related factors such as route of administration of recombinant Factor VIII on antibody development in Hemophilia A is not completely understood. It has been shown for human interferon alpha that protein administration via sc route elicits higher total antibody titers compared to protein given via iv route (6). In the present study, we investigated the effect of route of administration (sc vs iv) of rFVIII on immunogenicity in Hemophilia A mice. The results indicated that the sc route of administration is more immunogenic than iv route of administration in terms of total antibody development but no such effect could be established for inhibitory titers.

EXPERIMENTAL PROCEDURES

Materials

Recombinant full length Factor VIII expressed in the Chinese Hamster Ovary (CHO) cell line (Baxter Biosciences, Carlsbad, CA) was obtained as a gift from the Hemophilia Center of Western New York. B-domain deleted Factor VIII either was obtained as a gift from the Hemophilia Center of Western New York (Refacto -Wyeth, St Louis, MO) or purchased from American Diagnostica (Greenwich, CT). FVIII deficient plasma was purchased from Trinity Biotech (Co Wicklow, Ireland). Monoclonal antibodies ESH 4 and ESH 8 were obtained from American Diagnostica Inc. (Greenwich, CT). The activated partial thromboplastin time and Bethesda assays were performed using COAG-A-MATE coagulation analyzer (Organon Teknika Corp Durham, NC). Other buffer salts were purchased from Sigma (Saint Louis, MO) and used without further purification.

Immunogenicity studies

The relative immunogenicity of Factor VIII administered via sc and iv route was evaluated in Hemophilia A mice. Murine models are valuable tools to measure relative immunogenic responses. Since the immune response observed in Hemophilia A mice model is qualitatively similar to that observed in humans, this animal model has been routinely used to investigate relative immunogenicity of Factor VIII preparations (8,9). Immunization of 8-12 weeks old Hemophilia A mice bearing a targeted deletion in exon 16 of the Factor VIII gene consisted of four intravenous (i.v by penile vein) or subcutaneous (sc) injections of Factor VIII at weekly intervals. Blood samples were obtained at the end of the 6th week by cardiac puncture. Samples were collected in a 10:1(v/v) ratio of acid citrate dextrose (ACD) (85mM sodium citrate, 110mM D-glucose and 71mM citric acid). Plasma was separated by centrifugation and stored at -80°C until analysis. All studies were performed in accordance with the guidelines of Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo, The State University of New York.

Detection of Total Anti-Factor VIII Antibodies

Total anti-Factor VIII antibody titers were determined by ELISA as described previously (10). Briefly, Nunc-Maxisorb 96 well plates were coated with 50 µl of 2.5 µg/ml of Factor VIII in carbonate buffer (0.2 M, pH 9.4) and incubated at 4°C overnight. The plates were then washed six times with 100 µl of phosphate buffer (PB; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 14 mM NaCl, 2.7 mM KCl, pH 7.4) containing 0.05% Tween 20 (PBT). Nonspecific protein binding sites were blocked by incubation with 200 µl of PB buffer containing 1% bovine serum albumin (PBA) for 2 hours at room temperature. The plates

were then washed 6 times with PBT and 50 µl of serial dilutions of mouse plasma samples or standard solutions was added and incubated at 37°C for 1 hour. Standards consisted of 25 to 150 µg/ml of ESH8, a monoclonal murine anti-human Factor VIII antibody that binds to the C2 domain of Factor VIII. The plates were washed 6 times with PBT and incubated at room temperature for 1 h with 50 µl of a 1:1000 dilution of alkaline phosphatase/goat anti-mouse Immunoglobulin (Ig, IgM+IgG+IgA, H+L) conjugate in PBA. The plates were washed 6 times with PBT and 100 µl of 1 mg/ml p-nitrophenyl phosphate solution in diethanolamine buffer (consisting of 1M diethanolamine, 0.5 mM MgCl₂) was added. The plates were incubated at room temperature for 30 min and the reaction was quenched with the addition of 100 µl of 3 N NaOH. The alkaline phosphatase reaction product was determined by absorbance at 405 nm using a Spectramax plate reader (Molecular Devices Corporation, Sunnyvale, CA). Antibody titers were expressed as follows: linear regression was performed on the absorbance values obtained with ESH8. Half the difference between the maximum and minimum predicted absorbance was calculated as the plate specific factor (PSF), i.e., $PSF = \frac{1}{2} \times (M1 - M2)$ where; M1 = Maximum predicted OD of standard in the linear range and M2 = Minimum predicted OD of standard in the linear range. A linear regression of the plot of absorbance values of samples vs. log dilution (over the range of 1:100 to 1:40,000) was used to calculate the dilution that gave an optical density equal to the PSF. The dilution so obtained was considered the antibody titer of the sample.

Measurement of Inhibitory Anti-Factor VIII Antibodies Titers

Neutralizing (inhibitory) anti-Factor VIII antibodies were detected using the Nijmegen modification of the Bethesda assay (10,11). Plasma samples were diluted (1:8 to 1:16,000) and were mixed with normal human plasma. Residual Factor VIII activity was measured in duplicates, using the one stage aPTT assay. By definition, one Bethesda Unit (BU) is the neutralizing activity that produces 50% inhibition of the Factor VIII activity. The point of 50% inhibition was determined by linear regression of those data points falling within the range of approx. 20-80% inhibition. The titer values in Fig 1.b were expressed as BU/mL.

Statistical Analysis

P-value was determined by two-tailed t-test using Minitab15 software (Minitab Inc.) Retrospective power analysis and sample size determination was performed using SAS 9.1 analyst software (SAS Inc.)

RESULTS AND DISCUSSION

Product related factors of protein therapeutics contribute to its immunogenic response. The route of administration of the protein plays an important role in the elucidation of antibody response against administered protein (6,12). The effect of such product related factors on antibody development against Factor VIII was evaluated in Hemophilia A mice.

The total and inhibitory titers following iv and sc administration of full length Factor VIII is shown in Table 1. The high titer levels observed may be due to the administration of human protein in a non-tolerarized mice model. As is clear from the table, the total titers observed for animals that were given Factor VIII by sc route is higher than that observed for animals given protein by iv route. The total titer levels of 10978 ± 1153 (n = 31) were observed for animals given full length Factor VIII by sc route but it reduced to 4915 ± 723 (n = 13) for iv route of administration. Similar conclusion was drawn for other therapeutic proteins such as Interferon alpha and human growth hormone (6,13). It is possible that following sc administration of therapeutic proteins, proteolytic degradation at the site of injection may generate several epitopes and their presentation to more antigen presenting cells such as dendritic cells (DC) that are recruited at the site of injection contributes to the observed

increase in titer levels. Further, absorption of large proteins from the site of injection involves lymphatic uptake that may further increase its exposure to APC cells (14).

Based on the observed high titer levels of binding antibodies, it is anticipated that the induction of inhibitory or neutralizing antibody response will be higher for sc route of administration compared to iv administration. However, the inhibitory titer levels for both route of administration were comparable as the statistical significance between these two treatment groups could not be established. Inhibitory titers of 604 ± 66 ($n = 13$) were observed for full length Factor VIII given by sc route and 587 ± 54 ($n = 23$) was observed for animal given Factor VIII by iv route. Similar results were observed for B-domain deleted Factor VIII (data not shown). It is possible that the lower magnitude of immune response taken together with less number of animals used may not yield a meaningful statistical analysis (may take over 3400 mice to establish significance for inhibitory titers compared to 7 required for total titers Table 1). Nonetheless, much higher binding antibody titer levels observed for sc did not translate into induction of higher inhibitory titers. At this juncture, it is not clear whether this observation may be generalized across all types of immunogenic response including breaking of tolerance. It is appropriate to mention here that this observed immunogenic response is for a human protein that is a foreign antigen to mice and it may be relevant with the therapeutic use of Factor VIII. There are several possible explanations for this observation. (a) High binding antibody titers can facilitate the induction of inhibitory antibody titers by the mechanism of epitope spreading (15). The impact of epitope spreading in the induction of neutralizing (inhibitor) antibody development in Hemophilia A is not well understood. It is possible that the mechanism of epitope spreading and its efficiency may be different for these two routes of administration. For sc administration it may be delayed and inefficient compared to iv route of administration and may take longer exposure (than presented here) for the induction of neutralizing antibody response (15). (b) FVIII may be a weak antigen in eliciting neutralizing antibody response and the route of administration may not play a critical role (12) (c) It is possible that stability of Factor VIII in sc tissue may have partly contributed to this observation. The inhibitory antibodies may develop against conformational epitopes at the active site. The loss of such epitopes at the injection site due to enzymatic degradation of the protein may lead to inefficient processing and elicitation of neutralizing antibodies (15). Further investigations that could shed light on the mechanism of inhibitor development are certainly necessary.

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Abbreviations

rFVIII	recombinant human Factor VIII
sc	subcutaneous
iv	intravenous

Figure 1. Total and inhibitory antibody titers following sc and iv administration of full length factor VIII in hemophilia A mice.

Table 1
Effect of route of administration of recombinant Factor VIII on total and inhibitory titers in Hemophilia A mice

Titers	Group		n	Mean±S.E.M	p value	N (for Power 0.95)
Total	FL rFactor VIII	IV	13	4915 ± 723	< 0.01	7
		SC	31	10978 ± 1153		
Neutralizing	FL rFactor VIII	IV	13	587 ± 54	> 0.05 (0.84)	3421
		SC	23	604 ± 66		