Research Article

Role of Glycosylation in Conformational Stability, Activity, Macromolecular Interaction and Immunogenicity of Recombinant Human Factor VIII

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Abstract. Factor VIII (FVIII) is a multi-domain glycoprotein that is an essential cofactor in the blood coagulation cascade. Its deficiency or dysfunction causes hemophilia A, a bleeding disorder. Replacement using exogenous recombinant human factor VIII (rFVIII) is the first line of therapy for hemophilia A. The role of glycosylation on the activity, stability, protein–lipid interaction, and immunogenicity of FVIII is not known. In order to investigate the role of glycosylation, a deglycosylated form of FVIII was generated by enzymatic cleavage of carbohydrate chains. The biochemical properties of fully glycosylated and completely deglycosylated forms of rFVIII (degly rFVIII) were compared using enzyme-linked immunosorbent assay, size exclusion chromatography, and clotting activity studies. The biological activity of degly FVIII decreased in comparison to the fully glycosylated protein. The ability of degly rFVIII to interact with phosphatidylserine containing membranes was partly impaired. Data suggested that glycosylation significantly influences the stability and the biologically relevant macromolecular interactions of FVIII. The effect of glycosylation on immunogenicity was investigated in a murine model of hemophilia A. Studies showed that deletion of glycosylation did not increase immunogenicity.

KEY WORDS: factor VIII; glycosylation; hemophilia A; immunogenicity; inhibitor development.

INTRODUCTION

Human factor VIII (FVIII) is an essential cofactor in the blood coagulation cascade that anchors the activated factor IX and its substrate, factor X, on the surface of blood platelets (1). Synthesized as a single chain precursor, FVIII is subjected to limited proteolysis in the Golgi apparatus (2). It is secreted as a heterodimer consisting of a heavy (A1-A2-B) and a light (A3-C1-C2) chain (3). The presence of multiple proteolytic sites within the B domain leads to the generation

of heterogeneous FVIII compositions that contain heavy chains ranging from 90 to 210 kDa with a light chain 80 kDa (3,4). Apart from the limited proteolysis, FVIII is subjected to glycosylation (3). The native full length protein has 25 potential N-glycosylation sites, including 19 sites in the B-domain and three each in light (two in A1, one in A2) and heavy (two in A3, one in C1) chains (4). Of these, 21 sites were identified as carrying oligosaccharides with 16–17 potential glycosylation sites occupied in the B-domain (5,6). In spite of the presence of one Asn-Xxx-Ser/Thr consensus sequence for N-linked glycosylation, the A2 domain of FVIII does not carry any oligosaccharides. The A1 domain and the light chain carry two N-linked sugar chains each (4,5). FVIII binding to phosphatidylserine (PS)-containing platelet membrane surfaces is a critical step in the blood coagulation cascade and is necessary for the proper formation and anchoring of the tenase complex (7).

Hemophilia A is a bleeding disorder caused by the deficiency or dysfunction of factor VIII. Replacement using recombinant human factor VIII (rFVIII) is first-line therapy for hemophilia A. Immunogenicity, the development of antifactor VIII antibodies, is a clinical complication in the management of the disease. Immune response can be assessed as a measure of all the antibodies that bind to a protein (total) or only those antibodies to the protein that specifically abrogate activity (inhibitors). Inhibitory antibodies are routinely assessed in the clinic with between 15% and 30% of hemophilia A patients developing measurable levels of FVIII inhibitors during the course of treatment (8,9). Several product- and process-related factors have been shown

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ABBREVIATIONS: aPTT, Activated partial thromboplastin time; BPS, Brain phosphatidylserine; BSA, Bovine serum albumin; CHO, Chinese hamster ovarian cell line; DEA, Diethanolamine buffer; Degly FVIII, Deglycosylated recombinant human factor VIII; DMPC, Dimyristoylphosphatidylcholine; ELISA, Enzyme-linked immunosorbent assay; Endo, Endoglycosidase; FVIII, Factor VIII; PB, Phosphate buffer; PBA, Phosphate buffer with 1% bovine serum albumin; PBT, Tween 20 containing phosphate buffer; p-NPP, p-nitrophenylphosphate; PS, Phosphatidylserine; rFVIII, Recombinant human factor VIII; SEC, Size exclusion chromatography; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

to contribute to the development of total antibody responses—the presence of aggregates, route and frequency of administration, and glycosylation are a few such factors.

Glycosylation plays critical role in structure, function, and resulting immune response of several proteins (10–16). It has been reported that carbohydrates in glycoproteins are important for protein folding (12,13,17), stability (11,18), macromolecular interactions, and activity (19–21). Furthermore, alteration of glycosylation can affect the immunogenicity of therapeutic proteins as it has been demonstrated in the case of interferon beta (22,23). Such information is not presently available for rFVIII. In order to elucidate the role of glycosylation on rFVIII stability and immunogenicity, we generated a deglycosylated form of rFVIII (degly rFVIII) with enzymatic digestion and compared it with that of the fully glycosylated rFVIII.

Human and murine FVIII have high sequence homology with 74% overall homology and up to 84–93% in conserved regions (24). Murine models of hemophilia A mount an immune response to administered FVIII preparations that is qualitatively similar to humans, making them an ideal preclinical model for studying relative immunogenicity (25). We used this model to compare the immune response elicited by normal rFVIII to the deglycosylated form. Biochemical analysis were performed to assess the changes caused by deglycosylation in terms of aggregation and impairment of physiological function. The results indicated that deglycosylation resulted in loss of activity, impaired lipid interaction, and decreased stability, but it did not result in higher immunogenicity when compared to the glycosylated forms.

EXPERIMENTAL PROCEDURES

Materials

Recombinant full-length FVIII expressed in the Chinese Hamster Ovary cell line (Baxter Biosciences, Carlsbad, CA) was obtained as a gift from the Hemophilia Center of Western New York. B-domain deleted FVIII 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). The endoglycosidases endo F1, endo F2, and endo F3 were purchased from Sigma (St. Louis, MO). The monoclonal antibodies ESH4 and ESH8 were purchased from American Diagnostica, Inc. (Greenwich, CT). FVIII deficient plasma, normal coagulation control plasma, and activated partial thromboplastin time (aPTT) reagents were purchased from Trinity Biotech (Co Wicklow, Ireland). P-nitrophenylphosphate (p-NPP) substrate and diethanolamine buffer were purchased from KPL, Inc. (Gaithersburg, MD). Other buffer salts were purchased from Sigma (St. Louis, MO).

Deglycosylation of rFVIII

rFVIII was subjected to enzymatic deglycosylation by incubation with endo F1, endo F2, and endo F3. These endoglycosidases have been shown to cleave oligomannose bi and tri-antennary complex oligosaccharide structures from glycoproteins under native conditions (26,27). Complex tetra-antennary structures that are found in low frequency (one of

several potential sugars on only two residues the B domain of FVIII) are not cleaved by endoglycosidase Fs (5,6). The reactions were carried out at pH 6.5 or in the buffer provided by the manufacturer for 1 h at 37°C, if not stated otherwise. The extent of deglycosylation was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4–15% precast gel) stained with Coomassie blue. Cleaved sugar residues were not separated from solution, so for subsequent concentration calculations, it was assumed that the mass of the two glycoforms remained equal (Fig 1).

Activity of Factor VIII and Its Glycoforms

Biological activity of FVIII glycoforms was determined using a one-stage aPTT assay (28). Briefly, the samples (full-length or deglycosylated rFVIII) were mixed with FVIII-deficient plasma as substrate. The clotting time following addition of platelin L reagent and CaCl_2 was measured using a Coag-A-Mate XM coagulation analyzer (Organon Teknika Corporation, Durham, NC). The activity of different FVIII glycoforms were extrapolated from a standard curve generated using glycosylated rFVIII standards. Data were presented as relative specific activity—the specific activity of the glycoform normalized against the specific activity of glycosylated rFVIII standards (Sigma, St. Louis, MO). Each measurement was repeated on four samples ($n=4$).

Lipid-FVIII Interactions

The required amounts of dimyristoylphosphatidylcholine and brain phosphatidylserine were dissolved in chloroform. A thin lipid film was formed on the walls of a glass tube by removing the solvent in a Buchi-R200 rotoevaporator (Fisher Scientific). Multilamellar liposomes were prepared by rehydration of the lipid film with Tris buffer (25 mM Tris, 0.3 M NaCl, 5 mM CaCl_2 , pH=7.0) at 37°C (29). The liposomes were extruded eight times through double stacked 100 nm polycarbonate membranes using a high-pressure extruder (Lipex Biomembranes, Inc.) at a pressure of ~200 psi. The size distribution of the particles was monitored using a Nicomp model CW380 size analyzer (Particle Sizing System). Lipid recovery was estimated by assay of inorganic phospho-

Fig. 1. SDS-PAGE analysis of endoglycosidase digestion products of rFVIII. Lane 1 molecular weight standard; lane 2 full-length glycosylated rFVIII; lane 3 digestion of rFVIII with endoglycosidases endo F1, endo F2, and endo F3. Position of light and heavy chains are indicated by “LC” and “HC.” No bands were detected below 50 kDa

rous using the method of Bartlett (30). The lipid-rFVIII interactions were investigated by incubating each rFVIII glycoform in the presence of different concentrations and compositions of liposomes at 37°C for 30 min.

Sandwich Enzyme-Linked Immunosorbent Assay

The ability of FVIII glycoforms to bind to artificial lipidic membranes and the extent of those interactions were evaluated based on the ability of liposomes to compete with antibodies against the C2 domain of rFVIII in a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (31). Briefly, 96-well plates were coated overnight with ESH4 or ESH8 as capture antibodies, nonspecific binding was blocked with bovine serum albumin, liposomal-rFVIII complexes were incubated in the wells, a mixture of rat anti-human rFVIII polyclonal and goat anti-rat IgG-alkaline phosphatase-conjugated antibodies was added, and optical density of the p-NPP substrate was read at 405 nm with a SpectraMax plate reader (Molecular Device Corporation, Sunnyvale, CA). Washing with Tween 20 containing phosphate buffer was performed between each step.

High Performance Size Exclusion Chromatography

Size exclusion chromatography (SEC) experiments were carried out using a Biosep-SEC-S-4000 4.6×300-mm column (Phenomenex, Torrance, CA) maintained at a 30°C using a Shimadzu CTO-10A column oven (32). The chromatography system consisted of a Waters 510 HPLC pump, a Shimadzu SIL-10A autoinjector, equipped with a sample cooler unit, and two HPLC detectors connected in series (Shimadzu RF-10A XL fluorescence detector and Shimadzu SPD-10A UV detector). The chromatograms were recorded and data analyzed using a Shimadzu CR-8A integrator. All components, except for the HPLC pump, were connected to a Shimadzu SCL-10A controller. The experiments were carried out under isocratic conditions at 0.4 mL/min using Tris buffer. The time delay between the two detectors was ~0.08 min. Complete aggregation of the rFVIII glycoforms was achieved prior to injection by heating the sample at 80°C for 120 s. Samples were stored at 4°C on the sample cooler rack prior to injection onto the column. Excitation and emission wavelength for the fluorescence detector were set to 285 and 335 nm, respectively. The UV detector was set to monitor absorbance at 215 nm.

Murine Model of Hemophilia A

The relative immunogenicity of full-length and deglycosylated rFVIII was assessed in a murine model of hemophilia A. Immunization of 8–12 weeks old C57BL/6J mice bearing a targeted deletion in exon 16 of the FVIII gene consisted of four subcutaneous injections of factor VIII at weekly intervals. Each injection consisted of 2 µg of full length or degly rFVIII in 100 µL of Tris buffer. Blood samples were obtained at the beginning of the sixth week by cardiac puncture. Samples were added at a 10:1 (v/v) ratio to acid citrate dextrose (85 mM sodium citrate, 110 mM D-glucose, and 71 mM 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 at the University at Buffalo, The State University of New York.

Measurement of Inhibitory Anti-Factor VIII Antibodies Titers

Neutralizing (inhibitory) anti-FVIII antibodies were detected using the Nijmegen modification of the Bethesda assay (33). Plasma samples were diluted (1:8 to 1:32,000) in FVIII deficient plasma and mixed with normal human plasma. Residual FVIII activity following a 2-h incubation at 37°C 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 approximately 20% to 80% inhibition.

Statistical Analysis

Statistical analysis was carried out by one-way ANOVA, followed by Dunnett's post hoc analysis or student's *t* test using the Minitab 14 software application (Minitab Inc., State College, PA).

RESULTS

Generation and Characterization of Deglycosylated rFVIII

Degly FVIII was generated by digesting the oligosaccharides in the presence of endoglycosidases F1, F2, and F3 as previously reported for other glycoproteins (26,27). The digestion products, as well as the native rFVIII, were characterized by SDS-PAGE. Several bands were visible on the SDS-PAGE for the native full length rFVIII (Fig. 1). The 80-KDa polypeptide corresponds to the light chain. The several polypeptides with molecular weights ranging from 90 to 210 KDa were attributed to the heavy chain of the native rFVIII. The heterogeneity in the heavy chain is due to proteolytic processing of the B domain. A molecular weight shift was observed for lane 3 and was due to deletion of oligomannose residues and complex bi- and tri-antennary sugar structures. The largest molecular weight shifts were recorded for the highest molecular weight heavy chains that carry the maximum number of oligosaccharides (2,4,6).

Effect of Glycosylation on Biological Activity of FVIII

A one-stage aPTT activity measurement of fully glycosylated and degly rFVIII was performed to confirm retention of activity during the deglycosylation process. Deglycosylation of rFVIII with endoglycosidases resulted in substantial loss of activity showing only 62±13% activity compared to the native rFVIII at 94±14% (*p*<0.05). To account for the effect of traces of endoglycosidases present in the degly rFVIII preparations, the specific activity of rFVIII was measured immediately after addition of the endoglycosidases. The activity in the presence of Endo F1, F2, and F3 was found to be 91±8% (*p*>0.05 vs. control).

Table I. Lipid Binding Properties of rFVIII and Degly FVIII

Lipid identity	Lipid concentration (μmol/ml)	ESH4 % binding to rFVIII	ESH4 % binding to degly	ESH8 % binding to rFVIII	ESH8 % binding to degly
Free protein Phosphatidylserine	0.0	100.0±8.0	100.0±13.9	100.0±16.6	100.0±9.8
	0.1	35.2±2.2	80.0±20.1	—	—
	0.4	31.3±5.5	47.2±4.1	—	—
	0.8	24.5±3.5	40.2±1.5	53.2±3.4	62.8±7.1
	1.6	20.0±2.9	33.0±3.3	59.5±2.4	67.4±14.5
Phosphatidyl choline	0.1	50.7±1.1	64.9±3.0	—	—
	0.4	47.7±2.8	69.5±2.0	—	—
	0.8	49.0±2.2	63.1±2.0	63.8±6.2	59.3±5.6
	1.6	54.3±3.8	59.2±1.4	69.3±15.9	65.1±2.6

Effect of Glycosylation on Protein–Lipid Interaction

To assess the affinity and specificity of different rFVIII glycoforms for PS liposomes, antibody binding in the presence of increasing concentrations of liposomes was monitored. The mouse monoclonal antibody ESH4, which binds to the same lipid-binding region as PS (2,303–2,332), was used as a capture antibody while a rat polyclonal antibody with an alternative binding site was used as probe antibody (34). A larger concentration of PS liposomes required to disrupt ESH4 binding for degly rFVIII than needed for the full-length protein would indicate a decrease in PS affinity. To account for the differences in antibody binding between different FVIII glycoforms, the binding of ESH4 antibody in the absence of liposomes was normalized to 100% binding, and lipid concentration-dependent changes were monitored. As shown in Table I, for native rFVIII, presence of lipid as low as 0.1 μmol resulted in over 60% loss of binding to the antibody, indicating the high affinity of native rFVIII for PS membranes ($p < 0.01$, student's t test). In contrast, degly rFVIII did not show significant change in antibody binding in the presence of 0.1 μmol of lipids ($p > 0.05$), and although the larger concentrations of PS liposomes altered the binding of ESH4, the magnitude of the decrease was smaller than that observed for rFVIII. The results indicate that the affinity of rFVIII towards PS containing membranes is significantly decreased following deglycosylation.

Two control experiments were also carried out to investigate the specificity of the rFVIII glycoforms to PS-containing membranes. In the first control experiment, the liposome composition did not contain PS. In the absence of PS, the decrease in ESH4 percent binding was found to be independent of increasing liposome concentration for both full-length and degly rFVIII, suggesting a mechanism other than competitive inhibition and emphasizing the specificity of FVIII glycoforms to PS. In order to further investigate the involvement of lipid binding region on the liposome binding properties, ESH4 antibody was replaced with ESH8 directed against epitope region 2248–2285 as the capture antibody (34). Some nonspecific binding was observed for both full-length and degly rFVIII, but lipid concentration-dependent decrease was not observed indicating that epitope 2303–2332 not 2248–2285 is involved in membrane binding.

Thus, the sandwich ELISA experiments suggested that binding of degly FVIII to PS is decreased compared to glycosylated rFVIII, but the lipid dose-dependence indicated that binding of degly rFVIII to PS is specific.

Size Exclusion Chromatography Studies

Gel filtration was conducted to test the hypothesis that removal of the sugar chains from the surface of rFVIII led to partial aggregation. The use of SEC to characterize the aggregation behavior of rFVIII has been described before (32,35). Due to the heterogeneity of the native glycoform, rFVIII eluted as a broad peak with a retention time of ~7.11 min (Fig. 2). The aggregated rFVIII species generated by thermal stress eluted as a sharp peak in the excluded fraction (5.12 min). Incubation at such elevated temperatures was sufficient to aggregate the protein as judged by the complete disappearance of the native peak. For degly

Fig. 2. SEC profiles of FVIII glycoforms (in 300 mM NaCl, 25 mM Tris, 5 mM CaCl₂, pH 7.0) prior and after incubation at 80°C. The elution times are indicated on the x axis in minutes. The temperature of the column was maintained constant at 30°C during analysis

rFVIII, two distinct peaks were observed prior to exposure to thermal stress. The peak around 7.43–8.11 min is presumably the full-length monomeric protein while the other species eluting at 5.20 min is the aggregated species. An increase in elution time for the monomeric species of degly rFVIII compared to that observed for glycosylated rFVIII (from 7.11 to 7.43–8.11 min) would be expected from the reduction in the molecular weight following enzymatic removal of saccharides. The analysis of the chromatograms for degly FVIII revealed that deglycosylation under the current experimental conditions resulted in partial aggregation of degly FVIII (aggregates 5.20 and native 7.43–8.11 min). Peak area and height ratio analysis indicates that ~24% of the protein is aggregated.

It is possible that the fluorescence detection can confound the estimation of percent aggregated material in the sample

due to self-quenching of the intrinsic tryptophan fluorescence in the aggregated material. In order to rule out such possibility, data were also acquired using a second detector set to monitor UV absorbance at 215 nm (data not shown). Analysis of the peak area and height ratio of the native (7.51–8.24 min) and aggregated protein (5.27 min) confirmed an estimation of ~22% aggregated protein present for degly rFVIII. Further assessment indicated that the aggregation of degly rFVIII was a time-dependent process as prolonged incubation at room temperature resulted in an increase of aggregated species (~40%, data not shown).

Effect of Glycosylation on Immunogenicity

Deglycosylation of proteins has traditionally been associated with an increase in immune response caused by the

exposure of epitopes previously protected by polysaccharides (22,23). The relative immunogenicity of FVIII and degly FVIII was investigated in hemophilia A mice. This animal model, due to innate deficiency, represents a suitable animal model. Further, it has been shown that the immune response observed in this animal model is qualitatively similar to that observed in humans (36). However, there was no statistically significant difference observed in the formation of inhibitory antibodies between the glycoforms (Fig. 3). Animals receiving full-length FVIII animals developed an inhibitory response of 493 ± 108 BU while degly FVIII induced an inhibitory response of 220 ± 86 BU.

DISCUSSION

Deletion or substitutions of Asn residues from the N-linked consensus sequences Asn-Xxx-Ser/Thr of lysosomal enzymes (β -glucuronidase, α -galactosidase, heparan N-sulfatase) resulted in substantial loss of activity (37–39). Furthermore, it has been reported that ~90% of the activity of hCG was lost as a result of the deletion of the N-glycan linked to the α 52 Asn residues (40). Purohit *et al.* indicated that the α 52 oligosaccharide chain played a critical role in the conformational integrity of hCG rather than having a functional role in hormone–receptor interactions (40). In the case of rFVIII, deglycosylation of the full-length protein led to ~40% loss of protein activity. Due to the inability of the endoglycosidases to remove tetra-antennary structures, the possibility of incomplete deglycosylation cannot be ruled out. Given that ~80% of the N-linked glycosylation sites were distributed within the B domain and deletion of this domain led to a homogeneous protein with a high specific activity and stability, it is possible that glycans present in B domain may not be critical for the clotting activity and possibly stability of the protein (1,2,6). However, the B domain has been shown to be important for intracellular trafficking and binding to asialoglycoprotein receptor (4). Therefore, oligosaccharides attached to the A1 domain and the light chain (A3 and C1 domains) may be responsible for the observed loss of activity (Fig. 4).

Previously, it has been shown that rFVIII–membrane interactions, mediated through the lipid binding region 2303–

Fig. 3. Results of inhibitory titer analysis for varied glycoforms of rFVIII determined by the Nijmegen modification of the Bethesda assay (33). Dots reflect individual values with bars drawn through the mean

Fig. 4. The molecular model and glycosylation of heavy and light chain of B domain deleted rFVIII. The lipid binding region (residues 2303–2332, lower portion of image) was shown as balls and sticks. The Asn residues 42, 239 (A1 domain), 1810 (A3 domain), and 2118 (C1 domain) that carry N-linked oligosaccharides were shown as space-filled spheres with conclusively resolved sugars as attached sticks. The figure was reconstructed using Polyview-3D (<http://polyview.cchmc.org/polyview3d.html>) based on the structure of B Domain Deleted rFVIII resolved at 3.7 Å (PDB code: 2R7E) (47)

2332, were critical for the proper function of rFVIII (41). A reduction of affinity for PS in the platelet membrane could alter clotting activity. In addition, we have shown that small conformational changes in the lipid-binding region 2303–2332 were responsible for the initiation of the aggregation process (42). Based on these data, we speculate that the N-linked glycosylation proximal to this epitope may play a critical role in the observed loss of activity and aggregation (Fig. 4). It is possible that removal of N-glycan at positions N-1823 (A3 domain) and N-2131 (C1 domain) may alter the conformation of the light chain and this leads to aggregation of the protein.

The sandwich ELISA studies indicated that binding of ESH8 to epitope 2248–2285 was independent of the glycosylation state of the protein, indicating that this particular epitope or its structure is not altered by the deglycosylation process. In contrast, noticeable differences were found in the case of affinity to the ESH4 mouse monoclonal antibody directed against the lipid-binding domain. Since ESH4 is a conformation-dependent antibody (American Diagnostica-product insert), the results suggested that removal of sugar chains has an impact either on the conformation of lipid-binding region epitope 2303–2332 or on the accessibility of this epitope. Site-directed mutagenesis studies are warranted

to test the hypothesis that deletion of polysaccharides attached to the light chain of rFVIII lead to a decrease in protein stability and biological activity.

Conformational analysis of the aggregates indicated that the aggregates are nonnative in nature (data not shown), consistent with the behavior of several beta-sheet proteins (32,43). These observations, carried out in solution, corroborate with that obtained using SEC, thus, ruling out the possibility that sizing matrix influenced the aggregation state of the protein. Nonnative aggregation of α -chymotrypsinogen occurs through nucleation and growth with competing nucleus sizes and negative activation energies (44). The presence of aggregates in the degly samples may be responsible for the observed reduction in activity and protein-lipid interaction.

Inhibitory titers are routinely assessed in the clinic as a measure of immune response towards FVIII formulations. Immunogenicity studies showed that the deglycosylated form of rFVIII appears to elicit no greater of an immune response than the fully glycosylated form. This result is puzzling as it was anticipated that deglycosylated form will be more immunogenic as it is observed for other therapeutic proteins (22,23). While aggregation has traditionally associated with an increase in the immunogenicity of several therapeutic proteins, it has previously been shown that nonnative aggregation of rFVIII results in a less immunogenic form, possibly due to participation of CD4+ epitopes in aggregate formation that reduces its accessibility. However, the aggregated protein acted as a distinct antigen (45,46). We speculate that the formation of aggregates caused by the deglycosylation process may be of nonnative conformation, thus, failing to elicit an increased inhibitor response. This observation may be relevant only for hemophilia A therapy where the protein is administered in a nontolerized system.

In conclusion, the present study indicates that the removal of N-linked oligosaccharides resulted in limited aggregation and interference with relevant biological interactions leading to a substantial loss of specific activity. The deglycosylated protein does not seem to elicit more immunogenic response compared to the fully glycosylated protein.

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