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The Comparative Immunogenicity of Human and Porcine Factor VIII in Hemophilia A Mice

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Summary

Inhibitory antibodies to factor VIII (fVIII inhibitors) are the most significant complication in the management of hemophilia A. The immunogenicity of fVIII may be driven in part by structural determinants within the fVIII molecule itself. Regions of non-identity between human and porcine fVIII possibly could drive differential immune responses. The goal of this study was to compare the overall antibody response and levels of antibodies to the individual fVIII domains in naïve hemophilia A mice immunized with human or porcine fVIII. Hemophilia A mice were immunized with human or porcine fVIII using a protocol that mimics human clinical use. Inhibitor and total anti-fVIII antibody titers were measured and the domain-specificity of antibodies from 1759 anti-fVIII hybridomas was determined. The overall immunogenicity of human and porcine fVIII was similar but significant differences in domain recognition were discovered. Anti-A2 and anti-C2 antibodies constituted the majority of inhibitors in both the human and porcine fVIII groups, similar to inhibitors that develop in humans. The proportions of anti-A2 or anti-C2 antibodies were not significantly different between the two groups. However, the specific inhibitory activity of anti-A2 antibodies was higher in the human fVIII group. Additionally, proportion of anti-C1 antibodies was significantly higher in the human fVIII group. In contrast, anti-A3 antibodies were more common in the porcine fVIII group. The differential immune response to human and porcine fVIII suggests that it may be possible to reduce the immunogenicity of fVIII by mutagenesis of the A2, A3 and C1 domains.

Keywords

Factor VIII; hemophilia therapy; coagulation inhibitors

Introduction

Inhibitory antibodies (inhibitors) to factor VIII (fVIII) develop in approximately 30 percent of patients with moderate or severe hemophilia A (1;2),(3;4). Inhibitor development is considered the most significant complication in the management of hemophilia A. FVIII

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inhibitors also occur as autoantibodies in nonhemophiliacs, producing a condition called acquired hemophilia A, which is the most common autoimmune bleeding disorder involving the coagulation system. Human fVIII inhibitors consist of a polyclonal IgG population in which IgG₄ is disproportionately high relative to the normal IgG subclass distribution. Despite the different immunological settings in which they arise, most inhibitors in either hereditary or acquired hemophilia A bind the A2 and/or C2 regions within the A1-A2-B-ap-A3-C1-C2 domain sequence of fVIII (5). However, human inhibitors targeting the A1, ap, A3 and C1 domains also have been observed (5–11), suggesting a broad spectrum immune response that has not been well characterized.

Hemophilia A mice produced by targeted disruption of the fVIII gene (12) have been used as a model system to study the immunogenicity of human fVIII (13). After serial intravenous injections of human fVIII using a dose per body mass similar to that used in humans, hemophilia A mice develop high titer inhibitors (13–16). The inhibitor response in hemophilia A mice includes antibodies that recognize the immunodominant Arg484-Ile508 A2 epitope that is recognized by human hemophilia A patients (16), although the extent to which other B cell epitopes in the murine model and human hemophilia are similar is unknown. On the whole, hemophilia A mice appear to provide a reasonable model system to study the immunogenicity of fVIII and potentially provide a model system to dissect the polyclonal immune response using B cell hybridoma technology.

Porcine fVIII has been used to treat fVIII inhibitor patients whose antibodies cross-react poorly with porcine fVIII. A commercial plasma-derived porcine fVIII concentrate, Hyate:C, was used for approximately 20 years until it was withdrawn because of concerns over the presence of viruses in the porcine blood supply (17;18). Additionally, a recombinant B – domain deleted form of porcine fVIII has been evaluated in a phase II clinical trial (19). Hyate:C and recombinant B domain-deleted porcine fVIII produce inhibitory anti-porcine fVIII antibodies in hemophilia A mice that have been pre-sensitized to human fVIII (20). However, the immunogenicity of porcine fVIII has not been studied in naïve hemophilia A mice. The comparison of the immunogenicity of human and porcine fVIII potentially is important because there may be species-specific determinants of immunogenicity that are intrinsic to fVIII structure. In this study, hemophilia A mice received recombinant human or porcine B domain – deleted intravenously using body mass – adjusted doses similar to those used in humans. A domain-specific ELISA was used to assign domain specificity to anti-fVIII hybridoma antibodies (21), resulting in the characterization of the relative immunodominance of fVIII domains and domain/inhibitor associations. Significant differences in the immunogenicity of human and porcine fVIII were observed, which may have therapeutic implications in the management of hemophilia A.

Materials and Methods

Materials

Citrated human hemophilia A plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical, Inc. (Overland Park, KA). Polyoxyethylene sorbitan monooleate (Tween 80) was purchased from Pierce (Rockford, IL). Goat anti-mouse IgG-alkaline phosphatase conjugate and Alkaline Phosphatase Substrate Kit containing and *p*-nitrophenylphosphate were purchased from Bio-Rad Laboratories (Hercules, CA). Goat anti-mouse isotype and subclass specific antibodies were purchased from Southern Biotech (Birmingham, AL). SP Fast Flow-Sepharose was purchased from GE Healthcare Life Sciences (Piscataway, NJ). Recombinant full-length human fVIII and B-domain deleted (BDD) porcine fVIII were gifts from Baxter Biosciences (Duarte, CA) and Ipsen Pharmaceuticals (Milford, MA), respectively.

Expression and purification of recombinant human, porcine and hybrid human/porcine fVIII

The cDNAs encoding BDD forms of human and porcine fVIII were prepared as described previously (22). The BDD single porcine domain (SPD) hybrid human/porcine fVIII constructs HP1, HP46 and HP20 and the single human domain (SHD) hybrid human/porcine fVIII constructs HP42, HP48, HP51, HP52, HP53, HP54 have been described previously (21;23;24). The SPD constructs HP58, HP71 and HP72 were prepared using previously published procedures (9). Expression of stably integrated fVIII cDNAs from baby hamster kidney-derived cells and purification of recombinant fVIII constructs was performed as described previously (22).

Immunization of hemophilia A mice with BDD human and porcine fVIII

Preparations of purified BDD human or porcine fVIII were stored in 0.4 M NaCl, 20 mM HEPES, 5 mM CaCl₂, 0.01% Tween 80, pH 7.4 in small aliquots at -80 °C. Samples were diluted to 4 µg/mL in sterile normal saline immediately prior to injection. To compare plasma antibody responses to human and porcine fVIII, forty hemophilia A mice were randomized to two equal groups and were immunized as described previously (16). Mice received six tail vein injections of 10 µg/kg fVIII at 7 d intervals, followed by a final injection of 25 µg/kg fVIII two weeks after the sixth dose. Blood was collected by tail snip into 1/10 volume 3.8% trisodium citrate by terminal cardiac puncture three weeks after the final injection and plasma was prepared by centrifugation at 3000g for 15 min at 4° C. Separate groups of 7 and 8 mice were immunized with human or porcine fVIII, respectively for production of hybridomas.

Production of anti-fVIII B cell hybridomas

Three days after the last injection of fVIII, cell suspensions from individual spleens were fused with NS-1 myeloma cells and hybridomas were characterized as described previously (21). Approximately 300 anti-fVIII positive hybridomas were identified per spleen in the initial screen. A maximum of 192 positive hybridomas from each spleen was expanded, re-screened for anti-fVIII antibodies, and the resulting positives were subjected to domain mapping, Ig isotype/subclass determination, and fVIII inhibitor assays.

Purification of anti-fVIII MAbs

Anti-fVIII hybridomas were cloned by limiting dilution, expanded and secreted anti-fVIII MAbs were purified by SP-Sepharose chromatography as described previously (21). Antibodies were judged to be greater than 95% pure by SDS-PAGE analysis. IgG concentrations were estimated using an extinction coefficient at 280 nm of 1.4 (mg/mL)⁻¹cm⁻¹. Yields of purified IgG ranged from 0.4 to 4 mg per 50 mL of culture medium.

Bethesda assay for inhibitory anti-fVIII antibodies

fVIII inhibitor titers were measured by a modifications (20;21) of the Bethesda assay (25) in which human hemophilia A plasma was reconstituted with BDD human or BDD porcine fVIII to a final concentration of 0.8 – 1.2 units per mL. Residual fVIII activity was determined and compared to the control incubation, which was defined as 100% residual activity. One Bethesda unit (BU) per mL is defined as the dilution of inhibitor that produces 50% inhibition of fVIII activity using the published reference curve for values between 25% and 75% residual activity (25). The reported values represent the means of at least two separate 2 h, 37 °C incubations.

ELISA for anti-fVIII antibodies

Anti-fVIII antibodies were measured by direct ELISA using plates coated with human or porcine fVIII as described previously (26). Purified MAbs (2 µg/mL), dilutions of plasma, or undiluted hybridoma supernatants were incubated in wells for 1 h. After washing, bound antibodies were detected using alkaline phosphatase – conjugated goat anti-mouse IgG as the secondary antibody and *p*-nitrophenylphosphate as the chromogenic substrate. Plasma ELISA titers were determined by nonlinear least-squares fits of plots of A_{405} versus the reciprocal of the sample dilution to the four-parameter logistic equation. The ELISA titer was defined as the reciprocal of the plasma dilution that produced an A_{405} of 0.22 above the baseline on the fitted curve.

Anti-fVIII antibody isotype/subclass and domain-specificity

Anti-fVIII Ig isotype/subclass determinations were done by direct ELISA using goat anti-mouse isotype-specific and subclass-specific alkaline phosphatase conjugated antibodies as described previously (21). Domain-specific anti-human fVIII antibody mapping was carried out by direct ELISA on undiluted hybridoma supernatants in half-area 96-well plates using purified SHD hybrid human/porcine fVIII as described previously (21). Domain-specific anti-porcine fVIII antibody mapping was carried out similarly using purified SPD hybrid human/porcine fVIII.

Statistical analysis

Differences in the mean proportions of domain-specific hybridomas for groups of mice immunized with human or porcine fVIII were analyzed using a two-tailed Student's *t* test. A *P* value of less than 0.05 was considered statistically significant.

Results

Comparative immunogenicity of BDD human and porcine fVIII in hemophilia A mice

Hemophilia A mice were immunized with human or porcine fVIII (25 per group) using a dose per body mass and dosing frequency similar to that used in humans. The antibody response was measured by Bethesda assay and by ELISA using the isologous antigen (i.e., human or porcine fVIII depending on the immunogen). Porcine fVIII produced a strong immune response, with inhibitor titers and ELISA titers in most mice exceeding 100 Bethesda units per ml and 10^3 , respectively (Fig. 1). This response was similar to the response to human fVIII (Fig. 1), which previously has been shown to produce high-titer antibodies in this model (13;14;16;21).

Domain-specific ELISA for anti-human fVIII and anti-porcine fVIII antibodies

Active, recombinant BDD hybrid human/porcine fVIII molecules containing single human or porcine A1, A2, *ap*, A3, C1 or C2 domains (Fig. 2) were expressed in BHK-M cells and purified. The SHD or SPD hybrid fVIII molecules were coated onto microtiter plates to create a domain-specific ELISA. Results using SPD hybrids and anti-porcine fVIII MAbs are shown in Fig. 3 to illustrate the method. Clear domain-specific signals are present except for the rightmost column, which contains a MAb developed against porcine fVIII that cross-reacts with human fVIII, and thus cannot be interpreted.

Domain specificity of anti-fVIII B cell hybridomas

B cell hybridomas were produced from spleens obtained from 7 mice immunized with human fVIII and 8 mice immunized with porcine fVIII. The robust immune responses to human and porcine fVIII resulted in the production of an average of nearly 300 hybridomas per spleen. An average of 126 and 110 anti-human fVIII and anti-porcine fVIII hybridomas,

respectively, were subjected to domain mapping. Domain specificity was assigned to approximately 70% of the hybridomas. The remaining hybridomas were not evaluable largely due to cross-reactivity. Figure 4 shows the distribution of antibodies to the different domains of human and porcine fVIII.

Anti-C1 antibodies were significantly more frequent in the human fVIII group ($p = 0.03$). In contrast, anti-A3 antibodies were significantly more frequent in the porcine fVIII group ($p = 0.03$). Additionally, there was trend toward significantly more frequent anti-porcine fVIII A1 antibodies ($p = 0.06$). Anti-porcine fVIII antibodies with dual specificity for the A1 and A3 domains were identified, similar to results reported previously for hemophilia A mice immunized with human fVIII (21). However, anti-porcine A1/A3 antibodies were significantly less frequent than anti-human A1/A3 antibodies ($p = 0.04$). There was no significant difference between the human fVIII and porcine fVIII groups in the proportion of anti-A2 or anti-C2 antibodies. Anti-*ap* antibodies were not significantly different between the groups, although the statistical power was low due to the small number of positive samples.

Ig isotype/subclass determinations were made on the anti-human fVIII and anti-porcine fVIII hybridomas. IgG1 and IgG2a antibodies dominated the immune response, each representing 40 – 45 % of the total antibodies in both the human and porcine fVIII groups (data not shown), consistent with previous studies in the murine hemophilia A model (15;27–29). This pattern is characteristic of a combined T_H1 and T_H2 response ((30), review). There was no significant association between domain specificity and Ig subclass in mice immunized with human or porcine fVIII, indicating that helper T cell responses are not strongly polarized with respect to domain specificity. As is typical of murine (and human) immune responses, κ light chain antibodies were dominant, averaging 98% and 95% of antibodies in the human and porcine fVIII groups, respectively.

Inhibitory activity of domain-specific anti-fVIII antibodies

Hybridoma supernatants were analyzed for inhibitory activity using the Bethesda assay. The relationship between specific activity, BU/ELISA A_{405} , which provides a measure of inhibitory activity relative to the amount of bound antibody, and domain specificity, is shown in Fig. 5A. Inhibitory activity was detected in 77/144 (53%) and 70/119 (59%) of anti-human fVIII and anti-porcine fVIII antibodies, respectively. Most of the anti-A2 and anti-C2 antibodies were inhibitory. However, there were several anti-human A2 antibodies with no detectable inhibitory activity, suggesting the presence of at least one additional human fVIII A2 epitope in addition to previously described inhibitory Arg484-Ile508 epitope (21;31). Higher anti-A2 specific activities were seen in the human fVIII group than the porcine fVIII group. Inhibitory anti-A1 and anti-A3 antibodies were identified in both groups. Inhibitory anti-C1 antibodies were identified only in the human fVIII group. Most, but not all, of the anti-A1/A3 antibodies had no detectable inhibitory activity. Overall, the inhibitor response in both groups was dominated by anti-A2 and anti-C2 antibodies, which accounted for 56/77 (73%) and 49/70 (70%) of the detectable inhibitory antibodies in the human and porcine fVIII groups, respectively.

The analysis of inhibitory activity was extended by purifying 25 anti-human fVIII MAbs and 21 anti-porcine fVIII MAbs (Fig. 5B). The higher proportion of MAbs with detectable inhibitory activity compared to the hybridoma supernatants was due to the higher available concentrations of purified MAbs, which increases assay sensitivity. Inhibitory activity was detected in 72% and 67% of anti-human and anti-porcine MAbs, respectively. Consistent with the hybridoma supernatant results, most of the inhibitory MAbs had anti-A2 or anti-C2 specificities. Inhibitory antibodies with anti-A1 or anti-A3 specificity were less frequent in

the MAb sample than in the hybridoma supernatant sample, possibly due to the small sample number in the former group.

Discussion

Although intravenous injections of human and porcine fVIII produce similar inhibitor titers and total anti-fVIII antibodies in naïve hemophilia mice (Fig. 1), they differ significantly with respect to the immunogenicity at the domain level. The higher immune response to the human C1 domain (Fig. 4) suggests that substitution of the porcine C1 into human fVIII might produce a less immunogenic fVIII molecule. Additionally, although the overall immune response to the human and porcine fVIII A2 domains is similar (Fig. 4), further epitope mapping at the subdomain level may reveal differences that can be exploited therapeutically. This is suggested by the observation that anti-human A2 antibodies have greater inhibitory activity than anti-porcine A2 antibodies (Fig. 5A), which indicates that there are two or more A2 epitopes that are detected by the domain-specific ELISA. Consistent with this, we recently identified a human A2 inhibitor epitope that is distinct from the well-known A2 epitope that is bounded by residues Arg484-Ile508 (21).

The C2 domain, along with the A2 domain, represents the most immunogenic domains in human fVIII in inhibitor patients. The strong immunogenicity of the porcine C2 domain in hemophilia A mice (Figure 4) is disappointing because it indicates that simple porcine C2 domain substitution will not reduce the immunogenicity of fVIII. However, in this study we have produced a large number of anti-C2 antibodies that may reveal significant differences between human and porcine fVIII using high-resolution mapping methods. We recently used 56 anti-human C2 MAbs in a competition ELISA and site-directed C2 mutants to develop a comprehensive map of the human C2 epitope surface recognized by hemophilia A mice (32). In addition to previously described antibodies that block the phospholipid binding site of fVIII (33;34), “non-classical” antibodies were identified that inhibit the activation of fVIII by thrombin and factor Xa. These antibodies also are present in the plasmas of most human inhibitor patients (35). Similar mapping of anti-porcine C2 antibodies may reveal differences in epitope specificity that can be exploited therapeutically.

In the immunization protocol used in this and other studies (13;14;16), which mimics dose and dosing frequency of fVIII in humans, most mice develop high titer inhibitors, whereas the incidence of inhibitors in previously untreated hemophilia A patients is only ~30%. Additionally, most fVIII inhibitor patients do not develop high-titer inhibitors (greater than 10 BU/mL) (1). The fVIII inhibitor response in the murine hemophilia A model is dose-dependent in a therapeutically relevant range (20). Thus, although the reason for the robust immune response in hemophilia A mice is not known, it may simply indicate that doses used are supra-normal and expose the murine immune system to relatively higher concentrations of fVIII. The immune response in hemophilia A mice represents a strong signal that is useful for testing differences between treatment groups. A less immunogenic fVIII molecule might significantly reduce the inhibitor titer but not the incidence of inhibitors in the murine hemophilia A model, yet reduce the incidence of inhibitor development in previously untreated human hemophilia A patients.

What is known on this topic

- Inhibitory antibodies to factor VIII are the most significant complication in the management of hemophilia A.
- Porcine factor VIII has been used to treat inhibitor patients whose antibodies cross-react poorly with porcine factor VIII.

- Although the epitope specificity of anti-human factor VIII antibodies has been extensively studied, anti-porcine factor VIII antibodies have not been characterized.

What this paper adds

- A comprehensive analysis of the overall antibody response and levels of antibodies to the individual fVIII domains in naïve hemophilia A mice immunized with human or porcine fVIII was obtained.
- Although the overall immunogenicity of human and porcine fVIII was similar, significant differences in domain recognition were discovered.

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Figure 1. Anti-fVIII antibody response in hemophilia A mice immunized with BDD human fVIII or BDD porcine fVIII

EL6 hemophilia A mice were randomized to receive six weekly tail vein injections of 10 µg/kg BDD human fVIII (n = 20) or BDD porcine fVIII (n = 20), followed two weeks later by a final injection of 25 µg/kg. Plasma was obtained 14 d after the final injection and analyzed by Bethesda assay (A) and ELISA (B) against the isologous antigen (see “Materials and Methods”).

Figure 2. Hybrid human/porcine fVIII molecules

BDD fVIII molecules containing human (white) or porcine (gray) regions as single human domains or single porcine domains are shown. The linker region represents a short peptide containing a PACE/furin recognition sequence. FVIII domain boundaries are defined using human fVIII amino acid sequence numbering as follows: residues 1-372 (A1), 373-740 (A2), 1649-1689 (light chain activation peptide, *ap*), 1690-2019 (A3), 2020-2172 (C1) and 2173-2332 (C2) (36).

Figure 3. Domain-specificity of anti-porcine fVIII MAbs

Purified domain-specific anti-porcine fVIII MAbs were used as primary antibodies in a ELISA using plates coated with human fVIII, porcine fVIII, or SPD hybrid fVIII molecules. MAbs 4-3, 4-39, 4-23, 4-5, 4-1 or 4-63, which bind to the A1, A2, *ap*, A3, C1 or C2 domains, respectively, were added to wells in columns 1 through 6, respectively. Anti-porcine fVIII MAb 4-17, which cross-reacts with human fVIII, was added to wells in column 7. MAb binding was detected using goat anti-mouse IgG-alkaline phosphatase conjugate as secondary antibody and *p*-nitrophenylphosphate as substrate (see “Materials and Methods”).

Figure 4. Domain-specificity of anti-fVIII antibodies

Supernatants from B cell hybridomas obtained from hemophilia A mice immunized with human fVIII (open bars, $N = 7$) or porcine fVIII (gray bars, $N = 8$) were analyzed by domain-specific anti-fVIII ELISA (see “Materials and Methods”). Shown are the relative proportions of assignable domain specificities and the standard errors of the mean proportions. Asterisks represent statistically significant differences between groups.

Figure 5. Inhibitory activity of anti-fVIII antibodies

A. Supernatants from anti-human fVIII hybridomas (open circles) and anti-porcine fVIII hybridomas (gray circles) were analyzed for total anti-fVIII antibodies by ELISA and for inhibitory anti-fVIII antibodies by Bethesda assay (see “Materials and Methods”). The ordinate axis represents the ratio of the inhibitory activity (BU/ml) and ELISA reading (A₄₀₅/ml). Numbers below the data points represent the numbers of hybridomas with no detectable inhibitory activity (BU/A₄₀₅ < 0.4). Numbers above the data points represent the numbers of hybridomas with detectable inhibitory activity. B. Specific inhibitory activities of purified anti-human (open circles) and anti-porcine (gray circles) fVIII MAbs were determined using the Bethesda assay and the concentration of purified antibody.