Comparison of the immunogenicity of different therapeutic preparations of human factor VIII in the murine model of hemophilia A

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ABSTRACT

Von Willebrand factor (VWF) has been proposed to reduce the immunogenicity of therapeutic factor VIII (FVIII) in patients with hemophilia A. Using FVIII-deficient mice, we compared the immunogenicity of different preparations of plasma-derived (pd) and recombinant (r) FVIII. Treatment of mice with pdFVIII induced significantly lower titers of FVIII inhibitors, as measured by ELISA and *in vitro* coagulation assays, compared with rFVIII. Furthermore, pre-incubation of rFVIII with excess VWF significantly reduced rFVIII immunogenicity. Our data confirm that pdFVIII induces lower levels of inhibitors than rFVIII, and that VWF is an immuno-chaperone molecule for FVIII.

Key words: factor VIII, FVIII inhibitors, von Willebrand factor, animal model, hemophilia A

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Hemophilia A is a rare genetic hemorrhagic disorder that affects 1 in 5,000–10,000 males. It results from the absence of endogenous pro-coagulant factor VIII (FVIII).[1] The management of bleeding episodes involves the intravenous administration of therapeutic FVIII to restore normal hemostasis. Sources of therapeutic FVIII are either pools of plasma from healthy donors or recombinant molecules produced by genetic engineering. In up to 35% of cases, the administration of exogenous FVIII to patients with hemophilia A leads to the development of anti-FVIII alloantibodies that inhibit the pro-coagulant activity of FVIII.[2] The occurrence of anti-FVIII antibodies, therefore, prevents further use of FVIII and is a major therapeutic challenge. Several risk factors associated with the development of FVIII inhibitors have been identified.[3,4] In particular, VWF has been proposed as a key chaperon molecule in reducing the immunogenicity of therapeutic FVIII in patients with hemophilia A. There is some evidence to suggest that the incidence of inhibitor development is lower when patients are treated with VWF-containing plasma-derived FVIII (pdFVIII) than when recombinant products without VWF are used.[5] In fact, studies in a murine model of hemophilia A have indicated that the immunogenicity of FVIII is reduced in the presence of VWF.[6]

We used the well-established model of FVIII-deficient mice to compare the immunogenicity of therapeutic preparations of pdFVIII and recombinant FVIII (rFVIII) that were commercially available in France at the time of the study.

Design and Methods

Therapeutic FVIII preparations

Factane® (FAC, LFB, Les Ulis, France) was used as pdFVIII containing VWF (20 IU VWF:RCo/100 IU FVIII). rFVIII used in the study were Advate® (ADV, Baxter, Maurepas, France), Helixate® (HEL, Bayer Healthcare, Puteaux, France) and Refacto® (REF, B-domain deleted rFVIII, Wyeth, Paris-la-Défense, France). Each FVIII was resuspended to 50 IU/mL in its excipient and kept in aliquots at -20°C until use. Specific activities were confirmed using chromogenic assays (Dade-Behring). Wilfactin® (WIL, LFB) was used as a source of VWF. Wilfactin® contains residual quantities of FVIII (10 IU FVIII/100 IU VWF:RCo).

Mice

Mice were 7–10 week-old inbred 129/- (H-2D^b background) exon 16 FVIII-deficient males and females (a gift from Prof Kazazian, University of Pennsylvania School of Medicine, Philadelphia). Animals were handled in agreement with local ethical authorities (Comité régional d'éthique p3/2005/002). Mice were treated by retro-orbital intravenous injection of 5 IU/ml FVIII in PBS at day 0, 14, 21 and 28. Blood was drawn by retro-orbital bleeding 5 days after the administration of FVIII. Decomplemented plasma was kept at –20°C until use. Groups of 5-8 mice were used in each set of experiments.

Titration of anti-FVIII and anti-VWF IgG

ELISA plates (Nunc) were coated with rFVIII (2 µg/mL, Recombinate®) or with VWF (2 µg/mL, Wilfactin®) overnight at 4°C, and blocked with PBS-1% BSA. Serum dilutions were then incubated for 1 h at 37°C. Bound IgG was revealed using a HRP-coupled monoclonal anti-mouse IgG and substrate. The mouse monoclonal anti-FVIII IgG mAb6 (a gift from Prof. J.M. Saint-Remy, KUL, Belgium) and anti-VWF IgG Ac418 (a gift from Dr. J.P. Girma, INSERM U777, Bicêtre, France) were used as standards. Results are shown as optical densities in arbitrary units.

Titration of FVIII inhibitors

Decomplemented plasma was incubated with a standard human plasma (Dade-Behring) for 2 hrs. at 37°C. The residual pro-coagulant FVIII activity was measured using a chromogenic assay following the manufacturers' recommendations (Dade-Behring). Bethesda titers, expressed in Bethesda units (BU)/mL, were calculated as described.[7] Bethesda titers are defined as the reciprocal of the dilution of plasma that produces 50% residual FVIII activity.

Results and Discussion

FVIII-deficient mice were treated with 1 IU of different FVIII preparations, 4 times over a period of 5 weeks. Mice were bled 5 days after the last injection. Titers of anti-FVIII IgG were measured by ELISA. All the therapeutic FVIII preparations induced FVIII-specific IgG in all the mice (Figure 1A). Full-length rFVIII preparations (mean±SEM in arbitrary units: 1260±365 and 1283±368 for ADV and HEL respectively) induced titers of anti-FVIII IgG similar to that induced by B-domain-deleted rFVIII (2154±957 for REF). Anti-FVIII IgG titers were significantly lower in mice treated with pdFVIII (330±145 for FAC) compared with mice treated with rFVIII (p<0.055). PBS-treated mice used as a control group did not spontaneously develop anti-FVIII IgG. Given this, inhibitory titers measured in the plasma of pdFVIII-treated mice (193±80 BU/mL, Figure 1B) were lower than those measured in the plasma of HEL-treated mice

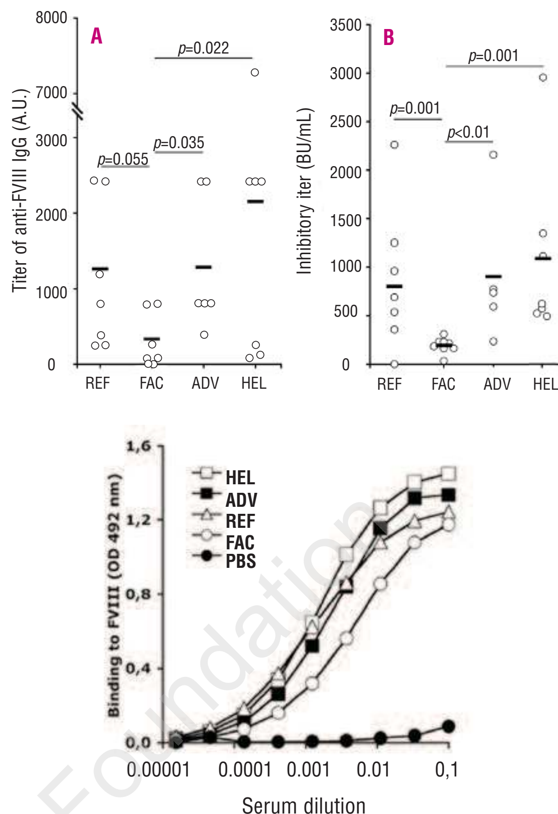

Figure 1. Anti-FVIII IgG in FVIII-deficient mice. FVIII-deficient mice were treated by intravenous administration of 1 IU human FVIII for therapeutic use. The FVIII preparations used were Helixate® (HEL), Advate® (ADV), Refacto® (REF), Factane® (FAC). Control mice were treated with PBS. After 4 administrations, mice were bled and the presence of anti-FVIII IgG in the serum was investigated by ELISA (panel A) or by chromogenic assay (panel B). Anti-FVIII IgG titers, defined as the inverse of the serum dilution yielding 50% of binding to FVIII, are shown on panel A for each mouse in arbitrary units (open circles). FVIII inhibitory titers, expressed as BU/ml (7), are shown on panel B (each mouse as an open circle). Means within groups of mice are shown as thick horizontal lines. The significance of the differences was assessed using the Mann-Whitney non-parametric test. Inset: raw data depicting the mean anti-FVIII IgG titers expressed for each group of mice in optical density (HEL: open squares, 6 mice; ADV: closed squares, 7 mice; REF: open triangles, 7 mice; FAC: open circles, 7 mice; PBS: closed circles, 6 mice). Data are from 1 of 2 independent experiments.

(1,089±886 BU/mL, p=0.001), ADV-treated mice (901±735 BU/mL, p<0.01) and REF-treated mice (802±704 BU/mL, p=0.001). Because the pdFVIII preparation used in the study contains VWF, we investigated the presence of anti-VWF IgG in the different groups of mice. As predicted, only pdFVIII-treated mice developed significant amounts of anti-VWF IgG, as assessed by ELISA (Figure 2).

We then investigated the protective effect conferred by VWF on the immunogenicity of FVIII in our experimental setup. rFVIII (ADV, 1 IU) was incubated alone or in the presence of 0.2 IU of pdVWF (WIL). Mice were then treated intravenously either with rFVIII alone, with rFVIII mixed with VWF, with VWF alone or with pdFVIII (FAC). Mice were bled 5 days after the fourth injection.

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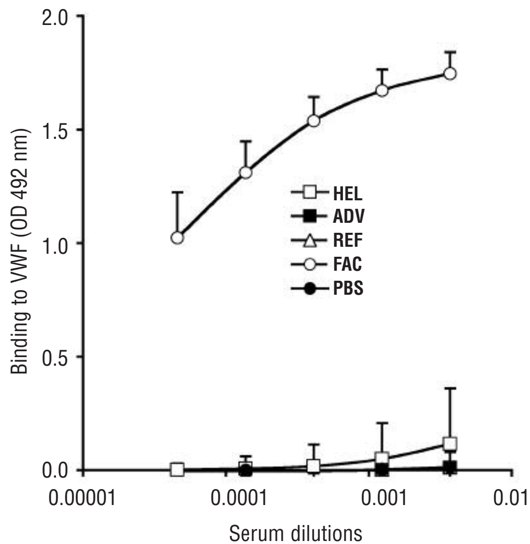

Figure 2. Titers of anti-VWF IgG in FVIII-deficient mice. FVIII-deficient mice were treated by intravenous administration of 1 IU human FVIII for therapeutic use, as explained in Figure 1. The FVIII preparations used were HEL (open squares, 8 mice), ADV (closed squares, 7 mice), REF (open triangles, 8 mice), FAC (open circles, 8 mice). Control mice were treated with PBS (closed circles, 5 mice). After 4 administrations, mice were bled. The presence of anti-VWF IgG was investigated in the plasma by ELISA. The data depict the IgG titers in arbitrary units (mean ± SD).

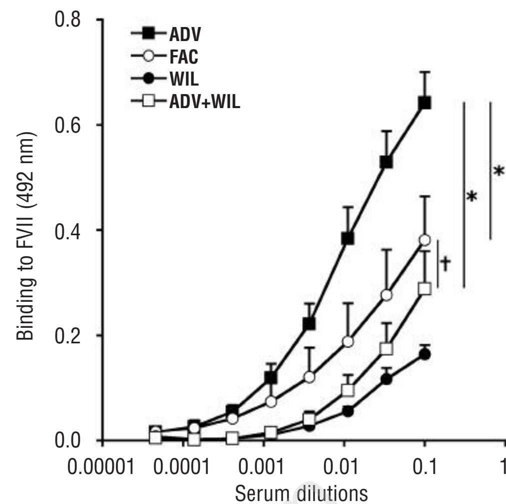

Figure 3. Protective effect of VWF. FVIII-deficient mice were treated by intravenous administration of 1 IU human FVIII. FVIII was injected alone (ADV: closed squares, 6 mice; FAC: open circles, 7 mice) or following 30 mins. pre-incubation in the presence of 0.2 IU pdVWF (ADV+WIL: open squares, 5 mice) at room temperature. Control mice received VWF alone (WIL: closed circles, 4 mice). After 4 administrations, mice were bled. The presence of anti-FVIII IgG was investigated in the plasma by ELISA. The data depict the IgG titers in arbitrary units (mean ± SEM). Differences were assessed using an ANOVA and the Fisher's PLSD post-hoc test (†: $p<0.01$; *: $p<0.0001$).

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Anti-FVIII IgG titers were measured by ELISA. A significant difference was found between the levels of anti-FVIII IgG induced by rFVIII and by pdFVIII ($p<0.0001$, Figure 3). Injection of rFVIII pre-incubated with VWF resulted in a significant reduction in the amounts of anti-FVIII IgG produced by the mice ($p<0.0001$). The occurrence of FVIII inhibitors upon intravenous administration of therapeutic FVIII to patients with hemophilia A remains the major treatment complication. Several risk factors favoring the development of FVIII inhibitors have been identified.[4] Among these, the nature of the therapeutic FVIII preparation has been highlighted. The relative risk of developing FVIII inhibitors when rFVIII is used, has therefore been estimated to be 2.4–3.2-fold greater than when pdFVIII is administered.[5] This study used the different therapeutic preparations of FVIII commercially available in France and confirmed that mice treated with rFVIII produce higher titers of anti-FVIII IgG than mice treated with pdFVIII.

The use of mice to study the immunogenicity of human proteins introduces a certain degree of bias and limitations. In particular, while only up to 35% of the patients develop FVIII inhibitors upon administration of FVIII, 100% of mice demonstrate an immune response to human FVIII. Similarly, the use of inbred mice with restricted MHC haplotype may limit the diversity of the anti-FVIII immune response. However, a recent study has reported an unrestricted repertoire of anti-FVIII IgG in H-2D^b FVIII-deficient mice.[8] In addition, while anti-VWF IgG have never been reported in patients treated with pdFVIII, we observed the development of VWF-specific IgG. This might be related to the sequence differences between the endogenous murine VWF and the exogenously administered human VWF (83% homology in amino acid sequences).

Three different therapeutic preparations were used to provide rFVIII: Helixate®, a full length FVIII molecule produced in baby hamster kidney (BHK) cell line; Advate®, a full length FVIII produced in Chinese hamster ovary (CHO) cell line; and Refacto®, a rFVIII, the B domain of which has been eliminated, produced in the CHO cell line. The three preparations of rFVIII induced similar levels of anti-FVIII IgG suggesting that the presence or absence of the B domain is not critical for the immunogenicity of FVIII in FVIII-deficient mice. Factane® was used as a source of pdFVIII. The administration of Factane® induced titers of anti-FVIII IgG that were 3.8–6.5- fold lower than that induced by the three rFVIII preparations. Therefore, the administration of pdFVIII induced lower titers of inhibitors than that of rFVIII.

A role for VWF in reducing FVIII immunogenicity has been previously suggested. The pre-incubation of rFVIII with VWF at molar ratios identical to that found in Factane® resulted in a significant decrease in the titers of anti-FVIII IgG induced by the intravenous administration of FVIII. This observation reflects previous findings in FVIII-deficient mice.[6] The VWF used in our studies is a plasma-derived preparation that contains trace amounts

of FVIII (0.05% on a mole-to-mole basis). Interestingly, the administration of pdVWF alone induced detectable levels of anti-FVIII IgG, indicating that a molar excess of VWF does not completely suppress FVIII immunogenicity. *In vitro* experiments have recently suggested that, unlike plasma-derived products, rFVIII concentrates derived from both CHO and BHK cell lines contain a fraction of FVIII:Ag molecules (approximately 20%) which is unable to associate with VWF.[9] Our results demonstrate that the *in vitro* pre-incubation of rFVIII with VWF reduced its immunogenicity to the level achieved with pdFVIII. These data suggest that the immunogenicity of rFVIII is not due exclusively to the reduced ability of rFVIII to bind to VWF, but that other mechanisms must be implicated. Molecular mechanisms that account for the protective effect of VWF on FVIII immunogenicity must still, therefore, be defined.

We have recently demonstrated that VWF protects in a dose-dependent manner FVIII from being endocytosed by human dendritic cells *in vitro*, thus reducing its presentation to cellular effectors of the immune system.[10] Whether similar mechanisms are pertinent *in vivo* remains to be confirmed. In contrast to previously published work,[5] a recent clinical study has reported that VWF-containing pdFVIII does not give a lower risk of developing inhibitory antibodies than rFVIII.[11] This discrepancy may be due to the heterogeneity of the different pdFVIII products used to treat the patients included in the study in relation to their VWF content. Indeed, the molar ratio of VWF to FVIII in the different plasma-derived FVIII preparations ranges from 0 in the case of Monoclate® or Hemophil-M® to 174:1 in the case of Haemate-P® (according to the manufacturers' communication).

Altogether, our data confirm that pdFVIII induces lower levels of anti-FVIII IgG than rFVIII, and confirm VWF as a key molecule in reducing the immunogenicity of therapeutic FVIII. The role of alternative immunomodulatory molecules such as transforming growth factor-beta 1 in reducing FVIII immunogenicity should, however, be considered.[12-14] As randomized clinical trials are difficult to implement in PUPs with hemophilia A, these results from comparative animal studies become particularly relevant.

Authors' contribution

SaD, SA, SuD, SC, ZT, SLD; performing the experiments : SaD, SuD, ANM, SVK, JB, SLD; analysis and interpretation of data : SaD, SA, ANM, SVK, JB, SLD; drafting the article: SuD, MHA, SVK, SLD; critical revision for important intellectual content : SA, MHA, SC, ZT; final approval of the version to be published: SaD, SA, SuD, ANM, SVK, MHA, SC, ZT, SL.

Conflicts of Interest

The authors reported no potential conflicts of interest.

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