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Oxidation of factor VIII increases its immunogenicity in mice with severe hemophilia A

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Abstract

The development of antibodies against therapeutic factor VIII (FVIII) represents the major complication of replacement therapy in patients with severe hemophilia A. Amongst the environmental risk factors that influence the anti-FVIII immune response, the presence of active bleeding or hemarthrosis has been evoked. Endothelium damage is typically associated with the release of oxidative compounds. Here, we addressed whether oxidation contributes to FVIII immunogenicity. The control with N-Acetyl cysteine of the oxidative status in FVIII-deficient mice, a model of severe hemophilia A, reduced the immune response to exogenous FVIII. *Ex vivo* exposure of therapeutic FVIII to HOCl induced a mild oxidation of the molecule as evidenced by the loss of free amines and resulted in increased FVIII immunogenicity *in vivo* when compared to native FVIII. The increased immunogenicity of oxidized FVIII was not reverted by treatment of mice with N-Acetyl cysteine, and did not implicate an increased maturation of professional antigen-presenting cells. Our data document that oxidation influences the immunogenicity of therapeutic FVIII.

Keywords: Hemophilia A, oxidative stress, FVIII immunogenicity, FVIII inhibitors

1. Introduction

The onset of an anti-factor VIII (FVIII) immune response, characterized by the development of antibodies targeting coagulation FVIII (FVIII inhibitors) represents the major complication of replacement therapy in patients with hemophilia A. While the pathogenesis of FVIII inhibitor development is not completely understood, several genetic and non-genetics risk factors have been suggested to affect the initiation of the anti-FVIII immune response [1]. Among the non-genetic risk factors, the presence of immuno-stimulatory events such as bleedings have been suggested to favor the development of FVIII inhibitors. In particular, an adjuvant effect of hemarthrosis on inhibitor development has been described in a rat model of severe hemophilia A [2]. While the conclusions of this study support that the presence of hemarthrosis at the time of FVIII infusion is associated with an increased incidence of inhibitor development, the underlying mechanisms are still unclear.

Bleeding episodes are consecutive to endothelium damage which leads to the release of several pro-inflammatory mediators, including reactive oxygen species (ROS), at the site of injury [3]. In addition to effects on immune and non-immune cells that are present in the vicinity of the bleeding site, ROS generate numerous chemical modifications on proteins (e.g., deamination, hydrolysis or oxidation), that influence their structure, function and immunogenicity [4–6]. Thus, Van Beers *et al.* demonstrated that the oxidative-dependent generation of aggregates of human interferon- β increases its immunogenicity, whereas the non-oxidative generation of aggregates does not [7]. Accordingly, the generation of aggregates involving a metal-catalyzed oxidation process increases the immunogenicity of interferon alpha2b [8]. Similar observations were generated in the case of model antigens that do not necessarily correspond to therapeutic proteins. In this case, the oxidative modification of type II collagen by hydroxyl radicals increases both its immunogenicity and

arthritogenicity in rats [9], and the oxidation of ovalbumin with HOCl increases its immunogenicity in OTII mice by facilitating endocytosis and processing [10].

Coagulation factors, including FVIII, are concentrated at the site of bleeding and are therefore likely to be exposed to pro-oxidative compounds. Moreover, FVIII is sensitive to oxidation [11] and the production of recombinant FVIII in oxygen free conditions preserves its pro-coagulant activity [12], probably by protecting FVIII from oxidative alterations. In the present study, we aimed at deciphering the participation of oxidative stress and the effect of oxidation on the immunogenicity of therapeutic FVIII.

2. Material and methods

2.1. Preparation of FVIII

FVIII (Helixate NexGen, CSL-Behring, Marburg, Germany) was dialyzed against HEPES buffer (150 mM NaCl, 10 mM HEPES, 2.5 mM CaCl₂ pH 7.4) for 2 hr at 4°C. FVIII was mixed with NaOCl (Sigma-Aldrich, Darmstadt, Germany) at various molar ratios and incubated at 4°C for 30 min. The NaOCl concentration was determined by absorbance measurement at 292 nm using a molar extinction coefficient of 350 M⁻¹ cm⁻¹. FVIII was then dialyzed against HEPES buffer for an additional 2 hr and concentration was determined by optical density at 280 nm. FVIII activity (FVIII:C) was determined using a chromogenic assay (Siemens, Marburg, Germany) using human plasma as standard. Loss of free amines was measured by fluorescamine fluorescence [13]. Briefly, 20 µl of native or HOCl-treated FVIII (1 µM) was added to 730 µl of borate buffer (200 mM, pH 8.5) and mixed while 250 µl of fluorescamine (20 µM) was added with vortexing. Following incubation at 25°C for 15 min, fluorescence was measured between 420 and 600 nm using an excitation wavelength of 390 nm. Carbonyl groups were quantified using a commercial ELISA kit (OxySelect Carbonyl ELISA).

2.2. Animals

Mice were 6- to 8-week-old C57Bl6/J-exon 16 knock-out mice. Animals were handled in agreement with local ethical authorities. Treatment with *N*-Acetyl-L-cystein (NAC, Sigma-Aldrich) was performed by dissolving NAC in drinking water at 142 g/l and changed every two days. ORAC values were determined in plasma, using a commercial kit and the antioxidant Trolox® as a standard (OxySelect™ ORAC Activity assay, CellBiolabs, Inc. San Diego, CA, USA). For FVIII treatment, mice were given 0.2 µg native or HOCl-treated FVIII intravenously in 200 µl PBS once a week for 4 weeks. Three days after the fourth administration of FVIII, blood was collected from the retro-orbital sinus. Serum was stored at -20°C until use.

2.3. Measurement of anti-FVIII IgG

ELISA plates were coated overnight at 4°C with recombinant FVIII (2 µg/ml, recombinant, Baxter, Maurepas, France). Plates were blocked with PBS 1% BSA. Serum in dilution was then incubated for 1 hr at 37°C. Bound IgG was revealed with a HRP-coupled monoclonal anti-mouse IgG antibody (Southern Biotech) and substrate. The mouse monoclonal anti-FVIII IgG mAb6 (a gift from Prof J. M. Saint-Remy, KUL, Belgium) was used as a standard.

2.4 Measurement of FVIII inhibitors

Mouse sera were decomplexed by incubation at 56°C for 30 min. Serial dilutions of decomplexed sera (ranging from 1:3 to 1:729) were incubated volume/volume with normal human plasma for 2 hours at 37°C. Residual FVIII activity was determined using a chromogenic assay. The serum dilution yielding 50% reduction of FVIII activity was used to represent the inhibitor titer and expressed in Bethesda units (BU)

2.5. Maturation of monocyte-derived dendritic cells (MO-DCs)

Monocytes from peripheral blood mononuclear cells of healthy donors were isolated using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) from heparinized buffy coats. To obtain immature monocyte-derived DCs, monocytes (0.5×10^6 cells per ml) were cultured in RPMI-1640 supplemented with penicillin/streptomycin and 10% FCS, in the presence of 2000 U/ml GM-CSF and 1000 U/ml IL-4 for 5 days. Five-days old immature MO-DCs (250,000/well in X-VIVO¹⁵) were seeded in 24-well plates and incubated with native or HOCl-treated FVIII (1 μ g) for 24 hr. LPS (1 μ g/ml) was used as a positive control. The expression of maturation markers was analyzed by flow cytometry using PE- or FITC-labeled antibodies against HLA-DR, CD40, CD80, CD86 and CD83 (all from BD bioscience).

2.6. Statistical analysis

All statistical analyses were performed using the GraphPad Prism 6.01 software (GraphPad Software, Inc, La Jolla, California, USA). Statistical significance was assessed using the non-parametric double-sided Mann-Whitney *U*-test or two-way ANOVA with Bonferroni post-test. Differences were considered significant when $p < 0.05$. ns: not significant.

3. Results and discussion

3.1. Treatment with N-acetyl cystein increases the anti-oxidant potential of plasma and decreases the immunogenicity of FVIII in FVIII-deficient mice.

The impact of bleeding on the immunogenicity of therapeutic FVIII has been the object of recent studies both in mice and in rats. While bleeding in mice was not associated with higher titers of anti-FVIII antibodies [14], recent study by Lovgren *et al.* reported an increased incidence of anti-FVIII IgG in a model of rats with hemophilia A [2]. In the present work, we

hypothesized that the release of ROS associated with vascular damages increases FVIII immunogenicity. To get an insight at the pro-oxidative status in FVIII-deficient mice, we first investigated the effect of the anti-oxidant compound, N-acetyl cysteine (NAC) *in vivo*. FVIII-deficient mice were fed with water supplemented with NAC for one week. The oxidant radical absorbance capacity (ORAC) was then determined on fresh plasma. As shown in figure 1A, supplementation of FVIII-deficient mice with NAC resulted in significantly increased ORAC values when compared to control mice. This demonstrates that treatment with NAC reduces the systemic oxidative status of hemophilic mice.

We then investigated the impact of the treatment with NAC on the development of the anti-FVIII immune response. Mice receiving or not NAC in drinking water were injected with FVIII once a week for four weeks. As depicted in figure 1B, the treatment with NAC significantly decreased the intensity of the anti-FVIII IgG titers. To further understand the role of oxidation on FVIII immunogenicity, we then addressed the effect of oxidation on FVIII.

3.2. Effects of hypochloric acid treatment on FVIII structure and activity

Several studies have described the deleterious effects of oxidation on protein activity, stability and immunogenicity. Hence, the oxidative modification of residues exposed at the surface of proteins (including amino-acid side chains and carbohydrates) generates neo-epitopes with a higher susceptibility to trigger activation of the immune system. Advanced oxidation protein products (AOPP) generated following exposure of albumin to HOCl were found to activate neutrophils and monocytes [15], and to potentiate the activation of T cells by AOPP-loaded dendritic cells [16]. Therefore, we first characterized the effect of mild oxidation by HOCl on FVIII structure and function. Increasing amounts of HOCl, ranging from 0 to 50 molar excess, were incubated with FVIII; unreacted HOCl was then removed by dialysis. The activity of FVIII was then assessed using a chromogenic assay. Incubation of FVIII with

HOCl resulted in a dose-dependent reduction of FVIII activity that reached 50% in the case of 50-fold molar excess of HOCl (Figure 2A). We then investigated the presence of oxidative modifications on FVIII following exposure to HOCl. Oxidation of amino groups by HOCl generally results in the progressive disappearance of free α and ϵ amino side chains, followed by the formation of aldehyde groups [17]. We determined the generation of carbonyl groups on HOCl-exposed FVIII, as compared to native FVIII. There was a marginal, yet not significant, appearance of carbonyl groups on the FVIII molecule (Figure 2B). We then determined the loss of free amines on FVIII following exposure to HOCl. Native or HOCl-exposed FVIII were incubated with fluorescamine, a compound that reacts with free amines. There was a dose-dependent and saturable reduction in the fluorescence emission spectrum of fluorescamine upon exposure of FVIII to HOCl (Figure 2C). Importantly, exposure of FVIII to up to 25-fold molar excess of HOCl did not alter the interaction of FVIII with von Willebrand factor (Figure 2D), and with a panel human and mouse monoclonal anti-FVIII antibodies (Figure 2E, F and data not shown).

3.3. Treatment of FVIII with HOCl increases its immunogenicity

The oxidation of self and foreign antigens has been repeatedly shown to increase antigen immunogenicity [7,8,18]. In order to determine the importance of oxidative alterations of FVIII, we exposed human recombinant FVIII to an excess of HOCl *in vitro* and administered FVIII to FVIII-deficient mice. Three days after the fourth injection of FVIII, the anti-FVIII IgG titers were 1.7-fold higher in mice that received FVIII exposed to a 25-fold molar excess of HOCl (1272 ± 127 $\mu\text{g/ml}$) as compared to mice treated with native FVIII (716 ± 97 $\mu\text{g/ml}$, $P < 0.001$, Figure 3A). Similarly, FVIII inhibitory titers were 1.8-fold higher in mice treated with FVIII exposed to 25-fold molar excess of HOCl (475 ± 159 Bethesda units) as compared to mice treated with native FVIII (262 ± 99 Bethesda units, $P < 0.05$, Figure 3B)

Since the intensity of the anti-FVIII immune response *in vivo* was decreased upon treatment with NAC, we wondered if supplementation of mice with NAC could also prevent the increase anti-FVIII IgG titer observed in the case of FVIII exposed to HOCl. As depicted in figure 3A and B, treatment of FVIII-deficient mice with NAC was ineffective in decreasing the intensity of the anti-FVIII immune response in the case of pre-oxidized FVIII. This suggests that the *in situ* oxidation of exogenously administered FVIII by the oxidative environment contributes to the immunogenicity of FVIII in FVIII-deficient mice, although this remains to be directly confirmed. Because the mild oxidation of FVIII induced upon exposure to HOCl results in an increased immunogenicity, we investigated whether FVIII oxidation has an impact on innate immune cells *in vitro*. To investigate a potential effect of oxidized FVIII on the maturation state of antigen-presenting cells, we used human MO-DCs from healthy donors. Five-day-old immature MO-DCs were incubated alone or with 1 μ g of FVIII exposed or not to 25 molar excess of HOCl. As depicted in figure 3C, the exposure of FVIII to HOCl did not alter the maturation profile of MO-DCs: indeed, neither the mean fluorescence intensities of HLA-DR, CD40 and CD80, nor the percentage of cells expressing CD83 and CD86 were modified. In contrast, LPS used as a positive control induced the maturation of MO-DCs, as indicated by the increase in MFI and % expression of the different maturation markers. Similarly, there was no change in the secretion profile of the cytokines IL-6, IL-8 and IL-10 (data not shown). This could be explained by the relatively low concentration of FVIII employed in our experimental setup that may not be sufficient to induce the maturation of dendritic cells, as compared to the high concentrations of albumin used in previous studies.

4. Conclusion

Taken together, our work describes the consequences of FVIII oxidation on its immunogenicity in FVIII-deficient mice. It also identifies the relevance of the systemic oxidative status as a risk factor for immunization against therapeutic FVIII. Oxidative modification of carbohydrate has been described to generate advanced glycation end-products (AGEs) that alter protein immunogenicity. Engagement of the receptor for AGEs (RAGE) was found to increase the secretion of pro-inflammatory cytokines such as IL-2 and TNF- α in monocytes [19]. In line with this, albumin containing AGEs was found to trigger a pro-inflammatory signaling cascade in RAW macrophages [20]. Whether such adducts originating from carbohydrates, are present on the FVIII molecule and whether they participate in triggering the anti-FVIII immune response remain to be determined.

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Author contributions

IP and SLD planned the work. IP, JDD and SD performed experiments. IP, JDD, BG, SD, AS and SLD analyzed the data. IP, SVK and SLD wrote the paper. SLD was the principal investigator and takes primary responsibility for the paper

The authors declare no conflict of interest

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Legend to Figure

Figure 1. Control of the oxidative status in FVIII-deficient mice reduces the immune response to FVIII. **Panel A.** FVIII-deficient mice (HemB6) were fed for one week with normal water or water supplemented with 0.142 g/l of *N*-Acetyl-L-cystein (HemB6+NAC). The oxidant radical absorbance capacity (ORAC) values were determined in the plasma using a commercial kit and the anti-oxidant Trolox® as a standard. ORAC values of individual mice are expressed as “ μM Trolox-equivalent”. **Panel B and C.** HemB6 mice were fed with NAC-containing or normal drinking water. One week after start of NAC feeding, mice received 1 IU FVIII (Helixate®, CSL-Behring, Marburg) intravenously once a week for four weeks. Three days after the fourth administration of FVIII, anti-FVIII IgG titers were determined in plasma by ELISA, using the monoclonal antibody mAb6 as a standard (Panel B). In parallel, inhibitor titers were determined using the Bethesda assay (Panel C). Symbols in panels A, B and C represent individual mice. Horizontal bars depict means \pm SEM. Statistical differences were assessed using the double-sided non-parametric Mann-Whitney *U*-test (* $p < 0.05$, ** $p < 0.01$).

Figure 2. Modification of FVIII by HOCl. **Panel A.** Exposure of FVIII with HOCl reduces FVIII pro-coagulant activity. FVIII (1 μM) was exposed to increasing amounts of HOCl for 30 min at 4°C and the residual FVIII activity was determined using a chromogenic assay. Results are depicted as mean \pm SEM and are representative of 7 independent experiments. **Panel B.** Detection of carbonyl groups was performed by ELISA. Results are depicted as fold increase in carbonyl content as compared to unexposed FVIII. Data are depicted as mean \pm SEM of 5 independent experiments. **Panel C.** Analysis of the loss of free amine groups by fluorescamine binding. Following incubation of FVIII alone or in the presence of HOCl, FVIII (20 nM) was incubated with fluorescamine (5 μM) in borate buffer. The emission of

fluorescence was recorded between 420 and 600 nm after excitation at 390 nm. The emitted fluorescence is expressed as arbitrary units (A.U.). The inset depicts the results obtained from 3 independent experiments (mean±SEM). Statistical comparison on panels A and B were performed using the double-sided non-parametric Mann-Whitney *U*-test (***) $p < 0.001$) between the conditions with 0 and 50 μM HOCl.

Figure 3. Exposure of FVIII to HOCl increases the immunogenicity of FVIII *in vivo*.

Panel A and B. Native FVIII or FVIII exposed to 25-fold excess of HOCl (FVIII_{HOCl}, 0.2 μg) were administered intravenously once a week for four weeks to FVIII-deficient mice. Three days after the last administration, blood was collected from the retro-orbital sinus and anti-FVIII IgG titres were determined in the serum by ELISA (Panel A) and using the Bethesda assay (Panel B). Results represent mean±SEM from 8 to 34 mice per group. The statistical significance of differences between groups was assessed using a one-way ANOVA with Dunn's multiple comparison test (**: $p < 0.01$). HOCl-exposed FVIII was devoid of endotoxin as assessed using the LAL assay. **Panel C.** Five-day old immature human MO-DCs from three individual donors were incubated alone or in the presence of native or FVIII exposed to 25-fold excess of HOCl (FVIII_{HOCl}, 1 μg) for 24 hours. Up-regulation of co-stimulatory molecules was determined by flow cytometry.

Figure 1

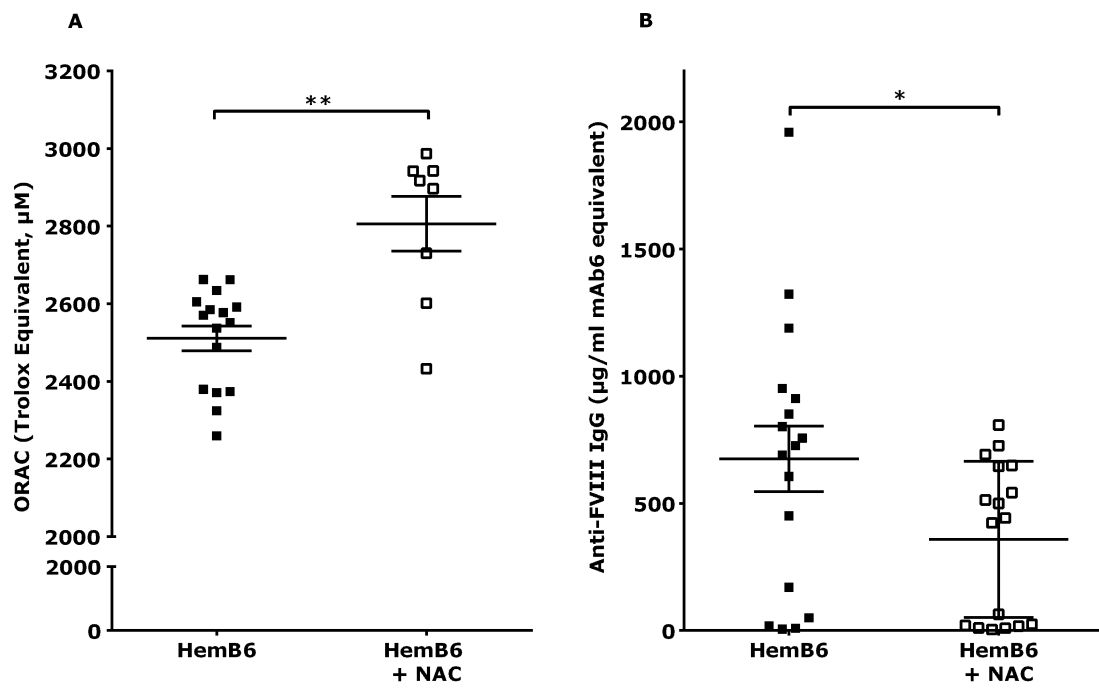


Figure 2

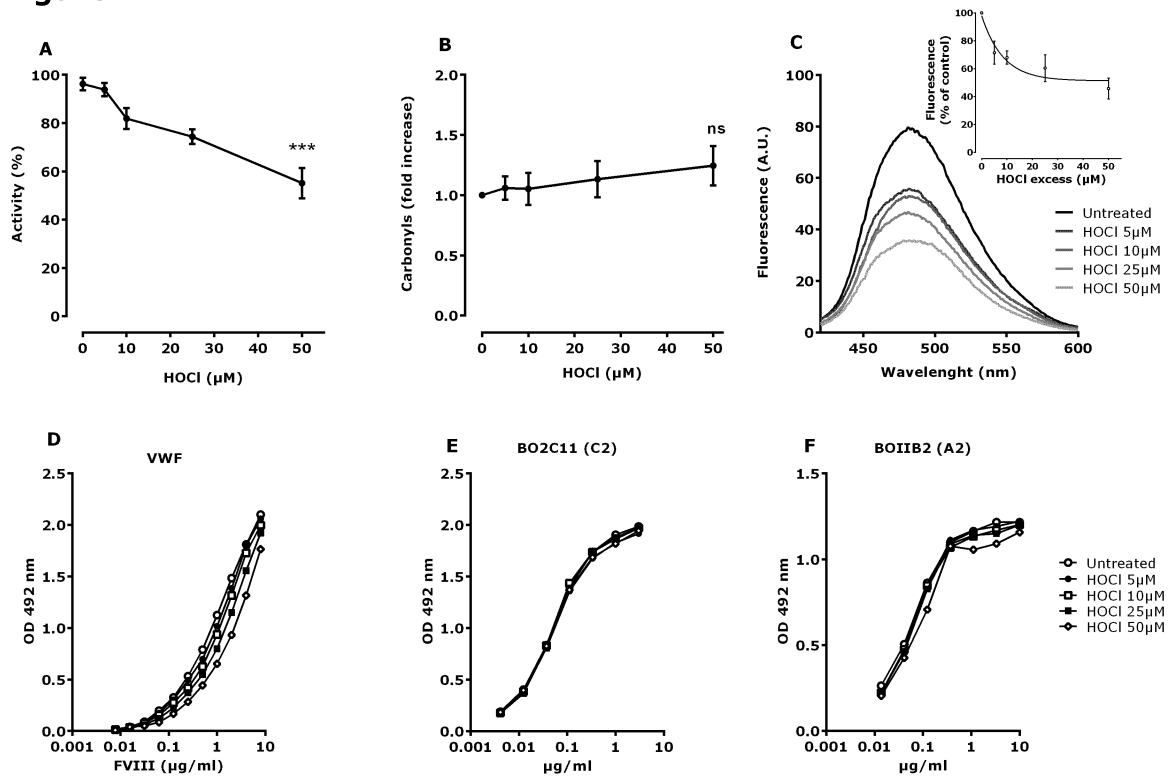


Figure 3

