Biochemical characterization and immunogenicity of Neureight, a recombinant full-length factor VIII produced by fed-batch process in disposable bioreactors

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A B S T R A C T

Hemophilia A is a X-linked recessive bleeding disorder consecutive to the lack of circulating pro-coagulant factor VIII (FVIII). The most efficient strategy to treat or prevent bleeding in patients with hemophilia A relies on replacement therapy using exogenous FVIII. Commercially available recombinant FVIII are produced using an expensive perfusion technology in stainless steel fermenters. A fed-batch fermentation technology was recently developed to produce 'Neureight', a full-length recombinant human FVIII, in Chinese hamster ovary (CHO) cells. Here, we investigated the structural and functional integrity and lack of increased immunogenicity of Neureight, as compared to two commercially available full-length FVIII products, Helixate and Advate, produced in baby hamster kidney or CHO cells, respectively. Our results demonstrate the purity, stability and functional integrity of Neureight with a standard specific activity of 4235 ± 556 IU/mg. The glycosylation and sulfation profiles of Neureight were similar to that of Advate, with the absence of the antigenic carbohydrate epitopes α-Gal and Neu5Gc, and with sulfation of Y1680, that is critical for FVIII binding to von Willebrand factor (VWF). The endocytosis of Neureight by human immature dendritic cells was inhibited by VWF, and its half-life in FVIII-deficient mice was similar to that of Advate, confirming unaltered binding to VWF. *In vitro* and *in vivo* assays indicated a similar immunogenicity for Neureight, Advate and Helixate. In conclusion, the production of full-length FVIII in a fed-batch fermentation mode generates a product that presents similar biochemical, functional and immunogenic properties as products developed using the classical perfusion technology.

1. Introduction

Hemophilia A is a X-linked recessive bleeding disorder consecutive to the lack of circulating pro-coagulant factor VIII (FVIII) [1]. Treatment and prevention of bleedings in patients with hemophilia A rely on replacement therapy using either plasma-derived or recombinant FVIII [2]. FVIII is among the most expensive protein therapeutics on the market owing to the low yields of production of the glycoprotein in the case of recombinant products and to the different steps required to eliminate potential viral agents [3]. The poor stability of FVIII [4,5] is an additional constraint that participates in increase of production costs. Production processes for recombinant FVIII (both full-length or B domain-deleted) are all based on expensive perfusion technology in stainless steel fermenters [6–8]. The use of continuous perfusion technology allows the growth of FVIII-producing cells to high concentrations while ensuring a relatively short protein residence time [9,10]. Shorter residence times were proposed to allow efficient production and less disruption of the recombinant FVIII (rFVIII) protein [11], thus minimizing the generation of potentially immunogenic FVIII degradation products. To address the need for better supply of affordable rFVIII at global level, efforts have focused on cell line development, process improvements and optimization of expression rates in commercial mammalian cell culture processes. Nevertheless, expression does typically not exceed 0.1 mg FVIII/L cell culture supernatant for a full-length FVIII [12].

We recently developed a fed-batch fermentation technology based on disposable fermentation technology to produce a full-length recombinant human FVIII. Compared to perfusion technology, fed-batch

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processes have significant advantages, such as ease of process validation and characterization, better lot definition and consistency, ease of downstream processing, overall reduced time to product approval, reduced medium consumption, reduced waste generation, less manpower requirements [9,13], easier technology transferability and less automation requirements. Current state-of-the-art single-use disposable technologies have been recognized as an increasing trend in biopharmaceutical manufacturing due to several advantages like reduction of the risk for cross-contamination, elimination of cleaning steps and an increase in flexibility, ease for handling and an estimated reduction in costs between 20 and 40% [14–16]. In the present work, the new full-length FVIII, referred to as Neureight, was compared to available full-length recombinant products, and the immunogenicity was assessed in *in vitro* and *in vivo* assays.

2. Material and methods

2.1. Sources of FVIII and VWF

Neureight is a recombinant full-length FVIII produced in eukaryotic CHO cells using a fed-batch process in a disposable setting in the absence of any stabilizing agents such as of VWF or human serum albumin. The purification of Neureight involves a step of affinity chromatography using the VIIIselect matrix (GE Healthcare, Piscataway, NI, USA) followed by traditional ion exchange, a hydrophobic interaction chromatography steps and virus reduction steps such as detergent treatment and nanofiltration. Helixate and Advate were purchased from CSL-Behring (Marburg, Germany) and Shire (Vienna, Austria), respectively. For *in vitro* studies, the three full-length FVIII were reconstituted in their respective excipients and dialyzed against DMEM-F12 for 2 h at 4 °C. FVIII were then aliquoted and stored at −80 °C until use. FVIII:Ag and FVIII:C in aliquots were assessed by Asserachrom (Stago, Asnières sur Seine, France) and by chromogenic assay (Dade-Behring, Marburg, Germany), respectively. Total protein content was measured by Bradford using bovine serum albumin as a standard. FVIII levels are indicated as International Units (IU) per ml based on the FVIII:Ag levels. Wilfactin (LFB, Les Ulis, France) was used a source of VWF. It was dialyzed in DMEM-F12 during 2 h at 4 °C, and aliquoted and stored at −80 °C.

2.2. Electrophoretic and Western blot analyses

SDS-PAGE was performed using 4–12% Bis-TrisNuPage gels (Life Technologies, Carlsbad, California, USA) followed by a staining with a Silver Express Stain Kit (Life Technologies). Western blotting was carried out using an anti-FVIII antibody GMA-012 (Green Mountain Antibodies, Burlington, VT, USA) which recognizes the A2 domain epitopes at residues 497–510 and 584–593, followed by an alkaline phosphatase conjugated anti-mouse antibody (Sigma-Aldrich Chemie GmbH, Munich, Germany).

2.3. Glycan profiling

MALDI-TOF MS analysis of N-glycans was performed after deglycosylation and derivatization of the separated and purified N-glycans. The permethylated glycans were further purified by a C18 cartridge and lyophilized, solubilized with 50% v/v methanol:water and mixed with MALDI mass spectrometry matrix before analysis. Positive ion reflectron MALDI mass spectra were acquired on a VOYAGER DE PRO (AB Sciex). Relative intensities (%) of N-glycans were determined based on the peak heights of the corresponding deisotoped monoisotopic mass ions. Interpretation of glycan structures corresponding to monoisotopic masses was performed using ExPASyGlycoMod tool and GlycoWork Bench.

2.4. Identification and assignment of sulfation sites, glycosylation sites and glycopeptides

Tryptic digestion and detection of the sulfo- and glycopeptides by MS and tandem MS (MS/MS) was performed to elucidate the sulfation and glycosylation sites and the corresponding glycans. The yielded tryptic glycopeptides were purified and enriched for the respective analysis by MALDI-TOF MS where positive ion linear MALDI mass spectra were acquired in different mass ranges and with multiple acquisition conditions on a MALDI-TOF/TOF Autoflex III spectrometer (Bruker Daltonics). Further, the separated glycopeptide-enriched fractions were deglycosylated, and the yielded peptides were also analyzed by MALDI-TOF MS where positive ion reflectron and linear MALDI mass spectra were acquired in different mass ranges and with multiple acquisition conditions on a MALDI-TOF/TOF Autoflex III spectrometer (Bruker Daltonics). This was done for determining which of the theoretical N-glycosylation sites are actually occupied with N-glycans.

For identification of sulfation sites, the obtained deglycosylated peptides were also injected on a nano-HPLC system coupled to an ion trap nano-electrospray mass spectrometer (LTQvelos, ThermoScientific). Acquisition of mass data was performed in a mass range of 300 to 2000 Da excluding monocharged ions. Proteome Discoverer ThermoElectron (v: 1.4) was used to analyze acquired LC-MS/MS data files.

2.5. Preparation of monocyte-derived dendritic cells

Blood from healthy donors was anonymously obtained from Etablissement Français du Sang Ile-de-France (Ivry-sur-Seine, France). Monocytes from peripheral blood mononuclear cells were isolated using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) from heparinized buffy coats of healthy donors. Purified monocytes were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, L-glutamine and antibiotics in the presence of 500 IU/10^6 cells of IL-4 (R&D Systems, Lille, France) and 1000 IU/10^6 cells GM-CSF (ImmunoTools, Friesoythe, Germany). After 5 days, the non-adherent immature dendritic cells (DCs)-enriched fraction was harvested and the immature status was confirmed by the expression of surface phenotypic markers (data not shown).

2.6. Incubation of FVIII with MO-DCs

For maturation experiments, immature 5-day old MO-DCs ($2.5x10^5$ cells/well) were incubated in 500 µl of X-VIVO^{15} (Lonza) with the different FVIII preparations at 1 or 10 IU/ml for 48 h. Surface expression of CD80, CD83, CD86, CD40 and HLA-DR was investigated by flow cytometry on 10,000 acquired cells per condition. As a positive control for induction of maturation, we used LPS at 100 ng/ml. Cells incubated alone were used as a negative control.

For FVIII endocytosis experiments, immature MO-DCs (4×10^5 cells/well) were incubated for 120 min with the FVIII at 50 to 400 IU/ml in 200 µl of X-VIVO^{15} at 4 °C or 37 °C. Intracellular FVIII was recognized after permeabilization with 0.5% saponin, using the FITC-labeled monoclonal antibody (anti-A2 FVIII domain mAb77IP52H7, 10 µg/ml, a kind gift from LFB, Les Ulis, France). 10,000 cells were acquired for each condition for flow cytometry analysis. Percentages of cells positive for FVIII were then calculated. Uptake was quantified as the difference in the % cells at 37 °C and 4 °C. When indicated, FVIII (400 IU/ml, 215 nM) was pre-incubated in X-VIVO^{15} with VWF (87.5 IU/ml, 3.6 µM) for 20 min at room temperature, prior to incubation with MO-DCs. mAb77IP52H7 does not interfere with the interaction of FVIII with VWF (not shown).

2.7. FVIII-specific T-cell activation assay

Five-day old immature MO-DCs derived from monocyte of a healthy

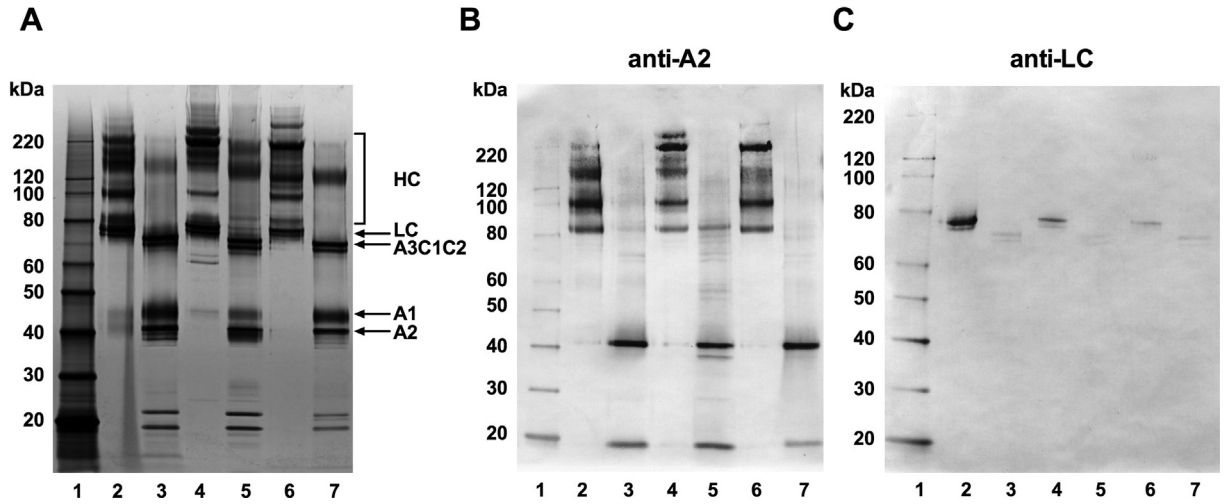

Fig. 1. Purity of Neureight and sensitivity to thrombin cleavage. Molecular weight markers (lane 1), Neureight (lanes 2–3), Helixate (lanes 4–5) and Advate (lanes 6–7) were separated by 4–12% SDS-PAGE under non-denaturing conditions (200 ng/lane) directly (lanes 2, 4 and 6) or after incubation with thrombin (2 IU) for 30 min at 37 °C (lanes 3, 5 and 7). Gels were either silver stained (Panel A) or transferred onto nitrocellulose membranes (Panels B and C). Blotted proteins were recognized using an anti-A2 domain monoclonal IgG (Panel B, GMA-012) or an anti-LC monoclonal IgG (Panel C, GMA-8018) and a labelled secondary anti-mouse IgG. Identity of the bands is indicated in the right of each panel (HC: heavy chain; LC: light chain; A1 and A2: A1 and A2 domains of the heavy chain; A3C1C2: thrombin-cleaved light chain).

donor with the HLA class II DRB1*0101/0301 haplotype, were cultured in RPMI-1640 medium supplemented with 10% FCS, 2-ME, L-glutamine and antibiotics in 96-round bottom plates, with the 1G8-A2 FVIII-specific HLA-DRB1*0101-restricted mouse T cell hybridoma [17] (10^4 DCs:10^5 T cells) in the presence of the different FVIII preparations at 3, 10 and 30 IU/ml. Twenty four hours later, supernatants were collected and the Il-2 secreted by activated T cells was measured by ELISA.

2.8. FVIII-deficient mice

FVIII-deficient mice were 8 to 10-week old males on a C57BL6 background (a gift from Prof Kazazian, University of Pennsylvania School of Medicine, Philadelphia) and were generated by in-house breeding. Our animal facility uses conventional SPF housing conditions where a maximum of 5 mice are kept in ventilated type IIL cages containing normal bedding, vegetal nests and cardboard tunnels at a temperature of 22 °C and 12-hour cycles of light/darkness. Mice were maintained on *ad libidum* normal diet A03 (SAFE, Augy, France) as well as water by automatic distribution. Mice were checked every day. Mice were sacrificed at the end of each experiment following decerebration under anesthesia (2–4% isofluorane). Mice were handled in agreement with French ethical authorities (Comité National de Réflexion Ethique sur l'Expérimentation Animale, Groupe hospitalier Pitié-Salpêtrière, Paris, France, authorization # 02058.04).

2.9. Half-life of FVIII

FVIII (1 IU) in 100 µl was administered intravenously to anesthetized (2–4% isofluorane) FVIII-deficient mice. The residual FVIII:Ag levels were measured at different time points (n ≥ 6 mice per time point and per FVIII product) using a FVIII-specific ELISA assay (Asserachrom, Stago). The data are expressed as the percentage of initial FVIII:Ag level (measured 5 min after administration) versus time. The half-lives were calculated using Graphpad prism by fitting the data to the two-phase decay model with the plateau constraint set to 0.

2.10. Anti-FVIII immune response in FVIII-deficient mice

Anesthetized FVIII-deficient mice were injected retro-orbital with 1 IU of each FVIII in its respective excipient at day 0, 7, 14, and 21. Blood was drawn under anesthesia by retro-orbital bleeding 5 days after the 4th administration of FVIII. Decomplemented plasma (56 °C for 30 min) was kept at -20 °C until use. For titration of anti-FVIII IgG, ELISA plates (Nunc) were coated with FVIII (2 µg/mL, Recombinate®) for 1 h at 37 °C, and blocked with PBS-1% BSA. Plasma diluted in PBS-1% BSA was 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) was used as standard. Results are shown in µg/ml mAb6-equivalent. For measurement of FVIII inhibitory titers, mouse plasma samples diluted in veronal buffer were incubated with a standard human plasma (Dade-Behring) for 2 h at 37 °C. The residual procoagulant FVIII activity was measured using a chromogenic assay following the manufacturer's recommendations (Dade-Behring). Bethesda titers, expressed in Bethesda units (BU)/mL, are defined as the reciprocal of the dilution of plasma that produces 50% residual FVIII activity.

3. Results

3.1. Production of Neureight

Neureight was produced using a novel fed-batch disposable fermentation concept in a 400 L scale using a high producer CHO cell line. Expression of recombinant full-length FVIII reached up to 30 IU/mL cell culture supernatant. The process was completely serum-free and did not contain animal-derived stabilizer. Purification of Neureight was carried out by applying several chromatographic steps including affinity chromatography. The purity and structural integrity of Neureight were compared to that of commercially available recombinant FVIII products, Helixate and Advate upon separation by SDS-PAGE with or without digestion with thrombin (Fig. 1A). The three recombinant FVIII demonstrated protein bands between 80 and 200 kDa, that correspond to the FVIII precursor, as well as the separated light chains (LC) at 80 kDa, and the separated heavy (HC), characterized by different molecular weight bands ranging from 90 kDa and higher that correlated to the variable lengths of the B domain, with the A1-A2 domains lacking the B domain as the smallest HC species at 90 kDa. Minor species were also detected between 40 and 50 kDa for Neureight, and to a lesser extent in Helixate and Advate, which can be correlated to the A1 and A2 domains as inferred from the thrombin digested profiles. Digestion of FVIII by thrombin generated the expected 73, 50, and 43 kDa

Table 1
FVIII:C and FVIII:Ag levels.

	FVIII:Ag* (IU/ml)	FVIII:C° (IU/ml)	Proteins† (μg/ml)	Specific activity‡ (IU/mg)
Neureight	4032 ± 2530	1971 ± 424	498 ± 133	4235 ± 556
Helixate	3451 ± 411	1629 ± 891	449 ± 290	3923 ± 1180
Advate	4271 ± 480	1717 ± 747	310 ± 77	6171 ± 824

Mean ± SD of 3 different lots for each product after dialysis against DMEM-F12.
°FVIII:C was measured using a chromogenic assay.
* FVIII:Ag was measured using Asserachrom.
† Protein concentration was measured in a Bradford assay using OVA as a standard.
‡ Specific activity reflects the IU of FVIII:C per mg of proteins (Bradford assay).

polypeptide fragments that correspond to the activated LC, and to the A1 and A2 domains of the HC, respectively. Thrombin digestion of Neureight confirmed the absence of contaminating protein in the preparation. Indeed, all the protein bands on the gel were sensitive to thrombin cleavage and demonstrated changes in migration profile, thus showing that they belong to FVIII.

The identity of the three products was further confirmed by Western blotting using monoclonal anti-A2 and anti-LC IgG (Fig. 1B and 1C, respectively). After dialysis against DMEM-F12, specific activities were 4235 ± 556, 3923 ± 1180 and 6171 ± 824 IU/mg for Neureight, Helixate and Advate, respectively (Table 1, means ± SD of 3 different lots), further substantiating the proper structure and function of Neureight.

3.2. Glycan profiling

N-glycan profiles of Neureight revealed both high mannose type glycan forms (Man)5(GlcNAc)2 to (Man)9(GlcNAc)2 and complex type glycans (Table 2). The majority of complex structures were fucosylated and sialylated. The level of sialylated N-glycans was determined with a percentage relative abundance of 59% for Neureight, as compared to 86% for Advate. High mannose type glycans were determined with a percentage relative abundance of 11% in the case of Neureight, compared to 4% in that of Advate. Importantly, the N-glycan profiles did not show the presence of the antigenic epitope Neu5Gc or of the non-human antigenic epitope galactose-α1,3-galactose (Gal-alpha-Gal) on N-glycans of the proteins. Neu5Gc was also undetectable in O-glycan profiling.

Table 3
Identification and assignment of sulfation sites.

Sulfation (Y)	Peptide position	Number of sulfates	Theoretical average mass (Da)	Neureight	Advate
				Measured average mass (Da)	
Y718, Y719, Y723	714–733*	1 2	2425.6 2505.6	2424.9 2505.7	2425.2 2505.8
Y1680	1674–1689	1	2079.1	2079.1	2079.1

* The tri-sulfated peptide was not detected as it is too unstable to be seen in the negative MALDI mode, but is likely to be present. The fact that mono-and di-sulfated peptides are also visible in the linear positive mode is a good indication that these ions are generated by spontaneous fragmentation of the tri-sulfated molecule.

3.3. Identification and assignment of sulfation sites, glycosylation sites and glycopeptides

The occupation sites and the type of glycan structures found for each of the 16 identified N-glycosylation sites were similar between Neureight and Advate (Table 2). For both molecules, the N41 glycosylation site was mainly occupied with complex bi-antennary glycans, mostly core-fucosylated and asialylated structures, the N943 and N2118 glycosylation sites both contained only high mannose type glycans, the N239 and N1066 sites presented with high mannose types and non-sialylated complex glycans, and the N1412 site was occupied by sialylated bi-antennary structures. For both Neureight and Advate, the N1255 glycosylation site was occupied only by high mannose-type glycans, while the N1259 glycosylation site was occupied by a variety of bi- and tri-antennary complex glycans with and without sialic acid (not shown). Tyrosine (Y) sulfation was identical between Advate and Neureight in terms of determined sites of tyrosine sulfation: the 714–733 peptide harboring the three tyrosine sulfation sites Y718, 719 and 723 was sulfated (Table 3). Both molecules showed sulfation of Y1680 (see Table 3), which is critical for the FVIII-VWF interaction.

3.4. Induction of maturation of monocyte-derived dendritic cells (MO-DCs)

In order to assess the potential immunogenicity of Neureight, we compared the capacity of the three FVIII products to induce the maturation of human immature MO-DCs. Incubation of 5-day old MO-DCs with LPS, used as a positive control, resulted in the overexpression of CD80, CD83, CD40 and HLA-DR (Fig. 2). In contrast, and as described previously [18], Advate and Helixate failed to induce the maturation of MO-DCs. Likewise, Neureight did not alter the immature profile of MO-DCs, irrespective of the FVIII concentration used in the assay. The results also reflect the lack of contamination of Neureight with endotoxins.

Table 2
Identification and assignment of glycosylation sites and glycopeptides.

Site (N)	Isolated peptide	Neureight		Advate	
		Mass range of detected glycopeptides (Da)	Description	Mass range of detected glycopeptides (Da)	Description
N41	37–47	2588.79–3349.53	complex bi-antennary glycans mostly core-fucosylated and asialylated structures	2589.49–3349.63	complex bi-antennary glycans mostly core-fucosylated and asialylated structures
N943	926–946	3851.20–4015.48	high mannose type structures (Man 8–9)	3850.76–4013.48	high mannose type structures (Man 8–9)
N239	231–240	2408.80–3058.59	high mannose type and non-sialylated	2409.13–3058.97	high mannose type and non-sialylated
N1066	1061–1067	2289.88–2815.15	complex structures	2288.14–2815.46	complex structures
N1412	1407–1422	3867.55	Sialylated biantennary structure	3866.54	Sialylated biantennary structure
N2118	2117–2136	3411.34–3897.38	high mannose type structures (Man 6–9)	3412.72–3898.33	high mannose type structures (Man 6–9)

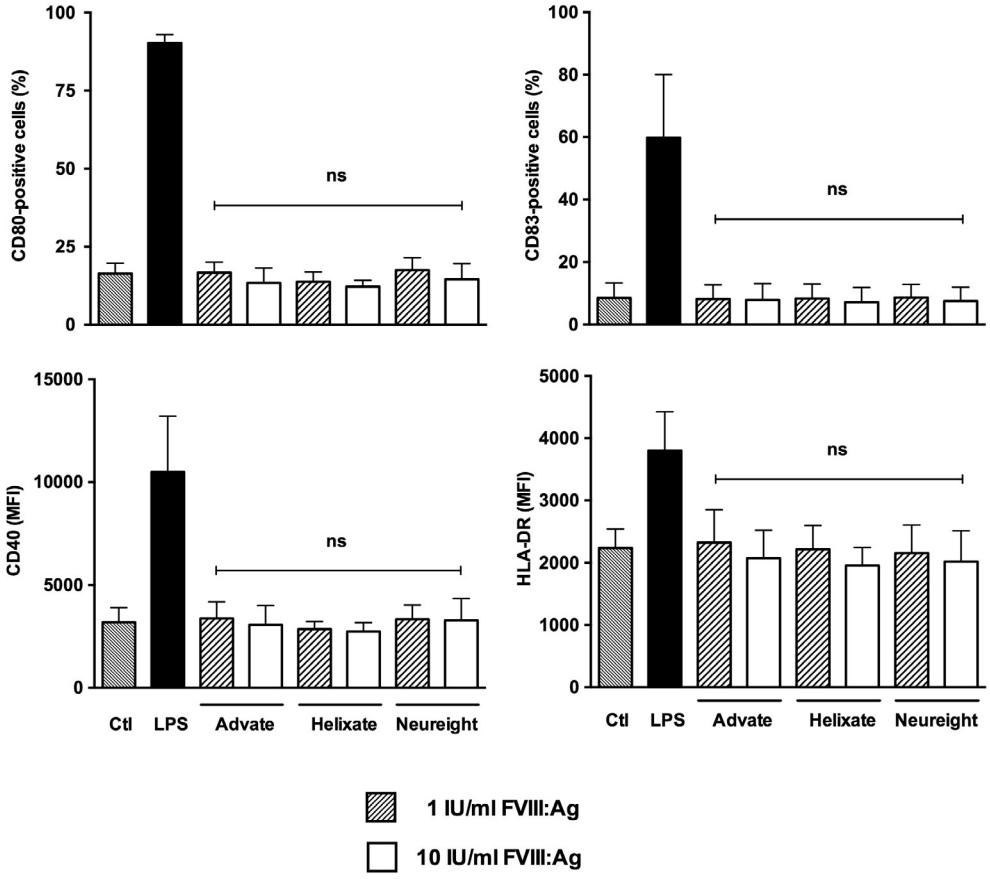

Fig. 2. Induction of maturation of MO-DCs by different recombinant FVIII products. Neureight, Advate and Helixate were incubated at 1 or 10 IU/ml (FVIII:Ag) with 5-day old immature human MO-DCs for 48 h. The expression of CD80, CD83, CD86, CD40 and HLA-DR was measured by flow cytometry and is expressed as percent of positive cells (CD80, CD83) and mean fluorescence intensity (MFI, CD40, HLA-DR). LPS was used as a positive control for the induction of MO-DC maturation. The results represent means and SEM of 3 independent experiments. Differences were not statistically significant (ns) as assessed using a two-tailed non-parametric Mann-Whitney test.

3.5. Endocytosis of Neureight by MO-DCs

The endocytosis of Neureight, Advate and Helixate by 5-day old immature MO-DCs was measured as a function of the concentration of FVIII. The 3 FVIII products were endocytosed in a dose-dependent manner (Fig. 3A). VWF has been reported to inhibit the endocytosis of FVIII by dendritic cells [19,20]. We thus compared the protective effect of VWF on FVIII endocytosis in our assay. The three FVIII products (400 IU/ml) were pre-incubated alone or with a 30-fold molar excess of VWF. FVIII was endocytosed by 37 ± 10, 40 ± 4 and 27 ± 4% of the cells in the case of Advate, Helixate, and Neureight, respectively (Fig. 3B). When the FVIII products were pre-incubated with VWF, percentages of FVIII-positive cells were reduced to 8 ± 2, 12 ± 2 and 14 ± 4%, respectively. VWF thus reduced the endocytosis of FVIII by 70 ± 15, 68 ± 9 or 49 ± 15% in the case of Advate, Helixate, Neureight, respectively (Fig. 3C). Differences between products were not statistically significant. Normal binding of Neureight to VWF was further substantiated by the observation of unaltered circulating half-life in FVIII-deficient mice, as compared to Advate (Fig. 4A).

3.6. Activation of FVIII-specific CD4+ T cells

To test whether the endocytosis of Neureight by MO-DCs leads to the activation of T cells *in vitro*, we took advantage of a FVIII-specific mouse T-cell hybridoma that is restricted to the human HLA-DRB1*0101 allele, and can be activated by human MO-DCs with the HLA-DRB1*0101 haplotype or by splenocytes from HLA-DRB1*0101-transgenic SureL1 mice [17]. The three different FVIII products were presented by human MO-DCs to T cells in a dose-dependent manner and to similar levels (Fig. 3D), suggesting that Neureight is endocytosed to a similar extent as Helixate and Advate by antigen-presenting cells, at FVIII concentrations below 100 IU/ml.

3.7. Immunogenicity of Neureight in FVIII-deficient mice

The three recombinant FVIII products were administered intravenously once a week for 4 weeks to FVIII-deficient mice at a quantity close to replacement doses in the human (50 IU/kg). After 4 administrations of FVIII, there was no statistical difference between the levels of anti-FVIII IgG induced by the three recombinant FVIII products (1816.0 ± 519.9, 1896.0 ± 478.9 and 680.7 ± 329.9 µg/ml for Advate, Helixate and Neureight, respectively, Fig. 4B). Levels of FVIII inhibitors, measured by the Bethesda assay (Fig. 4C), were statistically different between mice treated with Advate (123.3 ± 50.4 BU/ml, mean ± SEM) and mice treated with Neureight (15.7 ± 10.2 BU/ml, $P < .01$). There was however no significant difference between Helixate (120.2 ± 43.6 BU/ml) and Neureight.

4. Discussion

The objective of the present work was to validate the structural and functional integrity and lack of increased immunogenicity of Neureight, a recombinant full-length FVIII produced in CHO cells using a novel fed-batch fermentation mode based on a disposable concept. Neureight demonstrated a migration profile and sensitivity to digestion by thrombin, similar to that of Advate and Kogenate, two commercially available full-length FVIII produced using the classical perfusion technology. Importantly, thrombin digestion revealed the absence of contaminating non-cleavable protein bands in the three preparations, indicating similar degrees of purity of the end products. Importantly, the cell culture supernatant was harvested at high cell viabilities (> 90%) which may contribute to the quality of Neureight by limiting the accumulation of harmful/toxic metabolic by-products and keeping low levels of apoptotic cells. The purity and functional integrity of Neureight was further confirmed by the determination of the specific

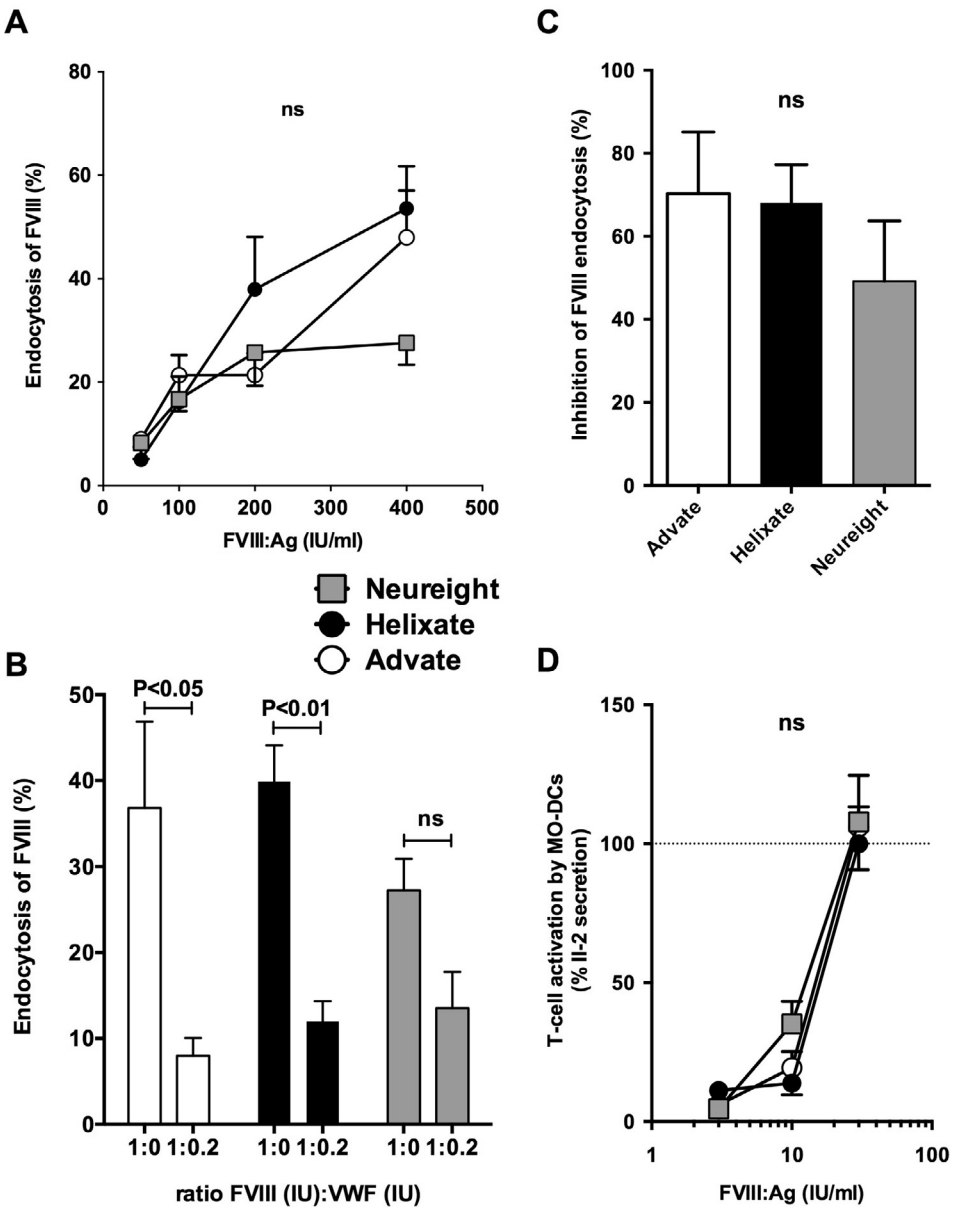

Fig. 3. Endocytosis and presentation of recombinant FVIII. Panel A. Immature five-day-old MO-DCs were incubated in X-VIVO[15] medium containing 50 to 400 IU/ml FVIII (FVIII:Ag) for 120 min at 37 °C or at 4 °C. The percent of cells having endocytosed FVIII was measured by subtracting the fluorescence measured at 4 °C from that measured at 37 °C minus. Results depict percent of positive cells. Error bars indicate SEM for 4 independent experiments with MO-DCs from 4 different healthy donors. ns: not significant as assessed using a two-way ANOVA. Panels B and C. FVIII (400 IU/ml FVIII:Ag) was pre-incubated for 20 min alone or in the presence of 87.5 IU/ml plasma-derived VWF at room temperature. Mixtures were then incubated at 4 °C or 37 °C in X-VIVO[15] medium containing 400 IU/ml FVIII:Ag. ns: not significant as assessed by the non-parametric Kruskal-Wallis test. In panel C, percent inhibition in the presence of VWF was calculated as the % FVIII-positive cells measured in the presence of VWF versus that measured in the absence of VWF. Results are mean ± SEM of 3 independent experiments. Panel D. Five-day old immature MO-DCs from healthy donors with the HLA class II DRB1*0101/0301 haplotypes, were cultured with the 1G8-A2 FVIII specific HLA-DRB1*0101-restricted mouse T-cell hybridoma in the presence of FVIII (1, 3, 10 and 30 IU/ml FVIII:Ag). Supernatant was collected after 24 h and Il-2 was measured in supernatant. Representative of three experiments. Data were normalized according to the IL-2 production measured for Helixate at 30 IU/ml FVIII:Ag and are depicted as % activation of the T-cell hybridoma. ns: not significant as assessed using a two-way ANOVA.

activities that were in the range reported for commercially available products, i.e., 4000–10,000 IU/mg for Advate and 2600–6800 IU/mg for Helixate [21].

Glycosylation is the most common post-translational modification observed in extracellular and integral membrane proteins. It impacts proper folding and efficient secretion of many extracellular proteins. Glycosylation also plays a significant role in determining the plasma half-life and biologic activity of several proteins. FVIII is extensively *N*-glycosylated, especially within the B-domain, with a variety of complex-, hybrid- and high mannose-type glycan structures [22]. As anticipated, the comparison of Neureight and Advate, that are both produced in CHO cells, by mass-spectrometry confirmed the heavily glycosylated profile of the two proteins with complex, hybrid- and high mannose-type glycosylations. The identical glycan structures were present at the same sites on both molecules, and there was a complete absence of the antigenic carbohydrate epitopes α-Gal and Neu5Gc [23]. Tyrosine sulfation is another post-translational modification that takes place in the *trans*-Golgi apparatus. Tyrosine sulfation of FVIII is required for full pro-coagulant activity as well as for optimal binding to VWF. Our study confirms the mono and di-sulfation of Neureight and Advate at the 714–733 peptide, while the tri-sulfated peptide was too unstable to be seen in the negative MALDI mode for both products. More importantly for proper binding to VWF, our study also confirms sulfation at Y1680.

The immunogenicity of a protein may result from different properties [24], including the capacity to be endocytosed by antigen-presenting cells, the capacity to provide maturation signals that trigger the maturation of the antigen-presenting cells, the capacity to intracellularly bind to MHC class II and be presented to T cells, as well as the presence in the organism of CD4+ T cells that are specific for the therapeutic protein-derived peptides and that are able to be activated (i.e., absence of active tolerance). Both full-length and B domain-deleted therapeutic FVIII products have been shown to be endocytosed by human MO-DCs. Although controversial, the endocytosis of FVIII implicates mannose-ending sugars and charged amino-acid residues in the C1 and C2 domains of FVIII [25–27]. Studies have ruled out a role for LRP and related receptors in the endocytic process by MO-DCs and implicated mannose-sensitive receptors such as CD206 [28,29]. In our hands, Neureight was endocytosed with a dose-dependency identical to that of Advate and Helixate, suggesting that the moieties implicated in FVIII internalization are identical irrespective of the method used to produce FVIII (fed-batch or perfusion approaches). Besides, the

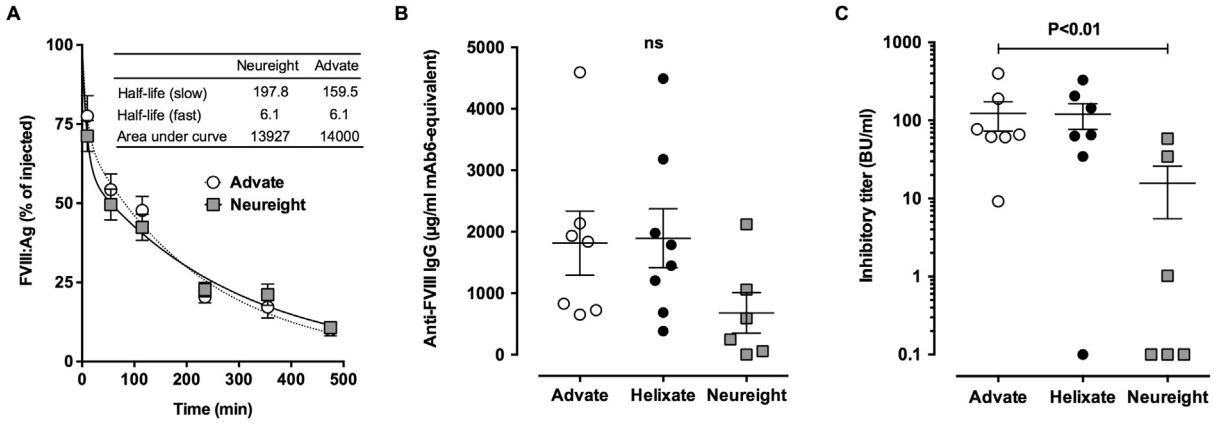

Fig. 4. Half-life and immunogenicity of FVIII in FVIII-deficient mice. Panel A. FVIII-deficient mice were injected with 1 IU FVIII:Ag (Advate: 6 mice; Neureight: 13 mice). Blood was collected 5, 15, 30, 60, 120, 240, 360 and 480 min after FVIII injection. FVIII in plasma was quantified by Asserachrom. Results are expressed in % of FVIII considering t = 5 min as 100%. Half-lives were fitted to two-phase decay non-linear regression curves using Graphpad Prism. Data depict means and SEM. Inset table: values for slow and fast half-lives expressed in min and area under the curves expressed in %*min. Panels B and C. FVIII-deficient mice (7–8 mice per group) received Advate, Helixate or Neureight (1 IU FVIII:Ag) intravenously once a week for 4 weeks. Mice were bled 5 days after the 4th FVIII administration. Anti-FVIII IgG and inhibitory antibodies were measured by ELISA (Panel B) and Bethesda assay (Panel C). Data depict means and SEM. Statistical significances were assessed with the non-parametric double-sided Mann-Whitney test (ns: not significant).

internalized Neureight was presented to a CD4+ T-cell hybridoma to a similar level as the two therapeutic proteins and both by mouse (not shown) and human antigen-presenting cells. The latter set of data indicates that the intracellular processing of Neureight does not differ from that of Advate and Helixate.

Previous work had documented that FVIII does not alter the maturation state of MO-DCs [18]. Indeed, incubation of Advate and Helixate with MO-DCs had no effect on the maturation state of the cells as assessed by the expression levels of CD80, CD83, CD40 and HLA-DR. Likewise, Neureight did not impart on the maturation profile of FVIII, suggesting that its binding to the surface of MO-DCs is identical to that of the other recombinant FVIII, and that it does not contain significant levels of endotoxins.

Interestingly, the levels of anti-FVIII IgG induced by Neureight in FVIII-deficient mice were identical to those induced by Advate and Helixate. In contrast, the inhibitory titers developed by the mice following administration of Neureight were significantly lower than that generated by the other FVIII proteins. An important difference between the *in vivo* model of severe hemophilia A and the *in vitro* predictive tests for FVIII immunogenicity used in the present work, is the presence or absence of VWF, respectively. VWF is a chaperon molecule that protects FVIII in the circulation from degradation by circulating enzymes, and transports FVIII to the site of bleeding. Controversial results suggest that VWF-containing FVIII may be less immunogenic in hemophilia A patients [2,30,31] and that VWF reduces the immunogenicity of FVIII in FVIII-deficient mice [32–34]. Here, we confirmed that VWF reduces the internalization of Helixate and Advate by MO-DCs *in vitro* [19]. Similarly, the endocytosis of Neureight was reduced, although without reaching statistical significance, in the presence of a physiological molar excess of VWF. The lack of statistical significance may be due to the somewhat lower amount of Neureight that is endocytosed, as compared to Advate and Helixate, when FVIII concentrations > 100 IU/ml are used in the assay. In addition, the half-life of Advate and Neureight were identical in FVIII-deficient mice, thus confirming the structural integrity of Neureight as well as its conserved ability to bind VWF. Taken together, our data predict that the immunogenicity of Neureight, at least as assessed using *in vitro* immunological assays or using the experimental model of severe hemophilia A, is similar to that of Advate and Helixate.

In conclusion, the production of full-length FVIII in a fed-batch fermentation mode generates a product that presents similar biochemical and functional properties as products developed using the classical perfusion technology. Further, the immunogenicity profile of the fed-batch fermentation product is not different from that of commercially available full-length products. Adoption of the fed-batch fermentation strategy should allow a substantial decrease in production costs in the range of 20–40% and permit the delivery of an efficient and safe recombinant FVIII product to developing countries.

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