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Manufacturing process used to produce long-acting recombinant factor VIII Fc fusion protein

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ABSTRACT

Recombinant factor VIII Fc fusion protein (rFVIIIFc) is a long-acting coagulation factor approved for the treatment of hemophilia A. Here, the rFVIIIFc manufacturing process and results of studies evaluating product quality and the capacity of the process to remove potential impurities and viruses are described. This manufacturing process utilized readily transferable and scalable unit operations and employed multi-step purification and viral clearance processing, including a novel affinity chromatography adsorbent and a 15 nm pore size virus removal nanofilter. A cell line derived from human embryonic kidney (HEK) 293H cells was used to produce rFVIIIFc. Validation studies evaluated identity, purity, activity, and safety. Process-related impurity clearance and viral clearance spiking studies demonstrate robust and reproducible removal of impurities and viruses, with total viral clearance >8–15 $\log_{10}$ for four model viruses (xenotropic murine leukemia virus, mice minute virus, reovirus type 3, and suid herpes virus 1). Terminal galactose-α-1,3-galactose and N-glycolylneuraminic acid, two non-human glycans, were undetectable in rFVIIIFc. Biochemical and in vitro biological analyses confirmed the purity, activity, and consistency of rFVIIIFc. In conclusion, this manufacturing process produces a highly pure product free of viruses, impurities, and non-human glycan structures, with scale capabilities to ensure a consistent and adequate supply of rFVIIIFc.

1. Introduction

Hemophilia A is an X-linked bleeding disorder, characterized by functional factor VIII (FVIII) deficiency. The mainstay treatment is FVIII replacement therapy. Following the widespread transmission of blood-borne viruses in the 1970s and 1980s related to the use of plasma-derived clotting factor concentrates [1], the FVIII gene was cloned and recombinant protein expression and purification techniques were developed. While the use of recombinant FVIII (rFVIII) and improved purification methodology contributed to significant improvements in the availability and safety of FVIII replacement therapy [2], periodic supply shortages and manufacturing quality breaches have continued into the 2000s. These issues reflected the relative difficulty in manufacturing rFVIII, a large, multi-domain glycoprotein with significant post-translational modifications.

To ensure the safety of rFVIII products, manufacturing processes should be evaluated for product quality and the capacity to remove viruses (regulations require demonstration of viral clearance using ≥3 viruses with differing characteristics and validation of ≥2 process steps that use different mechanisms for virus inactivation and or/removal) [3,4]. Additionally, processes should aim to mitigate potential immunogenicity associated with manufacturing rFVIII products in clonal cell lines [5]. Manufacturing processes for currently available rFVIII products have previously been described [6–8]. Recombinant FVIII Fc fusion protein (rFVIIIFc; Biogen,

Abbreviations: aPTT, activated partial thromboplastin time; α-Gal, galactose-α-1,3-galactose; BDD, B domain-deleted; FcRn, neonatal Fc receptor; FVIII, factor VIII; HC, heavy chain; HCP, host cell proteins; HEK, human embryonic kidney; HIC, hydrophobic interaction chromatography; ICH, International Conference on Harmonisation; IgG_1, immunoglobulin G_1; MCB, master cell bank; MMV, mouse minute virus; NGNA, N-glycolylneuraminic acid; RCB, research cell bank; Reo-3, mammalian orthoreovirus 3; rFVIII, recombinant factor VIII; rFVIIIFc, recombinant factor VIII Fc fusion protein; RT-PCR, reverse transcription polymerase chain reaction; SEC, size exclusion chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SuHV-1, suid herpes virus 1; UPLC, ultra-performance liquid chromatography; WCB, working cell bank; X-MLV, xenotropic murine leukemia virus.

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Cambridge, MA) is the first-approved, long-acting FVIII for adults and children with hemophilia A for the control and prevention of bleeding episodes, perioperative management, and routine prophylaxis to prevent or reduce the frequency of bleeding episodes; it has been designed to reduce the required infusion frequency of prophylactic treatment [9–12]. The presence of the Fc domain of human immunoglobulin G_1 (IgG_1) enables the fusion protein to bind to the neonatal Fc receptor (FcRn), part of an endogenous intracellular pathway that delays lysosomal degradation of Fc-containing proteins (ie, IgG) by cycling them back into circulation [13,14]. Fc fusion does not significantly alter the higher-order structure of FVIII or its functionality [9,10,15]. The phase 3 A-LONG study demonstrated an extended half-life of rFVIIIFc relative to rFVIII (~1.5-fold increase, 19.0 h), as well as the safety and efficacy of rFVIIIFc for the control and prevention of bleeding episodes [11,12].

The objective of this work was to describe the rFVIIIFc manufacturing process, and evaluate product quality and the capacity of this process to produce a product free from viruses and impurities.

2. Materials and methods

2.1. Manufacturing process: development of the rFVIIIFc cell line, cell bank, and cell line characterization

The coding sequences for human FVIII and the Fc region of the human IgG_1 (hinge and CH2 and CH3 domains) were obtained by reverse transcription polymerase chain reaction (RT-PCR) from human liver polyA mRNA and a human leukocyte cDNA library, respectively. HEK 293H cells (Invitrogen, Carlsbad, CA) were stably transfected with an expression vector containing two expression cassettes. One cassette expressed the native human FVIII signal sequence followed by a B domain-deleted (BDD) FVIII (S743 to Q1638 fusion) directly linked to the Fc region of human IgG_1 with no intervening linker. The second expression cassette held Fc with a heterologous mouse IgκB signal sequence [9,10].

Transfected HEK 293H cells were grown in serum-free medium. Clonal cell lines were derived and the optimal cell line was selected based on considerations for rFVIIIFc monomer productivity, rFVIIIFc activity (measured by chromogenic assay), superior cell growth properties, and stability. Cell lines with optimal characteristics were then sub-cloned by limiting dilution and further characterized to select the production clonal cell line for manufacturing.

The clonal cell line that was selected for manufacturing was expanded to create a research cell bank (RCB). The RCB was expanded to create the master cell bank (MCB) from which a working cell bank (WCB) was derived. The MCB and WCBs were tested for identity, purity, and freedom from adventitious agents. The transgene coding sequence, copy number, and gene integration patterns of the MCB and a cell bank produced from a cell culture that was propagated beyond the end of the manufacturing process were compared based on the International Conference on Harmonisation (ICH) guidelines Q5A, B and D [16–18]. The comparison was used to assess and confirm transgene integration and stability of the cell line over the course of the manufacturing process.

To characterize the resultant product from this cell line, rFVIIIFc was analyzed for the presence of two non-human glycans, terminal galactose-α-1,3-galactose (α-Gal) and N-glycolylneuraminic acid (NGNA). α-Gal was released with α-(1-3,4,6) galactosidase, labeled with 2-aminobenzoic acid, and analyzed with ultra-performance liquid chromatography (UPLC) with fluorescent detection. NGNA was released with 50 mM H_2SO_4, labeled with 1,2 diamino-4,5-methylenedioxybenzene, and analyzed with UPLC with fluorescent detection. Three currently available rFVIII products (Xyntha® [Wyeth Pharmaceuticals Inc, Philadelphia, PA], Advate® [Baxter, Westlake Village, CA], and Kogenate® [Bayer, Tarrytown, NY]) were also analyzed for the presence of α-Gal and NGNA using the same analytical methods.

2.2. Production of rFVIIIFc

One WCB vial was used to produce a single batch of rFVIIIFc in a multi-step manufacturing process (Fig. 1). The inoculum preparation phase includes thawing a WCB vial (Step 0) and expansion of culture in shake flasks (Step 1). Shake flasks were then pooled and used to inoculate the first seed train bioreactor (for further culture expansion; Step 2). The seed train bioreactors were operated in batch mode, with agitation, pressure, temperature, pH, and dissolved oxygen controlled. The expanded culture was used to inoculate a large-scale production bioreactor (Step 3). The production bioreactor (2000 L) was operated in fed-batch mode, during which agitation, pressure, temperature, pH, and dissolved oxygen were controlled.

Cells and cellular debris were removed by centrifugation (Step 4) and subsequent depth filtration steps (Step 5) to produce a clarified cell culture harvest containing the rFVIIIFc product. Detergent (Triton X-100; Step 6) was added to the clarified cell culture harvest as a virus inactivation step. The product was then captured and purified from the clarified cell culture harvest with a FVIII-specific affinity chromatography step using an VIIISelect column (GE Healthcare Life Sciences, Piscataway, NJ; Step 7). VIIISelect is an immobilized recombinant peptide-based affinity ligand specific for FVIII that is both highly effective and free of animal components, such as mouse monoclonal antibodies [19]. The VIIISelect adsorbent binds to the FVIII light chain portion of rFVIIIFc, and the product is desorbed and collected using a buffer solution at neutral pH, to ensure the integrity of rFVIIIFc is maintained. rFVIIIFc was further purified by charge-based separation using anion exchange chromatography (Step 8) followed by a flow-through anion-exchange membrane absorber (Step 9). The rFVIIIFc product was then filtered through a 15 nm virus filter (Planova™ 15N; Asahi Kasei Bioprocesses, Inc., Glenview, IL; Step 10) to purify based on size. The virus-filtered product was further purified using a final chromatography step (Hydrophobic Interaction Chromatography [HIC]; Step 11). rFVIIIFc was concentrated and buffer-exchanged using an ultrafiltration step (Step 12) to form the bulk product. The bulk product was formulated and filtered into bottles (Step 13) and stored at −70 °C to ensure stability prior to lyophilization and before final filling into individual drug product vials for use in clinical study.

2.3. Manufacturing process and impurity clearance validation studies

Process validation studies were performed to confirm identity, purity, quality, and activity of the rFVIIIFc product. A summary of the analytical tests used in these assessments is shown in Table 1. Non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with colloidal Coomassie blue, thrombin digest map, FVIII chromogenic assay and size exclusion chromatography were also employed in validation studies for purity assessment and identity confirmation. The presence of aggregated species (proteins that have undergone conformational changes during the manufacturing or storage processes resulting in misfolded protein species), was determined using size exclusion chromatography (SEC) [20]. Fc-binding activity was determined using an FcRn binding assay [21]. The specific activity of rFVIIIFc was assessed using both the two-stage chromogenic substrate and one-stage activated partial thromboplastin time (aPTT) clotting assays [9]. Safety determination was based on testing for the presence of bioburden and endotoxin.

Process Step No.	Step Description
Step 0	Thaw of one working cell bank vial
Step 1	Inoculum expansion (culture expansion in shake flasks)
Step 2	Seed train bioreactors (culture expansion in bioreactors)
Step 3	Production bioreactor
Step 4	Harvest by centrifugation
Step 5	Clarification (depth filtration)
Step 6	Virus inactivation (Triton X-100 addition)
Step 7[a]	Affinity chromatography (VIIISelect column)
Step 8[a]	Anion exchange chromatography (TMAE HiCap column)
Step 9	Anion exchange membrane (Mustang Q membrane)
Step 10[a]	Virus reduction filtration (Planova 15N)
Step 11	Hydrophobic interaction chromatography (Octyl Sepharose fast flow)
Step 12	Ultrafiltration/diafiltration
Step 13	rFVIIIFc product formulation, filtration, bottling, and storage

Fig. 1. Overview of the rFVIIIFc manufacturing process. [a]Viral clearance steps.

The purification process was designed to provide a high level of viral clearance for potential adventitious viruses. To demonstrate the capacity and robustness of the manufacturing process (and individual steps) to remove adventitious viruses, the purification process was evaluated for the capacity for removal of enveloped and non-enveloped viruses using four model viruses (xenotropic murine leukemia virus [X-MLV], mouse minute virus [MMV], mammalian orthoreovirus 3 [Reo-3; also known as reovirus serotype 3], and suid herpes virus 1 [SuHV-1; also known as pseudorabies virus]; Table 2). These viruses were selected to represent ranges of physiochemical properties of several human virus families, such as retroviruses, herpes viruses, reoviruses, and parvovirus. These studies were conducted according to the ICH Q5A Guidelines and the US Food and Drug Administration Points to Consider [16,22] and in accordance with Good Laboratory Practice [23].

The rFVIIIFc manufacturing process was also evaluated for the capacity to remove process-related impurities. Process-related impurity clearance validation studies were performed both at the manufacturing scale and in scaled-down spiking studies at the laboratory scale. Those performed at the manufacturing scale consisted of direct measurement of the impurities obtained from the manufacturing process intermediates, in which clearance was calculated from the amount removed during the process step. Scaled-down impurity clearance validation studies involved adding an impurity to the process intermediate and purifying the spiked intermediate using a scaled-down chromatography step. These studies were used when the impurity was below detectable levels in the manufacturing process intermediates and to demonstrate the capacity and robustness of the process to provide additional clearance.

Table 1
Analytical tests used to assess identity, purity, activity, and safety of rFVIIIFc.

Test	Method description
Polyacrylamide gel electrophoresis (non-reducing)	Polyacrylamide gel electrophoresis performed in the presence of sodium dodecyl sulfate (SDS-PAGE) under non-reducing conditions. Gels are stained using Colloidal Coomassie Blue staining
Size exclusion chromatography	Resolution of aggregated forms from the monomeric form of rFVIIIFc using high performance liquid chromatography (Sepax SRT SEC-300 column)
Coagulation activity	One-stage activated partial thromboplastin time (aPTT) clotting assay method performed in accordance with Pharmacopeia guidelines (USP<32> and Ph. Eur. 2.7.11)
Chromogenic activity	Colorimetric method performed in accordance with Pharmacopeia guidelines (Ph. Eur. 2.7.4)
FcRn binding	Neonatal Fc receptor (FcRn) competitive binding measured using an amplified luminescent proximity homogenous assay
Bioburden	Microbial enumeration test performed in accordance with Pharmacopeia guidelines (USP<61> and Ph. Eur. 2.6.12)
Endotoxin	Kinetic turbidimetric method in accordance with Pharmacopeia guidelines (USP<85> and Ph. Eur. 2.6.14)

FcRn, neonatal Fc receptor; Ph. Eur., European Pharmacopeia; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 2
Summary of rFVIIIFc viral clearance validation studies and virus clearance reduction factors.

Virus name	Virus type	Virus size (nm)	Virus reduction factor (LRV)				
			Detergent inactivation (log_{10})	Affinity chromatography (log_{10})	Anion exchange chromatography (log_{10})	Viral filtration (Planova 15N) (log_{10})	Total clearance (log_{10})[a]
Xenotropic murine leukemia virus (X-MLV)	Retrovirus	80–130	≥4.4[b]	2.4	2.7	≥5.6[b]	≥15.1
Suid herpes virus 1 (SuHV-1)	Enveloped DNA virus	120–200	≥4.4[b]	3.1	NP	≥4.0[b]	≥11.5
Reovirus type 3 (Reo-3)	Non-enveloped RNA virus	60–80	NP	2.8	NP	≥5.5[b]	≥8.3
Mouse minute virus (MMV)	Small DNA virus	18–22	NP	>4.6[b]	1.6	≥5.7[b]	≥11.9

NP, not performed; log_{10}, log reduction value of viral clearance.
The LRV of four viruses for the rFVIIIFc purification process steps are included.
[a] Total clearance (LRV) represents the summation of the steps evaluated for viral clearance for the four viruses evaluated in the studies.
[b] The ">" indicates virus levels were below levels of detection for the respective step.

3. Results

3.1. Cell line safety and characterization

The MCB and WCB were manufactured in accordance with current Good Manufacturing Practice procedures, with purity, safety, and identity test results demonstrating no detectable virus or adventitious agents. Testing with random amplified polymorphic DNA, isoenzyme analysis, and RT-PCR confirmed the cell bank origin; microbial and viral testing confirmed they were free of bacteria, fungi, mycoplasma, and adventitious viruses. Additionally, the cell line chosen resulted in a product free of the non-human glycans, terminal α-Gal and NGNA (Table 3).

3.2. Validation studies: assessment of product quality and process consistency

The rFVIIIFc manufacturing process generated product of consistent purity, quality, and activity. All manufacturing steps were successfully validated for consistency based on process performance and product quality data from four batches. The manufacturing process validation study demonstrated consistency through evaluation of controlled parameters, in-process controls, and product quality. Results from four validation batches are shown in Table 4. All batches demonstrated >97% purity, when measured by non-reducing SEC and SDS-PAGE (Fig. 2).

Table 3
Levels of A) galactose-α-1,3-galactose (α-Gal) and B) N-glycolylneuraminic acid (NGNA) in rFVIIIFc and three commercially available rFVIII products.

Sample	α-Gal		NGNA	
	Average % mol/mol (n = 3)	Standard deviation (n = 3; %)	Average % mol/mol (inter-day; n = 9)[a]	RSD[b] (inter-day; n = 9; %)[a]
rFVIIIFc	<LOD[c]	NA	<LOD[d]	NA
Xyntha	10.2	1.6	20.31 (0.73)	3.6
Advate	3.3	0.6	1.33 (0.14)	10.8
Kogenate	1.3[e]	0.8	5.99 (0.32)	5.3
Positive control	41.7	0.4	–	–

α-Gal, galactose-α-1,3-galactose; LOD, limit of detection; NA, not applicable; NGNA, N-glycolylneuraminic acid; rFVIIIFc, recombinant factor VIII Fc fusion protein; rFVIII, recombinant factor VIII; RSD, relative standard deviation.
[a] Analysis was done in triplicate on 3 separate days (total of n = 9).
[b] Inter-day (n = 3 days) RSD.
[c] LOD is 1.1% (0.1 pmol) for rFVIIIFc.
[d] LOD is 0.28% (2.5 fmol) for rFVIIIFc.
[e] 1.3% is at the LOD (1.3% [0.1 pmol]) but below the limit of quantification (2.6% [0.2 pmol]).

Table 4 also shows that the specific activity of rFVIIIFc was consistent among the validation batches, with batches possessing specific activity values of 1660–1770 IU/nmol of rFVIIIFc for the aPTT assay and values of 1420–1720 IU/nmol for the chromogenic substrate assay. Importantly, comparable ranges for specific activity were achieved using both the aPTT and chromogenic substrate assays. Additionally, results were comparable to the specific activity of native FVIII (1429 IU/nmol) [10] and specific activities previously reported for rFVIIIFc (1861 ± 154 IU/nmol and 2057 ± 298 IU/nmol using the one-stage aPTT and chromogenic substrate assays, respectively) and that reported for ReFacto® (1862 IU/nmol) [9]. In addition, potency of rFVIIIFc in binding to FcRn was consistent across the batches. No bioburden was detected in any of the batches, and endotoxin levels were all below detectable levels. Overall, results from analytical testing demonstrated that the manufacturing process consistently produced a highly pure and active rFVIIIFc product.

3.3. Validation studies: virus- and process-related impurity clearance

Virus removal studies demonstrated significant clearance of viruses possessing different physical and chemical properties. The overall total clearance for the rFVIIIFc purification process was ≥15.1 log_{10} for X-MLV, ≥11.5 log_{10} for SuHV-1, ≥8.3 log_{10} for MRV-3, and ≥11.9 log_{10} for MMV. The detergent virus inactivation step, the VIIISelect affinity chromatography step, the anion exchange chromatography step, and the virus filtration step (Planova 15N) each contributed to this viral clearance, with the most substantial removal of model virus achieved with the use of the 15N Planova nanofilter (Table 2).

In addition to providing robust removal of viruses, the rFVIIIFc purification process also provided robust and reproducible clearance of process-related impurities. Reductions in HEK 293H host cell proteins (HCP), HEK 293H host cell DNA, and Triton X-100 are shown in Table 5. Levels of VIIISelect ligand leachate were below detectable levels during the manufacturing process and in the final product, demonstrating that only minimal, sub-detectable levels may leach during the manufacturing process. A reduction factor of 0.8 log_{10}, obtained by performing scale-down spiking studies of the VIIISelect ligand, further illustrates the robustness of the rFVIIIFc manufacturing process to remove any residual VIIISelect ligand that may be present following the VIIISelect chromatography step. Additionally, levels of host cell DNA were below detectable levels (<1 pg DNA/mg rFVIIIFc) in the final product, well below the level that is considered acceptable by the World Health Organization [24].

Table 4
Product quality results from four validation batches.

Product attribute	Test method	Results			
		11-011	11-012	11-013	11-014
Identity	Non-reducing SDS-PAGE;	Comparable to reference standard			
	Thrombin digest map	Conforms to reference standard			
	FVIII chromogenic substrate assay	Meets biologic activity specification			
Purity	Non-reducing gel electrophoresis (%)	97.7	98.1	98.2	98.6
	Size exclusion chromatography (%)	>99.0	>99.0	>99.0	>99.0
Activity	Coagulation activity based on one-stage aPTT clotting assay specific activity (IU/nmol rFVIIIFc)[a]	1660	1700	1770	1660
	Activity based on chromogenic substrate assay specific activity (IU/nmol rFVIIIFc)[a]	1420	1620	1640	1720
	FcRn binding relative potency[b] (%)	127	127	120	122
Safety	Bioburden (CFU/10 mL)	0	0	0	0
	Endotoxin (EU/mL)	<1.00	<1.00	<1.00	<1.00

aPTT, activated partial thromboplastin time; CFU, colony-forming units; ELISA, enzyme-linked immunosorbent assay; EU, endotoxin units; FcRn, neonatal Fc receptor; rFVIIIFc, recombinant factor VIII Fc; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WHO, World Health Organization.
[a] Coagulation activity is calibrated against the WHO international standard for FVIII. For comparison, the specific activity of rFVIII is 1429–1862 [9,10].
[b] One GMP batch manufactured using the same process, scale, and facility has been designated as a reference standard. Potency was determined against this reference standard.

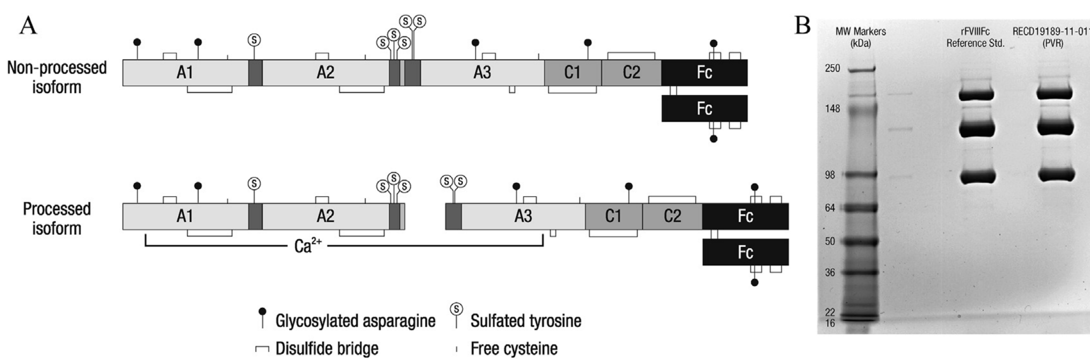

Fig. 2. **A)** Structural components of rFVIIIFc: the single-chain (SC) non-processed isoform and the processed isoform (FVIII light chain [A3, C1, C2] covalently linked to Fc dimer [LC–Fc/Fc]); **B)** Non-reducing SDS-PAGE analysis of rFVIIIFc validation batch (RECD19189-11-011) used for the determination of purity and identity. The three bands are indicative of the three components of rFVIIIFc: FVIII heavy chain (HC), the processed isoform, and the SC isoform. Non-reducing SDS-PAGE was conducted on a 4%–12% polyacrylamide gel in Bis–Tris buffer. Samples were denatured with SDS in the presence of 15 mM N-ethylmaleimide for 5 min at 75 °C. The gel was stained with Colloidal Coomassie. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MW, molecular weight; HC, FVIII heavy chain; LC–Fc/Fc, FVIII light chain covalently linked to Fc dimer; SC, single-chain.

4. Discussion

Many technologic advances have offered benefits to the hemophilia A population since FVIII was found to be enriched in the cryoprecipitate of fresh frozen plasma in 1964 [25]. This discovery led to the development of plasma-derived factor replacement therapies. However, there was widespread contamination of these products with hepatitis and HIV in the 1970s and 1980s [1]. Viral safety concerns accelerated the development of recombinant clotting factors (initial approval in 1992). However, plasma proteins added to stabilize the final formulation of first generation products (eg, albumin) and plasma-derived proteins used in the cell culture medium of second generation products continued to fuel concerns about viral transmission (additionally, supply shortages occurred through the early 2000s). The advent of third generation products, free of human and animal proteins, has ushered in a new era of theoretical safety; however, despite high theoretical safety, the hemophilia community has remained concerned about viral transmission. This illustrates the key importance of conducting manufacturing validation studies in recombinant products that demonstrate viral clearance and removal of other impurities.

Table 5
Summary of impurity clearance achieved throughout the rFVIIIFc manufacturing process for select process-related impurities.

Process-related impurity	Impurity clearance validation scale	Overall reduction factor (log_{10})[a,b]
HEK 293H HCP	Manufacturing	5.6
HEK 293H DNA	Manufacturing	>7.0
VIIISelect Ligand Leachate	Laboratory	0.8
Triton X-100	Laboratory	>8.9

HCP, host cell protein; HEK, human embryonic kidney; rFVIIIFc, recombinant factor VIII Fc.
[a] Overall reduction factor (log_{10}) is the sum of the reduction factor values for each of the process steps validated for removal of the respective process-related impurities.
[b] Reduction factor = log_{10} (impurity load of input/impurity load of output).

The rFVIIIFc manufacturing process validation studies described herein demonstrated the capacity of the manufacturing process to produce a product of consistent high quality and purity and to remove potential viruses and process-related impurities. This process uses a number of recently developed techniques to ensure product quality and purity, including a virus inactivation step, three different chromatography steps, and a 15 nm pore size virus filter to provide robust removal of viruses.

A key feature of rFVIIIFc's manufacturing process is the use of a human cell line and a process that is free from added human- or animal-derived components. The HEK 293H cell line has biochemical properties that are advantageous for recombinant protein expression, such as amenability to transfection, high efficiency, and effective translation of human protein processing and production [26–28]. This cell line has been selected to produce a number of human recombinant protein therapeutics, including drotrecogin alfa-activated protein C, a factor VIII product, and a factor IX product [5,27–31]. Another advantage of using a human cell line is this ensures no non-human glycan structures are incorporated into the expressed proteins, which need to be monitored and screened for during cell line development in cells lines derived from rodents [32,33]. Results of this work indicate that the use of a human cell line to manufacture rFVIIIFc yields a product free of the non-human glycan structures found in proteins expressed in hamster cell lines. It has been previously reported that all humans possess anti-NGNA antibodies and sometimes at high levels, approaching 0.1%–0.2% of circulating IgG [34]. Anti-α-Gal antibodies (IgE) have also been previously observed in humans [35]. As a result, α-Gal and NGNA are potentially immunogenic. In this analysis, neither NGNA nor α-Gal were detected in rFVIIIFc, but different amounts of both were found in the three commercially available rFVIII products: Advate, Xyntha, and Kogenate (Table 3), all produced with hamster cell lines. Although the impact of these antigens in vivo is not known, their absence may result in lower immunogenicity [5].

Similar viral clearance steps that include multiple chromatography steps, a virus filtration step, and a virus inactivation step have also been utilized to manufacture other rFVIII products. To our knowledge, this is the first reported use of a 15 nm nanofilter in the manufacturing process of a rFVIII product. The viral clearance resulting from the manufacturing process of rFVIIIFc can be compared with that of another rFVIII product produced in mammalian (CHO) cells [6]. The CHO-based rFVIII process achieved >11.4 logs for X-MLV, >14.0 logs for SuHV-1, >10.3 logs for Reo-3, and 5.2 logs for MMV. The process removed viruses to below detectable levels for three of the four model viruses evaluated in the studies (X-MLV, SuHV-1, and Reo-3). However, the process did not clear MMV to below detectable levels, achieving an overall clearance of 5.2 logs compared with >11.9 logs achieved using the rFVIIIFc manufacturing process. This may be due to the use of a larger (35 nm) pore size virus filter in the rFVIII manufacturing process, which was less effective at removing relatively small MMV viruses. MMV is a surrogate for parvoviruses, among the smallest human pathogens.

Reducing the pore size in nanofiltration is known to greatly enhance the effectiveness of viral clearance without affecting purified FVIII [36]; the small pore size (15 nm) of the Planova 15N virus filter provides an effective barrier for a wide range of large-size impurities. In the current study, the Planova 15N filter provided an extremely stringent level of clearance providing a total reduction factor $\geq$8.3 $\log_{10}$ for each of the model viruses tested and >15 $\log_{10}$ clearance of retroviruses. Overall, these results demonstrate that the rFVIIIFc manufacturing process can effectively clear retroviruses, in addition to a broad spectrum of adventitious virus types.

5. Conclusions

Over more than two decades, the safety of manufactured rFVIII proteins has improved dramatically compared with the previous manufacture of plasma-derived FVIII, but has not been without challenges. The rFVIIIFc manufacturing process employs multiple new methods including a unique cell line and state-of-the-art purification and viral filtration to consistently produce a novel, fully active, and highly purified product free from viral contaminants or impurities. Importantly, by utilizing a scalable and transferable process, the product can be produced within any of the manufacturer's large-scale manufacturing facilities, reducing supply risks [37].

Author contributions

Justin McCue composed the manuscript. Stephen Raso analyzed the data. All authors contributed to the interpretation of the data, manuscript revisions, and approval of the submitted version.

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Declaration of interest

All authors were employees of and held equity interest in Biogen at the time this research was conducted.

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