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Cell culture and purification process – purity and safety testing

This paper is intended to demonstrate that the Genetics Institute has developed a very safe and consistent recombinant factor VIII product. The process used will be described here.

The previous paper described the cell culture process in detail, the making of the product. In developing the cell culture process there is a rational choice of cell lines. We have cell banks that we have thoroughly characterized, and, more importantly, we have shown that the cells do not change during the production process or from one campaign to the other.

The cell banking system is really a two-part system: there is a master cell bank (Fig. 1), and there are working cell banks. The master working cell bank is a system in which we preserve the cells at –135°. Each time we begin a campaign we thaw a vial from the working cell bank; therefore, we always begin with the same starting material.

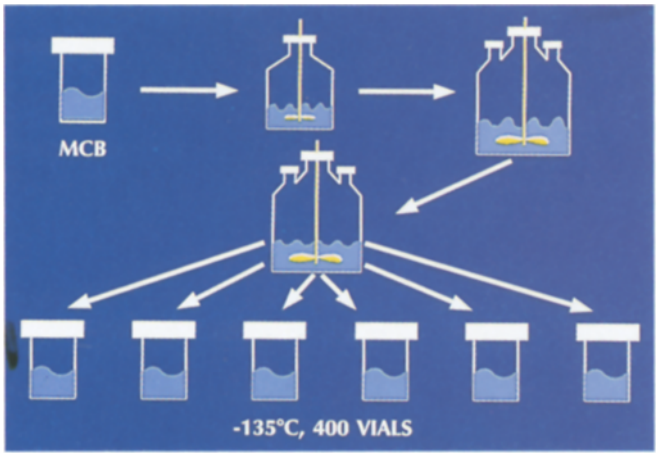

Fig. 1 Master working cell bank

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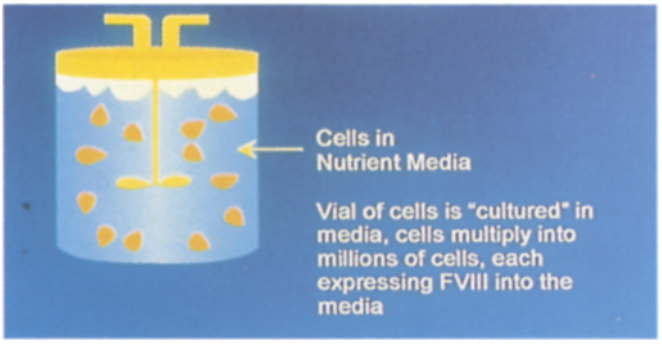

Fig. 2 Bioreactor

To perform the cell culture process on a large scale, we have to grow enough cells to inoculate our 2500-l bioreactors. With mammalian cell culture, generating sufficient inoculum is a relatively slow process; we have to grow the cells slowly, because they are very fastidious regarding cell density in a culture (Fig. 2). During inoculum development (Fig. 3) we use spinner flasks; we start at approximately 250 ml or less and go up ultimately to 8-l spinner flasks.

In the previous paper our bioreactors are described. Figure 4 shows our cell-culture area. Three 2500-l bioreactors are in the factor VIII producing suite, in addition there are harvest tanks. We use a batch re-feed process (Fig. 5), in which we start with a bioreactor with a full volume of medium; we remove most of the bioreactor volume, which is then used for further purification. The cell culture remaining in the bioreactor is essentially the inoculum for the next medium feeding. This process continues for approximately 15 cycles, a typical run. From one inoculum we normally generate about 15 separate large-scale bioreactor batches.

We typically combine three bioreactor batches, ending up with approximately 7000 l of conditioned medium. The medium at this point contains the CHO cells, which of course we no longer want, but we do want the product. We would like to remove the other

Fig. 3 Inoculum preparation

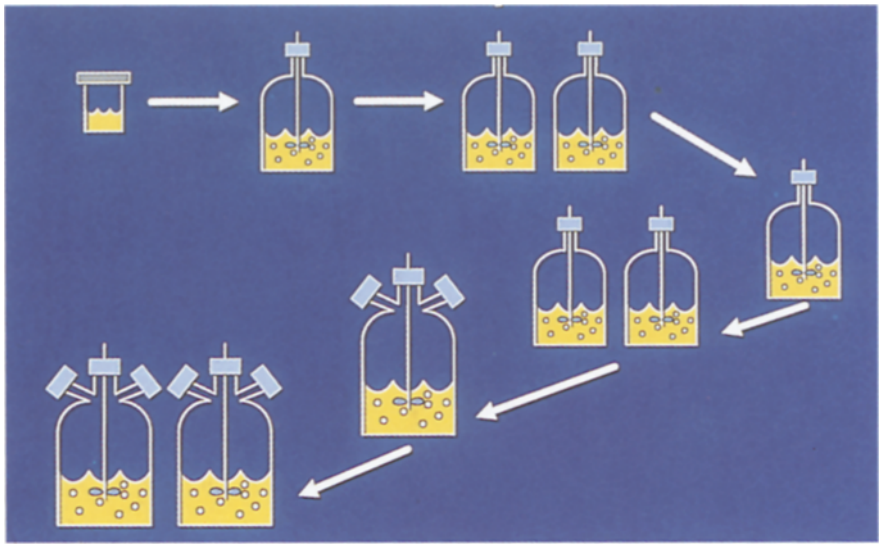

Fig. 4 Cell-culture area, with 2500-l bioreactors and harvest tanks

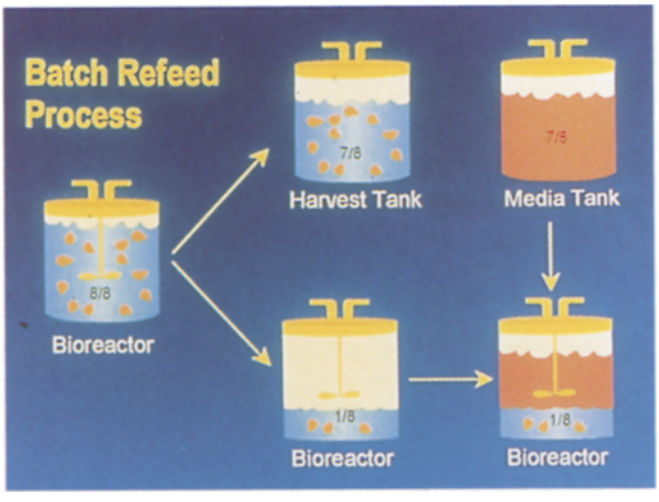

Fig. 5 Batch re-feed process

components from the system, i.e., rAHF, rvWF, medium components, and CHO (host cell) residues, so that we have a pure product. From the 70001 our goal is to come up with just 11 of a purified, concentrated, safe, and effective drug substance.

We would like to leave factor VIII as integral as possible; we would like to remove all of the residues so that we have a very safe product; and we would like to do this at a cost of goods that is acceptable to the patients. To achieve these goals, we have developed a purification process as shown in Fig. 6. The first step is filtration, in which we remove the cells by physical means with a a filter that is small enough to allow factor VIII to pass through. The next step is immunoaffinity chromatography, using the same monoclonal antibody that is used for purification of the plasma-derived factor VIII preparation Hemofil M. The basis for the separation is reactivity: the monoclonal antibody identifies a specific region, a specific epitope, on the factor VIII molecule, and achieves a very high degree of purification. Most of the residues flow through the immunoaffinity column (Fig. 7). Factor VIII is then eluted off. Two different ion-exchange steps follow. By changing the pH of the column load, we change the charge on factor VIII. The residues, having a different charge, pass through the column.

Filtration	Physical (size)	To remove cells
↓		
Immunoaffinity (monoclonal antibody) chromatography	Chemical (reactivity)	Binds epitope
↓		
Ion exchange 1 chromatography	Charge (ion exchange)	Binds positively charged molecules
↓		
Ion exchange 2 chromatography	Charge (ion exchange)	Binds negatively charged molecules

Fig. 6 Factor VIII (rAHF) separation process

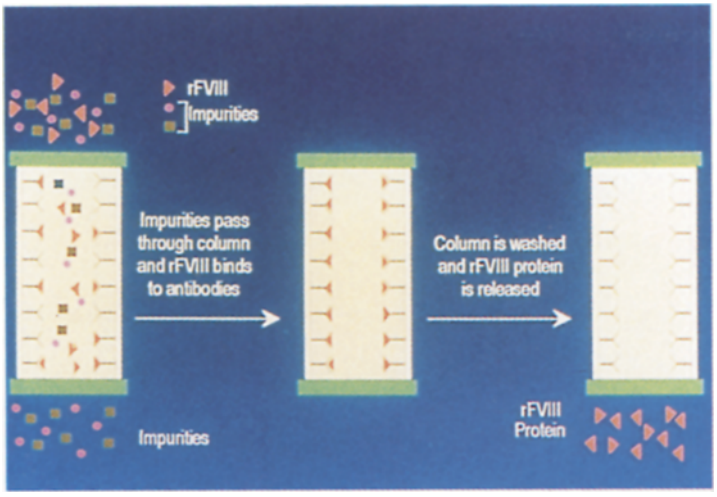

Fig. 7 Factor VIII is purified from medium components using a monoclonal antibody column and ion-exchange columns

During extraction:
1. Exposure to EtOH (65–70%) at pH 3 at 10–20° C for 4–7 h
2. Subsequent purification steps including:
   - Isoelectric precipitation
   - Gel filtration chromatography
   - Ion-exchange chromatography

Raw material is tested for:
BVD
IBR
PI 3
General cytopathic effect

Fig. 8 Purification of bovine insulin

The most important advantage of recombinant factor VIII is the absence of viral contaminants. Clearly, viral safety is a very important aspect of factor VIII. Of course viruses that are not present in the human source should not be introduced into recombinant factor VIII. We believe that we have eliminated problems that have occurred in the past as a result of human-source and raw materials, but we do not want to add any more problems to the situation. We therefore have worked out a five-point viral safety program.

The first point of this program is the choice of raw materials. We use several animal-sourced raw materials (but no serum, as already mentioned in the previous paper), and we have sourced our animal-based raw materials from certified herds. These three animal-sourced raw materials are subjected to solvent treatment steps and subsequent chromatography separation and are quite pure. It should again be emphasized that we do not use serum, so the general "gemisch" that one would get from serum, from fetal calf serum or whatever, is not present. All of our animal sources are highly purified and are derived only from cattle in Canada or the USA. One example of these animal-based raw materials is bovine insulin, which is exposed to low pH and high ethanol concentration and is purified via precipitation, gel filtration, etc (Fig. 8). The insulin is also tested for typical viruses. All other animal-sourced raw materials are also purified extensively, further assuring safety.

The next point in this program is the choice of cell line. Very few human viruses actually infect Chinese hamster ovary cells. Table 1 shows the results of a study by Dr. Lubiniecki and colleagues [1]. Human pathogenic viruses that were demonstrated to be nn were used to infect CHO cells. The viruses in fact were infectious to the reference cell, but most were not infectious to the CHO cells. Several viruses were infectious to the CHO cells, some in the parainfluenza line, mumps, and reo viruses. We used these virus lines as a base for our process validation studies.

We also make sure that the cells, both before and after cultivation, are free of any endogenous or adventitious viruses (Fig. 9) by means of a very extensive series of tests for microbial contaminants and various viral contaminants. With regard to retroviral particles, we determine whether the particles are infectious or defective, either before or after the campaign.

The fourth point in our program is "aseptic practices". The bioreactors are run in a very aseptic manner (Fig. 10). Therefore, human employees, who really are the worst source of viruses, are not able to infect the product or the CHO cells.

The fifth and probably most important point is "process validation". Even if we were able to get a virus into this system, our purification process has a very good capacity to remove it. This has been proven by validating the processes: we have chosen viruses from the various classes that could potentially infect the CHO system (Fig. 11); we scaled down the purification process to laboratory scale, we added these viruses to the scaled-down purification process, and we analyzed the removal of the virus through the system – just in case. We demonstrated a decrease of viruses of 7–13 logs. Therefore, we feel comfortable in claiming that we have a process that would not allow viruses in, would test against viruses, and, in the extremely rare chance that a virus actually infected the cells, would remove it. It can be assumed that it is a virus-free product.

Figure 12 shows the monoclonal antibody columns, two large columns acting as a single column in the cold room. The volumes get much smaller as you go through the process. In the end there is 1 l of drug-substance (Fig. 13), sufficient for thousands of patients, stored in a freezer at our site until it is fully tested and released for further processing.

Figure 14 is an overview of our testing program. The consistency of the process is demonstrated by quite a bit of testing. We test the working cell bank mentioned above; we assay the inoculum and the conditioned medium for microbial purity and endotoxin levels; we calculate the productivity of the bioreactor; we do post-production cell-line testing; we make certain that the chromatography buffers are contaminant free; we ana-

Table 1. Susceptibility of cell substrate to human viruses (*HFDL* human total diploid lung, *RMK* primary rhesus monkey kidney), *FA* fluorescent antibody staining, *CPE* cytopathic effect)

Virus	Reference cell line	Detection method	Growth in reference cell line	Growth in CHO-K1 cells	Growth in n-PA cell substrate
Adenovirus Group					
Adenovirus 3	HFDL	FA	+	0	0
Adenovirus 4	HFDL	FA	+	0	0
Adenovirus 7	HFDL	FA	+	0	0
Adenovirus 14	HFDL	FA	+	0	0
Adenovirus 21	HFDL	FA	+	0	0
Coronavirus Group					
Coronavirus 229	HFDL	CPE	+	0	0
Picornavirus Group					
Coxsackle A2	HFDL	CPE	+	0	0
Coxsackle A4	HFDL	CPE	+	0	0
Coxsackle A9	RMK	CPE	+	0	0
Coxsackle A10	HFDL	CPE	+	0	0
Coxsackle A16	HFDL	CPE	+	0	0
Coxsackle B1	RMK	CPE	+	0	0
Coxsackle B2	RMK	CPE	+	0	0
Coxsackle B3	RMK	CPE	+	0	0
Coxsackle B4	RMK	CPE	+	0	0
Coxsackle B5	RMK	CPE	+	0	0
Coxsackle B6	RMK	CPE	+	0	0
Echovirus 4	HFDL	CPE	+	0	0
Echovirus 5	HFDL	CPE	+	0	0
Echovirus 7	HFDL	CPE	+	0	0
Echovirus 9	HFDL	CPE	+	0	0
Echovirus 11	HFDL	CPE	+	0	0
Rhinovirus 1B	HFDL	CPE	+	0	0
Rhinovirus 2	HFDL	CPE	+	0	0
Rhinovirus 30	HFDL	CPE	+	0	0
Rhinovirus 47	HFDL	CPE	+	0	0
Rhinovirus 49	HFDL	CPE	+	0	0
Herpesvirus Group					
Herpes simplex 1	HFDL	FA	+	0	0
Herpes simplex 2	HFDL	CPE	+	0	0
Cytomegalovirus	HFDL	FA	+	0	0
Varicella-Zoster	HFDL	FA	+	0	0
Orthomyxovirus Group					
Influenza A	RMK	FA	+	0	0
Influenza B	HFDL	FA	+	0	0
Paramyxovirus Group					
Parainfluenza 1	RMK	FA	+	+	+
Parainfluenza 2	RMK	FA	+	+	+
Parainfluenza 3	RMK	FA	+	+	+
Respiratory syncytial	HFDL	FA	+	0	0
Mumps	Vero	FA	+	+	+
Measles	BS-C-1	FA	+	0	0
Reovirus Group					
Reovirus 1	RMK	CPE	+	+	+
Reovirus 2	RMK	CPE	+	+	+
Reovirus 3	RMK	CPE	+	+	+
Rotavirus	BS-C-1	FA	+	0	0
Togavirus Group					
Rubella	BS-C-1	FA	+		0
	BHK-21	FA	+	0	

Abbreviations Used: HFDL, Human Total Diploid Lung; RMK, Primary Rhesus Monkey Kidney; FA, Fluorescent Antibody Staining; CPE, Cytopathic Effect

lyze the eluates from the columns to make certain that the proper amount of factor VIII is in the system, and that the columns perform properly as in-process controls. Before we release the product to Hyland for further processing we have to go through a very rigorous set of release tests as well. Finally, the utilities in our production process are thoroughly analyzed to make certain that we are not adding any microbial contami-

- Microbial contaminants
  - Mycoplasma by both direct and indirect tests
  - Sterility for bacteria and fungi
- Adventitious viral contaminants
  - In vitro assay using 6 cell lines (human, bovine, primate and hamster)
- Retroviral contaminants
  - Transmission EM
  - Infectivity tets-S^+L^- for xenotropic viruses
  - Reverse transcriptase assay

Fig. 9 Purity testing of cells prior to and after cultivation

Fig. 10

Model virus	Type	Coat
Bovine diarrhea	RNA	Nonenveloped
Reovirus	RNA	Nonenveloped
Parainfluenza class 3	RNA	Enveloped
Murine xenotrope retrovirus	RNA	Enveloped
Simian virus (SV) 40	DNA	Nonenveloped

Fig. 11 Process validation with potentially infective viruses

Fig. 12 Monoclonal antibody columns in the cold room

Fig. 13 End-product – 1l, sufficient for 2000 patients

Drug Substance

1. Working cell bank
2. Inoculum-conditioned medium
3. Bioreactor-conditioned medium
4. Post-production cell line testing
5. Chromatography-buffers
6. Chromatography eluents
7. rAHF drug substance release testing
8. Environmental and utility testing programs

Drug Product

9. Acceptance testing
10. Sterility/endotoxin testing
11. Drug product release testing
12. U.S. FDA lot release

Fig. 14 Overview of testing program

nants or viruses to the general environment. When we finish our testing program, Baxter begins. They test our incoming product to make certain it is acceptable; they have their own sterility and endotoxin program for the final filled product. The FDA tests every lot of product before its release to the US market. Recombinate is an extremely well-tested product by the time it is received by the physician or the patient. As a matter of fact, Genetics Institute alone runs over 400 laboratory tests on every lot of factor VIII produced.

Figure 15 shows a gel electrophoresis of Recombinate concentrate before and Fig. 16 after thrombin digestion. The outside lanes are markers. The lanes show several lots of the recombinant factor VIII produced between 1986 (left lane) and today (right lane). It can be seen that the various protein bands show a very good consistency from one lot to the other, which is really the goal of a biological production process. A similar situation can be seen when the product is digested with thrombin and stained. One batch looks virtually identical to the next. Therefore, we feel comfortable in claiming that it is a controlled and consistent produc-

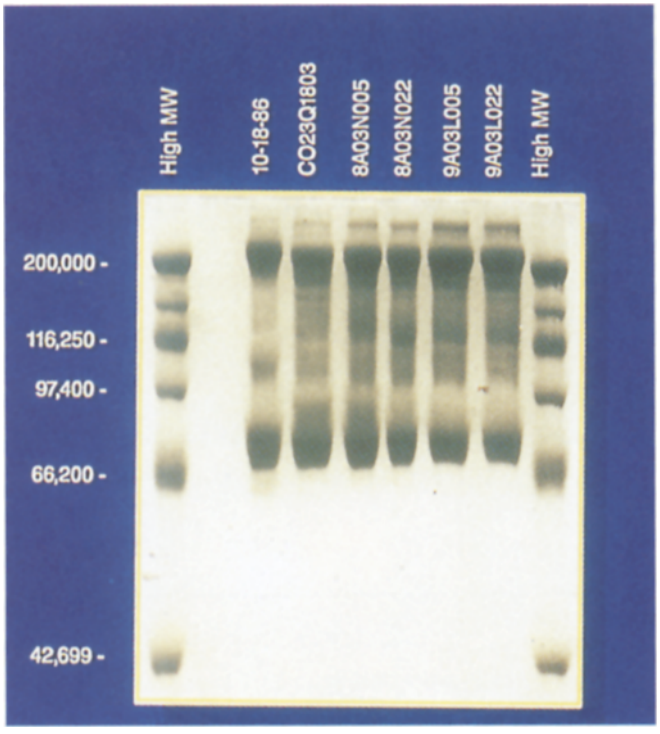

Fig. 15 Batches of bulk FVIII not digested with thrombin and stined with Coomassie blue; gel electrophoresis

tion process, that the product is virally safe, that it is extensively tested, and we are producing a high-quality, safe, and efficacious product.

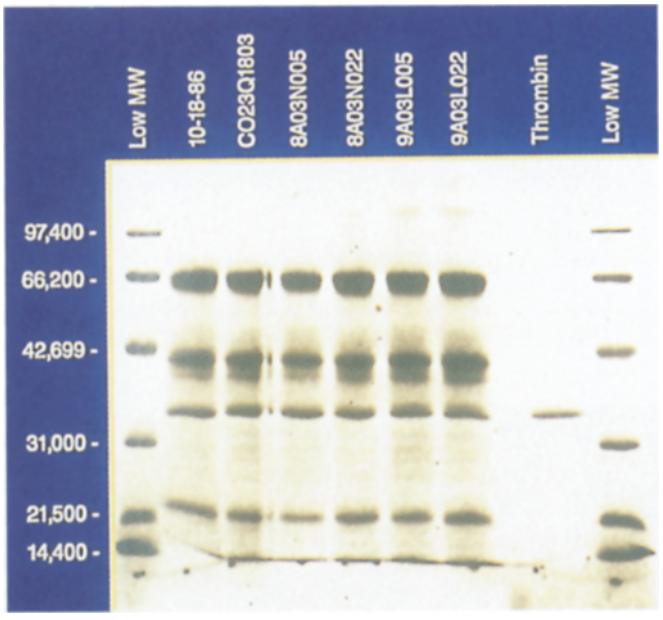

Fig. 16 Batches of bulk FVIII digested with thrombin and stained with Coomassie blue; gel electrophoresis

References

1. Wiebe ME, Becker F, Lazer R, May L, Casto B, Semense M, Fantz C, Garnick R, Miller C, Masover G, Bergmann D, Lubiniecki AS (1989) A multifaceted approach to assure that recombinant T-PA is free of adventitious virus. Dev Biol Stand 70:147–151