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Biotechnology in Lausanne: The *Rh* D Project

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Abstract. Hemolytic disease of the newborn is an often fatal condition of some newborn babies due to the immunogenicity of their *Rh* D positive erythrocytes in the *Rh* D negative mother. This condition can be prevented by injecting anti-*Rh* D antibodies. The current source of these antibodies is blood from immunized human donors. In order to avoid problems with limited supply and donor safety, the *Rh* D project was set up to develop recombinant monoclonal anti-*Rh* D antibodies as a possible replacement. In a multidisciplinary collaboration between the Zentrallaboratorium Blutspendedienst (ZLB) of the Swiss Red Cross, the Center of Biotechnology of the University and the EPFL (CBUE), and the Institute of Chemical and Biochemical Engineering (EPFL), co-funded by the Swiss National Science Foundation and ZLB, a candidate monoclonal anti-*Rh* D antibody has been selected, expressed in CHO cells, and a manufacturing process for large-scale production has been developed.

Introduction

At the Swiss Federal Institute of Technology Lausanne (EPFL), there are several laboratories active in various fields of biotechnology. Examples are the Laboratory of Biological Engineering (Laboratoire de génie biologique), headed by Prof. *P. Péringer*, the Laboratory of Polymers and Membranes (Laboratoire de chimie physique des polymères et membranes), lead by Prof. *H. Vogel*, and the Laboratory of Chemical and Biochemical Engineering (Laboratoire de génie chimique et biologique), directed by Prof. *U. von Stockar*. A few years ago, however, the Swiss Federal Institute of Technology and the University of Lausanne decided to strengthen their biotechnology efforts by creating the 'Center of Biotechnology of the University and the EPFL' (CBUE). This unit is jointly operated by the two schools and was designed to perform research that requires an intensive collaboration between life sciences and engineering sciences. It represents the only set-up in the Swiss academic world linking directly an institute of chemical engineering to a department of molecular biology. For the time being, it consists of three units: The Laboratory of Molecular Biotechnology, headed by Prof. *N. Mermod*, and the Laboratory for Cellular Biotechnology,

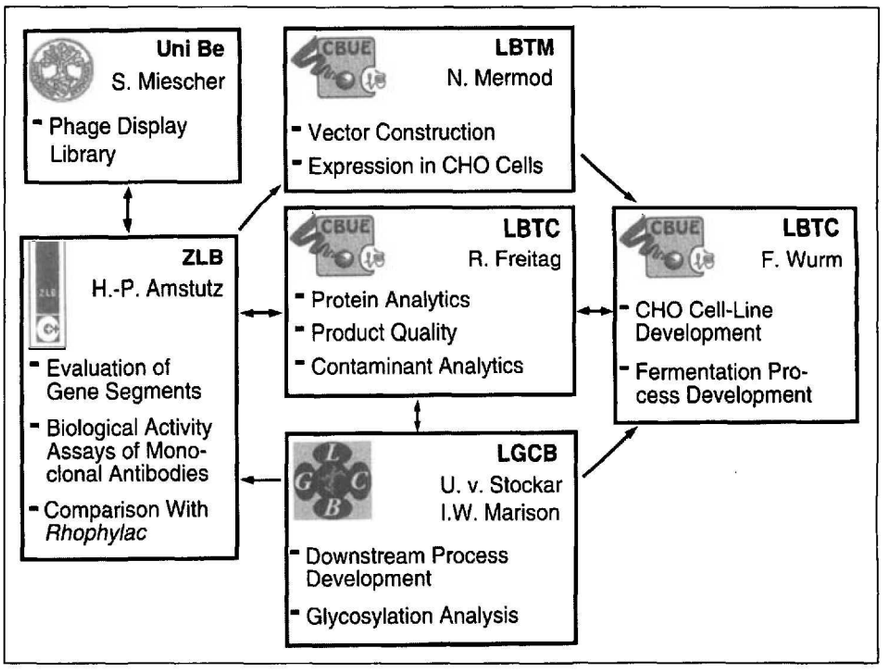

Fig. 1. *Schematic representation of the different laboratories and their responsibilities in the* Rh *D project*

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	Rhesus Phenotypes			Erythrocytes		*Rhesus* Partial D Variants							
antibody or F_{ab}	CCDDee	ccddee	CCddEE	Ccddee	ccdddEe	D III	D IVa	D IVB	D Va	D VI(J)	D VII	RH33	DFR
F_{ab} LD2-14	++++	–	–	nd	nd	++++	+++	+++	+++	–	+++	+++	–
IgG1-14 (Sup)	++++	–	+	+	±	++++	++++	++++	++++	++	++++	++++	++++
F_{ab} LD1-52	+++	–	–	–	–	+++	–	–	+++	–	++	–	–
IgG1-52 (Sup)	++++	–	–	–	–	++++	++++	++++	++++	–	++++	+	+++
F_{ab} LD1/2-6-3	+++	–	–	–	–	+++	+++	+++	+++	+	+++	+++	±
IgG1-3 (Sup)	++++	–	–	–	–	++++	++++	++++	++++	++++	++++	++++	++++
F_{ab} LD1/2-6-33	+++	–	–	–	–	+++	+++	+	+++	+	+++	±	±
IgG1-3 (Sup)	++++	–	–	–	–	++++	++++	++++	++++	++++	++++	+	++++

directed jointly by Prof. *R. Freitag* and Prof. *F. Wurm*.

The *Rh* D project, which will briefly be presented in this paper, is a typical example of applied research that can be carried out owing to the presence of a unit such as the CBUE.

The *Rh* D Problem

More than 80% of the population of Europe is *Rh* D positive, which means that a special protein, called *Rh* D protein, is inserted in the membrane of their red blood cells. This protein spans the membrane several times and presents to the outside of the red blood cells a number of loops which can be immunogenic in *Rh* D negative recipients of red blood cells. The problem arises when mothers who are *Rh* D negative carry a baby, which is *Rh* D positive. During the first pregnancy, which develops normally, it cannot be prevented that some of these *Rh* D positive red blood cells escape into the blood-circulation system of the mother. The immune system of the mother can recognize *Rh* D proteins as foreign and mount a strong immune response against it, resulting in the elimination of the few *Rh* D positive blood cells. But the mother is now immunized against *Rh* D proteins. When she carries a second fetus that is also *Rh* D positive, the immune system will synthesize large amounts of antibodies directed against *Rh* D proteins. These can easily cross the barrier of the placenta, penetrate into the fetus, and start to wage a war against its red blood cells. As a result, the baby may be born with very severe hemolytic anemia (low red blood-cell count). Brain damage and even death may be ultimate consequences of this condition.

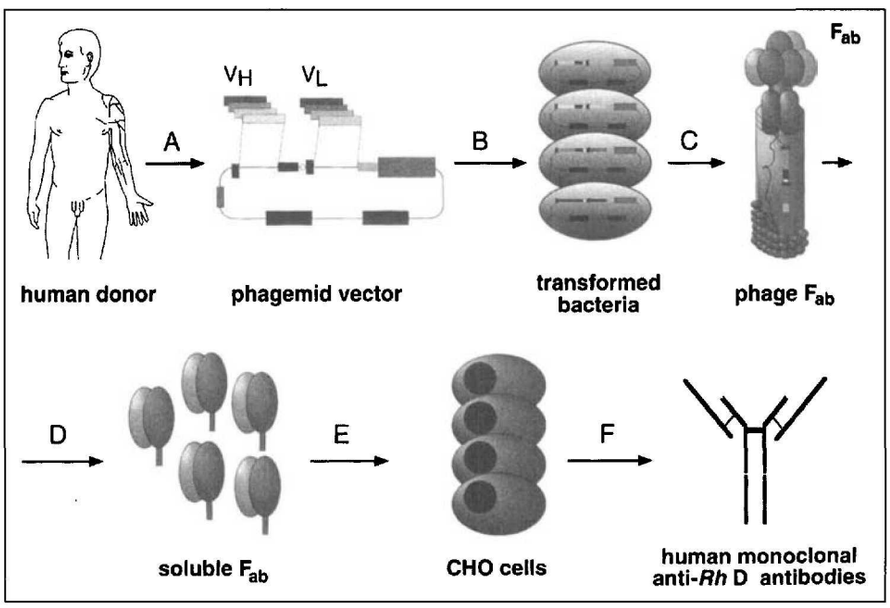

Fig. 2. *Representation of the strategy that was followed to obtain specific human monoclonal anti-*Rh *D antibodies.* RNA is extracted from the peripheral blood cells of a *Rh* D negative human donor immunized with *Rh* D positive erythrocytes. Heavy and light chain variable-domain genes are amplified by PCR and inserted in a phagemid vector. B) The phagemid vector is used to transform bacteria that are subsequently infected with phage and then produce phages expressing individual F_{ab} fragments on their surface. C) By positive and negative affinity selection using *Rh* D+ and *Rh* D– erythrocytes, phages expressing a *Rh D* specific F_{ab} are identified. D) The identified F_{ab} fragments are produced in soluble form in bacteria and their fine-specificity is analyzed using erythrocytes carrying different *Rh* D protein variants. E) The F_{ab} is cloned into a eukaryotic expression vector coding for a complete human antibody and used to transfect CHO cells. F) Clonal selection is performed to identify high-producer CHO cells which then produce human monoclonal anti-*Rh* D antibodies.

It has been found out in the sixties that this reaction of the mother's immune system can be prevented if she is given a dose of foreign antibodies against the D-protein during and just after the first pregnancy. What seems to happen is that these antibodies bind to the few fetal erythrocytes that circulate in the blood of the mother, and consequently, they are eliminated from her blood much faster than the

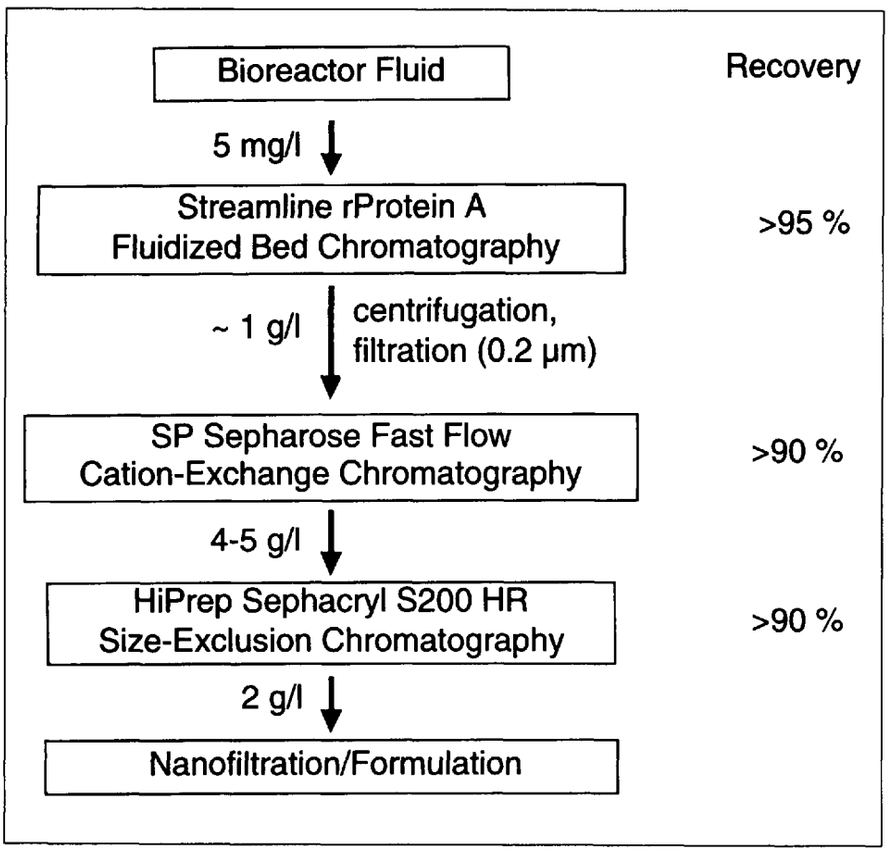

Fig. 3. *Schematic representation of the developed large-scale recovery and purification process*

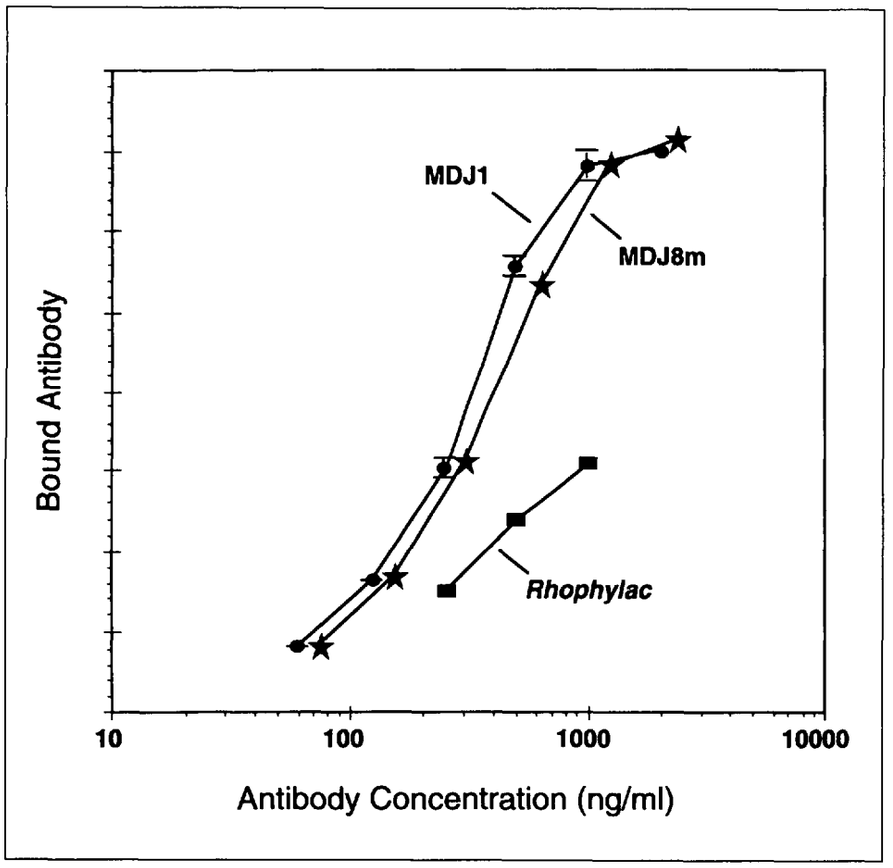

Fig. 4. *Binding activity on* Rh *D+ erythrocytes of* Rhophylac *and two IgG1-3 preparations obtained from different CHO clones as measured by FACS analysis*

time needed for immunization. Thus, the immunization of the mother is avoided, and she can give birth to a second *Rh* D positive baby without problems. The antibodies needed for this type of treatment are isolated either from the blood of mothers who have experienced the problem and therefore have these antibodies in their blood, or from *Rh* D negative individuals who volunteered to become immunized artificially. All donors have to be boosted frequently with red blood cells in order to maintain a reasonable anti-D antibody titer.

The source of these polyclonal antibodies is, however, rapidly running dry. The number of women who experienced the problem and therefore developed these antibodies is decreasing because of the treatment that is now generally applied. Immunizing volunteers becomes more and more difficult due to the need of injection of intact human cells. The pressure, therefore, mounts to develop a solution based on recombinant antibodies.

In Switzerland, a preparation of polyclonal anti-D antibodies is produced in the classical way and marketed under the name *Rhophylac*® by the ZLB of the Swiss Red Cross. A few years ago, the ZLB teamed up with several university laboratories in order to develop a recombinant product and a production process. The large research consortium that was organized to this effect was elected by the Swiss Priority Program for Biotechnology (SPP) to become the module on Medically Important Proteins and could, thus, benefit from a considerable financial support. The role of the different laboratories in this project is shown in *Fig. 1*.

Results

Fig. 2 sketches the strategy that was followed in order to develop the new product. Peripheral blood lymphocytes were prepared from an immunized anti-D donor and the genes coding for the F_{ab} fragments amplified by PCR. Based on the respective DNA, a phage-display library of immunoglobulin variable regions was constructed. A phage-display selection procedure was then applied in order to find phages that displayed F_{ab} fragments with interesting binding characteristics (for details, see [1]). The corresponding DNA was then recombined with the DNA coding for the rest of the antibody and further engineered for high-level expression in CHO (DG44) cells. Subsequent cell-line and process development enabled identification and isolation of CHO cell lines

with high productivity of the required IgG antibody.

Table 1 shows the fine-specificities of four of the F_{ab} fragments found in the phage-display library and the corresponding immunoglobulins obtained from the CHO cell culture, as detected in a hemagglutination test (for details, see [2]). In each pair of horizontal lines, the first one indicates specificity of the F_{ab} fragment from the phage-display library, and the second line the specificity of the cell-culture-produced complete antibody. As can been seen from the first five response columns, all the clones that were tested reacted very strongly against *Rh* D positive haplotypes, but showed no or only negligible reactivity against other *Rhesus* proteins, such as C or E. The selectivity against the various D variants known to exist in the population is shown in the last eight response columns in *Table 1*. As can be seen, clone number 3 reacted very strongly against all of the variants and was therefore selected for further development.

The target antibody IgG1-3 was produced in the cell lines MDJ1 and MDJ8. They were cultivated serum-free in roller bottles as well as in batch fermentations up to the 100-l scale.

A large-scale recovery and purification process was developed, which is schematically depicted in *Fig. 3*. The antibodies can be quickly recovered and concentrated to a high concentration by running the whole bioreactor fluid without pretreatment through a fluidized bed affinity-chromatography column. After centrifugation and filtration, the proteins are purified in a cation-exchange column, which was possible due to the fact that IgG1-3 is a protein with a comparatively high pI. The separation from aggregates *etc.* is carried out in a third step by size-exclusion chromatography. Finally, potentially present virus particles can be eliminated by nanofiltration.

The binding activity of the resulting immunoglobulins is shown in *Fig. 4* in terms of a binding-response curve against *Rh* D positive erythrocytes (for details, see [2]). The number of red blood cells carrying antibodies were measured by FACS analysis and the result plotted against the amount of antibody used to treat 1 ml of blood. It can be seen that the activity of the IgGs produced from the MDJ1 and MDJ8 clones was virtually identical and even better than the response of the polyclonal commercial product *Rhophylac*®. The F_c-dependent activity of the antibody was also tested in various assays, one of which is shown in *Fig. 5*. Macrophage activation was detected by the induced oxygen-radical production that could be measured by a chemoluminescence test. Again, it can be seen that the two selected clones were at least as good if not better than the commercial *Rhophylac*® preparation.

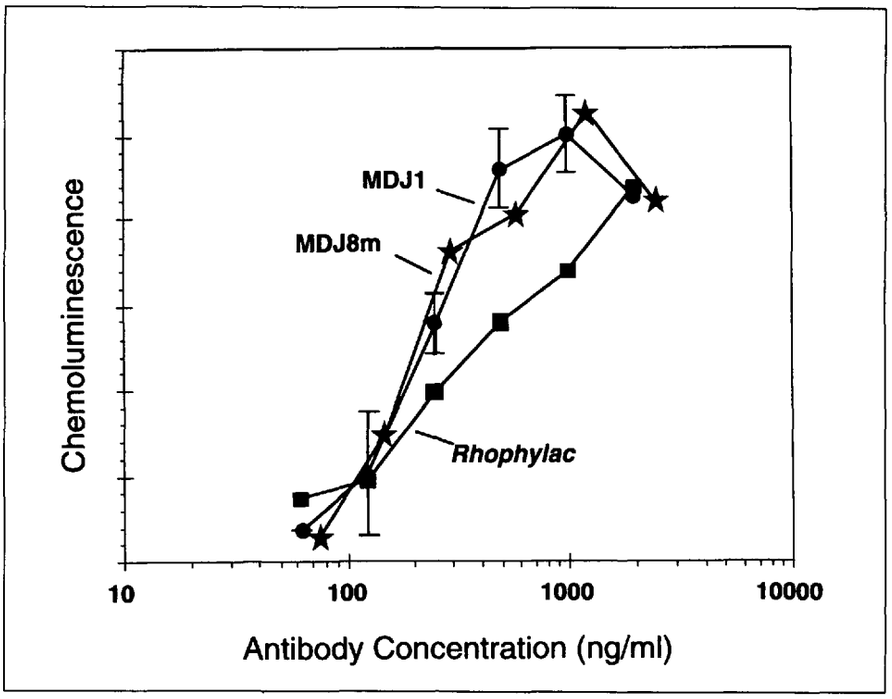

Fig. 5. *Macrophage activation by* Rhophylac *and two IgG1-3 preparations obtained from different CHO clones.* After binding to *Rh* D+ erythrocytes, oxygen-radical production by antibody-activated macrophages is measured by chemoluminesence.

Conclusion

The *Rh* D project demonstrates a successful collaboration between industry and university in biotechnology for the development of a commercial product and process in a short period of time. In this collaboration

- High-affinity recombinant human antibodies have been constructed and expressed in CHO cells. Antigen-specificity and F_c-mediated activities were comparable to approved polyclonal anti-D immunoglobulin (*Rhophylac*®).
- High-yielding, stable (1 year without selection), and clinically acceptable, suspension cell lines were obtained.
- A 10–100-l scale, serum-free and animal-protein-free production system (stirred tank) was established.
- A large-scale four-step recovery and purification process for therapeutic-grade recombinant antibodies was developed and applied up to the 50-l scale (overall yield 75%).
- Analytical method development and monitoring of glycosylation using high-pH anion-exchange chromatography, validated by LC-ES/MS, was performed.

Several hundred milligrams of anti-D immunoglobulins have been produced and purified so far for tests. It is planned to enter pre-clinical trials in late 1999. If all the tests turn out positive, clinical phase I will start in the year 2000.

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