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Historical reflections on cell culture engineering

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Abstract

Cell culture engineering has enabled the commercial marketing of about a dozen human therapeutic products derived from rDNA technology and numerous monoclonal antibody products as well. A variety of technologies have proven useful in bringing products to the marketplace. Comparisons of the technologies available 15 years ago are contrasted with those available today. A number of improvements in unit operations have greatly improved the robustness of the processes during the past 15 years. Further evolution of the technology is expected in several directions driven by commercial and regulatory pressures. Some problems remain for the next generation of cell culture engineers to solve.

Abbreviations: BSE – bovine spongiform encephalopathy; CHO – Chinese hamster ovary; CCL – continuous cell line; CJD – Jakob Creutzfeld disease; HIV – human immunodeficiency virus; Mab – monoclonal antibody; rDNA – recombinant DNA; tPA – tissue plasminogen activator; TSE – transmissible spongiform encephalopathy.

Introduction

This article will attempt to provide a historical perspective of cell culture engineering from the viewpoint of an industrial manager trained as a cell biologist who gets up in the morning to put novel biotechnology products on the market. The term 'novel biotechnology' is used to describe those technologies which came about as a result of scientific developments in the 1970s such as hybridoma preparation and what we know today as genetic engineering. This concept of novel biotechnology also serves to distinguish these newer tools from conventional biotechnology such as fermentation, which was successfully used for preparation of medicinals, vaccines, foods and beverages, and for sanitary engineering (Miller, 1995).

The author's conventional biotechnology experiences started in the 1960s with natural interferons and virus vaccines, and his exposure to novel biotechnology started in the 1970s with hyridomas, continued with recombinant DNA (rDNA) technology in cell culture in the 1980s, and rDNA monoclonal antibodies (Mabs) in the 1990s. In that time interval, many things have changed, mostly for the better, and this can be seen in the content of this series of meetings on cell culture sponsored by the Engineering Foundation since 1984. It would now be appropriate to describe some of what has changed, what enabled the changes, and what progress has been made as an industry in terms of putting novel products on the market.

Then

Compare snapshots of where the industrial use of cell culture technology stood 15 years ago (when the first Cell Culture Engineering meeting was being planned) and today. Then, no marketed products existed from continuous cell lines (CCLs). The first rDNA (rDNA) -derived product to be approved for marketing had just happened in 1982 (human insulin) using *Escherichia coli* as an expression system. There was substantial doubt that cell culture would survive as a manufacturing technology, and there was a feeling that cell culture as a source of viral vaccines (and its many

technological problems) would soon be superceded by *E. coli*-expressed rDNA products. The author joined Genentech, Inc at that time, when it was then very much a company in which *E. coli* was the predominant technology. An example of this occurred shortly after the author joined the company; when he was introduced to the head of process engineering, who asked, 'Tell me, what exactly is it that you cell culture people do with cow blood?' The nature of this question informed the author that he had a substantial education gap which would have to be closed in order to succeed!

The enthusiasm of the early days of microbial expression of rDNA proteins also reflected a general lack of appreciation for the need for large proteins to be correctly folded in order to be active, and for the ability of animal cells to fold and process highly complex proteins in ways that microbes could not do well. A famous biochemical engineer stated at a prestigious biochemical engineering meeting of the times that he had a quarter kilo of tissue plasminogen activator (tPA) in a jar which he held up for public view, which had been expressed in *E. coli*, and all of it was enzymatically inactive. In 1984, Genentech, Inc. entered human clinical trials with rDNA-derived tPA expressed in Chinese hamster ovary (CHO) cells, which was enzymatically and biologically active because it was properly folded and processed. Despite this technical success which enabled small scale human trials, it was far from clear in 1983 that cost–effective large scale animal cell technology was possible to implement for preparation of rDNA proteins. An established biochemical engineering consultant offered the opinion that six major technological miracles were needed to make it work at large scale.

Even if the technological miracles happened, it was also far from clear whether the regulatory community would approve the product for market. Major concerns existed over perceived risks of tumorigenicity for recipients of products derived from CCLs (Lubiniecki, 1987; Petricciani, 1985; Hopps, 1985). In fact, one senior regulator of the time confided to the author (years afterwards) that when he first heard the author describe the results of characterization of the CHO cells expressing tPA (basically, CHO cells are tumorigenic in nude mice and express retrovirus-like particles), he feared that the desire to investigate these products in humans would rekindle the spirited debates of 1954–1975 on the use of CCLs to prepare biologicals for human use. The then-recent discovery of human immunodeficiency virus (HIV) and its transmission to recipients of blood and plasma products and their derivatives (Curran et al., 1985) fueled concern in the regulatory community over the possibility of iatrogenic transmission of pathogenic viruses, especially retroviruses. There was also theoretically-based concern in a few ex-US regulatory agencies over the possibility of transmissible spongiform encephalopathies (TSEs) as a potential contaminant of medical products based on earlier studies of ovine scrapie and human kuru. These concerns were heightened because the unknown causative agent seemed unusually resistant to chemical or physical inactivation agents. This particular concern became more than theoretical a few years later in 1989 when bovine TSE or bovine spongiform encephalopathy (BSE) was discovered. Another problem was that no 'Points to Consider' document or ex-U.S.A. equivalent existed at that time for rDNA products, for monoclonal antibodies, or for products derived from CCLs. In short, there was no regulatory roadmap for the development of tPA as a biological product for human use. The only tools available were those of science and common sense (which fortunately were up to the task).

Now

If one were now to fast forward into the present, in 1998, most of these situations which have just been described look very different. Currently, there are 15 licensed/approved rDNA products plus 10 Mabs for *in vivo* diagnostic or therapeutic use in man which are derived from CCLs (Tables 1 and 2). In addition, there are four Mabs licensed for use in preparation of natural or rDNA-derived biologicals (Table 2). In addition, there are over 100 purified protein products created by novel biotechnology methods which are currently in clinical trials. Other things have also changed.

Some of the early Cell Culture Engineering meetings devoted substantial time to discussion of which technology was the best suited to prepare the products of novel biotechnology. In general, there were a variety of systems to choose from; sometimes it seemed as though there was at least one system for each presenter at the meeting. If one revisits these debates about bioreactor design and strategy, it is now possible to conclude that many of the beliefs about the adequacy of some of the proposed systems were perfectly correct based on the fact that multiple systems are actually used to prepare commercial supplies for regulated distribution (Table 3). Clearly, it is not only possible to use many of these systems, it is also possi-

Table 1. Licensed/approved rDNA biologicals expressed by mammalian cells

Product	Protein	Cells	Year[a]	Licensed
Activase/Actilyse	tPA	CHO	1987	Broadly
Epogen/Procrit/Eprex	Epo	CHO	1989	Broadly
Epogin/Recormon	Epo	CHO	1990	Japan, Europe
Saizen	hGH	C127	1989	Broadly
GenHevac B Pasteur	HBsAg	CHO	1989	France
HB Gamma	HBsAg	CHO	1990	Japan
Granocyte	G-CSF	CHO	1991	Japan, Europe
Recombinate	F VIII (80+90 kDa)	CHO	1992	Broadly
Kogenate	F VIII (80+90 kDa)	BHK-21	1993	Broadly
Pulmozyme	DNase I	CHO	1993	Sweden, U.S.A., Switzerland
Cerezyme	Glucocerebrosidase	CHO	1994	U.S.A., Austria, New Zealand
Gonal-F	FSH	CHO	1995	Sweden, Finland
Puregon	FSH	?	1996	Denmark
Novo Seven	F VIIa	BHK	1996	Switzerland, Europe
Avonex	IFN β	CHO	1996	U.S.A.
Bene Fix	FIX	CHO	1997	U.S.A.

[a] First licensure/approval.

Table 2. Licensed/approved MAb biologicals

Category	Product	Immunogen	Indication	Year	Licensed
Therapeutic	OKT3	CD3	GVHR	1986	Broadly
	Centoxin	Endotoxin	Sepsis	1990	Europe[a]
	ReoPro	Platelet IIb/IIIa	MI	1994	U.S.A.
	Panorex	?	Colorectal Cancer	1995	Germany
	Zenapax	IL2R	GVHR	1997	U.S.A.
	Rituxan	CD20	Non-Hodgkin B cell lymphoma	1997	U.S.A.
In vivo diagnostic	Oncoscint O/V	CEA	Cancer	1990	Europe, U.S.A.
	Myoscint	Myosin	Cardiac muscle necrosis	1989	Europe, U.S.A.
	Prosta Scint	?	Prostate cancer	1996	U.S.A.
	Verluma	?	Small cell lung cancer	1996	U.S.A.
	CEA-Scan	CEA	Colorectal cancer metastases	1996	U.S.A.
Preparative	Roferon A	IFNα2A	Purification from cell lysate	1986	Broadly
	Monoclate	Factor VIII	Purification from plasma	1987	U.S.A.
	MonoNine	Factor VIII	Purification from plasma	1992	U.S.A.
	Kogenate	Factor VIII	Purification from conditioned medium	1993	U.S.A.
In vitro diagnostic	✇100	Various	Various	1980	Broadly

[a] Withdrawn from marketing in 1993.

ble to conclude that they are adequately cost-effective otherwise the products would probably not remain in commercial distribution. However, this does not mean that the bioreactor systems are all equal, or that any one of them is capable of making all the products – there are no data to support such conclusions. It should also be remembered that some of the technologies discussed in the past are no longer used, and some of the companies founded upon them have disappeared. A few of these now defunct companies were Endotronix, Helix Biocore, Verax, Invitron, Damon Biotech, and BioResponse. In general, it seems to be true that the large volume products tend to be made from some form of suspension culture, whether perfused or batch. It also seems clear that simpler technologies perform more consistently and robustly at large scale than more complicated technologies, again giving an advantage to batch suspension culture as typically the simplest of all the major technologies in current use at large scale.

Several technologies have been critical to the development of robust cell culture technology, upon which the current rDNA and Mab product portfolio depends. Key examples include the development of 0.1 micron cartridge filtration in the 1970s to effectively remove mycoplasma contamination from animal serum. Prior to this, large scale cell culture meant either periodic contamination or use of irradiation (often ineffective) or alkylation agents (effective but not without risk) to reduce mycoplasma infectivity. Contamination by small bacteria such as pseudomonads can also be effectively controlled by use of 0.1 micron filters. Another major advance in the 1980s was the development of serum-free medium by a number of companies to support large scale growth of hybridomas and CHO cells. Developments in sanitary design technology such as diaphragm valves and steam block devices, steam-in-place, and clean-in-place equipment also improved the robustness of large scale cell culture. Additionally, widespread use of affinity chromatography and ion exchange resins capable of base sanitization and the availability of excellent analytical tools for proteins have added further robustness and reliability to these processes and products.

On the regulatory front, substantial progress has also been made since 1983. At this time, there is no serious concern by most regulators over potential tumorigicity risks from purified products with low levels of redsidual cellular DNA derived from CCLs. World Health Organization, which pioneered scientific risk assessment for residual cellular DNA (Petricciani and Regan, 1986), recently advocated a standard of 10 ng DNA per dose (WHO Expert Committee on Biological Standardization, 1998). This is 1000-fold higher than what was acceptable in 1983. Fermentation and purification processes for some products would be designed in a substantially different manner in 1998 than they were in 1983, and some would now have substantially higher yields from process designs based on the current recommendations. Many regulatory bodies now publish useful guidance documents for novel biotechnology products, and have undertaken efforts with industry trade associations to harmonize the regulations in the United States, Europe, and Japan for these products as part of the International Conference on Harmonization (Lubiniecki, 1997). Six such harmonized guidance documents have been prepared to date for novel biotechnology products, of which five are official in those geographic areas.

HIV concerns in 1983 turned out to be well-founded. Over 300 000 US residents have died from HIV infection and sequellae; over 6000 acquired HIV infection from contaminated blood and plasma-derived products. Recently, regulatory officers and health ministry officials in several countries have been imprisoned for a perceived lack of timely governmental action to protect the blood and plasma supply and hence the public health. Product liability settlements for HIV contamination of plasma-derived factor VIII alone has totaled over $1 billion. Naturally, such actions tend to focus regulatory attention as well as industry concern on preventing recurrence of this type of problem. This concern has also carried over to the use of human plasma proteins for preparation of other products, including use in cell culture, purification, and formulation of novel biotechnology products. For example, products for approval in the European Community may use only blood or plasma products which have come from materials tested as if the materials were to be used for blood donation *by the currently recommended test*. Advances in test technology lead to periodic changes in the nature of the recommended test, which may affect products in preparation, storage, or distribution which have been prepared with blood and plasma derivatives tested under the previous version of the recommended test. Rejection of product lots or product recalls from commercial distribution have resulted from such events without substantial warning.

Recently, concerns over transmission of BSE have been heightened by discovery of a new variant of Jakob Creutzfeld disease (CJD) in relatively young residents of the United Kingdom and France, pre-

sumed to be associated with the recent epizooitic of BSE in that geographic area (Will et al., 1996). Biochemical evidence is also beginning to appear which supports the similarity of the agents causing both the human and bovine diseases (Bruce et al., 1997; Hill et al., 1997). The European Union has created legislation to ban the use of specified raw materials (essentially, neurological tissue) from medicinal products for human or animal use. No distinction is currently made over whether the specified raw materials are product excipients or used earlier in bulk manufacturing. This concern not only applies to animal–derived proteins used for cell culture, but may also be applied to gelatin and tallow derivatives used for most orally administered pharmaceuticals. At this time, there is some question as to when and how this legislation will be implemented.

In addition to concerns over BSE and new variant CJD, new information suggesting the possibility of iatrogenic transmission of CJD by blood and perhaps plasma products donated by CJD patients prior to diagnosis has led to regulatory actions. The US Food and Drug Administration has withdrawn from distribution over 1600 lots of plasma derivatives containing materials derived from CJD patients or their close relatives during the past several years, and *any other products made with those 1600 lots of plasma derivatives*. Since plasma derivatives are prepared typically from large pools of up to 40,000 donors, even a rare disease like CJD with an incidence rate of one per 10^6 per year can potentially affect many product lots. Thus, regulatory concerns over CJD and BSE have changed the debate over the use of human and animal–derived raw materials (e.g., serum, transferrin, albumin) from a theoretical discussion in 1983 to the intensely practical problem of many product lots being recalled or destroyed in 1998.

In summary, the environment in which novel biotechnology products are developed, manufactured, and marketed has changed significantly in the past 15 years. Technologies have become proven, and many regulatory issues have been largely put to rest. A few old problems like HIV still provide concerns for human cell lines, and for cell culture performed with human raw materials like transferrin. CJD and BSE have provided a further basis for concern over human and animal–derived raw materials, especially those processed under relatively mild conditions.

Table 3. Technologies used to prepare commercial products

• Roller Bottles	Epo, hGH, HBsAg
• Microcarriers	Glucocerebrosidase
• Suspension	tPA, FVIII, FIX, alpha IFN, several MAbs
• Perfused suspension	FVIII, several MAbs
• Hollow fiber	*In vivo* diagnostic MAb
• Ascites	OKT3

Table 4. Licensed/approved purified natural proteins

Product	Protein	Cells	Year	License
Welferon	IFNα	Namalva	1985	Europe, Japan
Several	IFNβ	HDF	1980	Japan, Germany
Abbokinase	Urokinase	HDF	1990	Japan

Cell culture products in commercial distribution

As mentioned above, a number of purified protein products from novel biotechnology have been commercialized in the past 15 years. Table 1 shows that there are currently 15 licensed/approved rDNA products expressed in cell culture representing 12 nominal molecular entities distributed under 20 tradenames. Table 2 shows that there are six therapeutic Mabs, 5 *in vitro* diagnostic Mabs, and 4 preparative Mabs which have been licensed/approved. In 1997, the first chimeric and first humanized Mabs were licensed; these were created and developed specifically to overcome the human immune response to murine Mab determinants which limited the therapeutic utility of some early Mab investigational products used in a repeated dose regimen. Also in 1997. the first cellular therapy product (Carticell) was licensed. Table 3 shows that there are several cytokine preparations and one enzyme prepared from CCLs. In addition to these products of novel biotechnology, it should be remembered that there are also 12 viral vaccines still made from conventional biotechnology.

The first Mab product, OKT3, was licensed in 1986, and the first cell culture rDNA product, tPA, was licensed in 1987. Currently, about half of all commercialized rDNA products are expressed in cell culture, and well over half of those in the clinic are expressed in cell culture. Cell culture technology has been employed to express some very large and com-

plicated proteins, including, for example, Factor VIII (the size of the encoded protein is 330 kDa) and Mabs (heterotetramers) and tPA (17 disulfide bonds) which cannot be expressed in active form in microbial systems at commercially feasible levels. Cell culture technology has proven adequately robust and efficient to create these commercial markets, and is no longer thought of as a curious technique associated vaguely with cow blood and headed towards extinction.

The slow but steady growth of cell culture biotechnology and biotechnology products has resulted in significant sales. In 1996, an estimated $ 3.5 billion worth of rDNA and Mab products were sold, and about $ 1.0 billion worth of conventional viral vaccines were sold. This represents about 2% of the worldwide pharmaceutical sales (around $ 275 billion). In 1986, only the conventional viral vaccines were in commercial distribution. Of today's figure of $ 3.5 billion, $ 2.2 billion comes from a single product, erythropoeitin. The preparation and properties of many of these cell culture-expressed rDNA products have been reviewed recently elsewhere (Lubiniecki and Lupker, 1994).

It is also true that modern cell culture technology is capable of providing products which are safe in terms of risk of viral contamination. Previous generations of conventional biotechnology products (viral vaccines and plasma derivatives) were not free of such problems (Parkman, 1996). So far, there have been no product failures due to process safety concerns, reflecting a combination of cell bank characterization, raw material certification, process validation, quality control testing, and GMP compliance. While there have been a handful of incidents where viral contamination of in process materials was detected, these viral contaminants were not detectable in final product (Garnick, 1996; Burstyn, 1996). Such episodes have stimulated development of rapid PCR-based screening technology to ascertain the presence of specific viral contaminants prior to harvesting a given production fermentor (Garnick, 1996). Thus, current technology is quite robust and provides safe products.

Future trends

The recent clinical success of chimeric and humanized Mabs in overcoming the human anti-mouse antibody (HAMA) response problem, which limited treatment efficacy, is an example of ingenuity and science triumphing over natural limitations. It paves the way for additional products of this type in the future. There are also a number of truly human Mabs currently in clinical trial. One human Mab, Centoxin, was licensed in Europe in 1991, but was subsequently withdrawn for safety problems. This product was prepared using so-called trioma technology. Other technologies employed for creating human Mabs currently in clinical trial include immunization in mice whose immunoglobulin gene repertory has been knocked out and transgenically reconstituted with human immunoglobulin regions. Additional innovations will undoubtedly arise.

A number of investigational products are currently being prepared in transgenic animals. It seems likely that technical barriers to economical production systems in animals will be overcome. What is less clear at this time is what quality systems will be required to assure product quality, especially from an adventitious agent standpoint. It will take time to evaluate these methods and gain experience, but in principle there is no known reason why this cannot occur. Work is also ongoing with plant expression systems, but current product titers per unit biomass appear somewhat lower than those obtained in transgenic animals or in cell culture.

Genetic therapies based on genetic modification of cultured cells and/or genetic modifications of cells *in situ* by rDNA-modified viruses or plasmids continue to be developed. Successes are being reported with various systems, but so far, plasmid expression seem to be transient. It is not yet clear whether these approaches for therapeutic applications will lead to therapies, or whether the therapies will lead to products. Prophylactic applications such as vaccines are also under study, and here product efficacy may not be limited by transient expression.

The recent regulatory concern over CJD, TSEs, and HIV will probably continue to shift medium component selection away from animal and human materials toward chemically defined synthetic medium components. Required proteins such as insulin can be obtained from microbially-expressed microbial sources. Synthetic chemicals and plant-derived materials can substitute for natural products derived from animals or humans. While not yet regulatory requirements, concern over these risk factors can take on an unpredictable aspect. Those who have already removed the putative risk factor from their processes and products will be in a superior competitive position to those who have not yet done so if/when regulatory controls are imposed in the future.

Another trend among newer products, especially Mabs, is that doses are becoming larger. Most of the early rDNA products, especially the growth factor and hormone products, were administered in sub-milligram amounts. Many of the current new products are administered in tens or hundreds of milligrams, and a few are nearing the marketplace with regimens requiring over a gram of protein. This trend seriously moves the bar for process economics and process quality. The former puts pressure on cost of goods to keep selling price at appropriate levels, while the latter calls for higher purities for contaminants with fixed specifications per dose (e.g., DNA, endotoxin). For example, a regulatory specification of not more than 350 Endotoxin Units (EU) per 70 kg adult per 8 hr (or less) of treatment means a product specification of 350 EU mg^{-1} for a 1 mg hormone dose, but a product specification of 0.35 EU mg^{-1} for a 1 gram dose of Mab. Consistently attaining less than 0.35 EU mg^{-1} probably means having a target of 0.1 EU mg^{-1} or less, which requires substantial attention to sanitary equipment design, raw material specifications, and processing conditions to achieve on a routine basis. These trends will continue to drive the search for ever more efficient ways to grow cells, express protein, and purify protein drugs.

A final thought is that current cell culture technology allows the manufacture of every biopharmaceutical for which efficacy has been shown. In other words, there is no case where the technology has failed to deliver a commercializable product at an affordable price. Despite concerns whether cell culture technology would be cost effective, no product with good clinical data was ever kept off the market by high production costs. As stated above, this has been true in an era where doses for most biopharmaceuticals were at or below a mg per day. However, the current trend is for higher doses, especially for some Mabs, some of which are studied at doses of one gram per day. It is hoped that cell culture engineering methods will continue to improve and to be capable of providing the biopharmaceuticals of the future in a cost effective manner.

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