The cellular

Far removed from Hooke's images of microscopic cork, modern cell technology brings biologicals to market and drug production "to life".

BY MARK S. LESNEY

HOOKE'S IMAGE OF CORK: NATIONAL LIBRARY OF MEDICINE

Ever since Robert Hooke saw the many-chambered architecture of cork under the microscope in 1665, the cell has been at the forefront of biological study. Although cheese, yogurt, and winemaking have always utilized microbes to do work, the industrialization of biotechnology in the 1970s found exciting new occupations for cells in the service of humankind—the production of biotechnology products or biopharmaceuticals, from antibodies to insulin.

Currently, the vast majority of biopharmaceuticals are produced through bacterial fermentation. But more and more, the use of eukaryotic cell systems, including yeast and mammalian cells, is seen as the most likely method for routinely producing the most complex biomolecules in specific architectures that will make them immediately useful for pharmaceutical needs.

From then 'til now

In the beginning, all proteins for therapeutic purposes had to be purified from farm or laboratory animals, or from human cadavers. The rise of cell culture systems rapidly allowed the production of vaccines and antitoxins in vitro. The development of monoclonal antibodies from murine hybridoma culture in the mid-1970s created a dynamic new therapeutic avenue. Isolation and humanization of the specific antibody genes allowed fermentation in other cell systems. For example, Herceptin (Genentech, *www.genentech.com*) was created by the humanization of an anti-breast-cancer antibody that was genetically engineered into Chinese hamster ovary (CHO) culture for mass production.

In the area of recombinant protein drugs, the first cell systems for mass production were bacterial cultures. The first such drug approved by the FDA was human insulin for treatment of diabetes—Humulin (1987). Others rapidly followed, and today, there are more than two dozen approved recombinant therapeutic or vaccine proteins—whether produced in bacterial, yeast, or mammalian cell lines—and hundreds more potential drug candidates are in the discovery and development pipeline, many already in clinical trials.

© 2003 AMERICAN CHEMICAL SOCIETY

SOLUTION

Vive la différence!

Despite the commonalities of genomes across the kingdoms of life, all cells are not created equal when it comes to how they treat proteins. Bacterial cells are incapable of post-translationally processing proteins that require glycosylation, phosphorylation, or a variety of assisted foldings. In an attempt to get around such difficulties, almost since the beginning of the genetic engineering revolution, a (literally) growing group of non-*E. coli*, eukaryotic cell types has been developed for producing recombinant proteins—each with different benefits and problems (*1*).

Fungi. Not surprisingly, because the properties of fungi allow easy fermentation culture and vat processing similar to *E. coli*, a variety of yeasts, including *Saccharomyces* and *Pichia* species, have proved useful for industrial-scale recombinant protein production. Yeast glycosylation patterns differ from those of humans in their tendency to add high-mannose structures, which, while nonimmunogenic, are likely to have a shorter half-life. The filamentous fungus *Neurospora* is also proving itself a viable cell line for producing a wide variety of proteins, both human and animal, as shown by the work of researchers at Neugenesis (*www.neugenesis.com*).

Recently, S. R. Hamilton and colleagues created a humanized pathway by adding five active protein genes to *Pichia pastoris*. The genes coded for mannosidases I and II, *N*-acetylglucosaminyl transferases I and II, and the UDP-*N*-acetylglucosamine transporter. Together, these new proteins enable the production and secretion of a human glycoprotein with appropriate complex *N*-glycosylation (*2*).

Human cells. Although human cells are not the most widely used for recombinant protein production via fermentation, in many cases, using an appropriate cell line is the critical first step for producing sufficient proteins for research purposes and analysis. In other cases, a human cell line may be suited for industrial-scale protein production under the most "natural" possible conditions of physiology and glycosylation. Human cell line *N*-glycans terminate in *N*-acetylneuraminic acid (sialic acid). If these are absent, the liver is signaled to remove the glycoprotein from circulation. Other animal cells, such as CHO, are close but not identical in their patterns.

CHO cells. These are perhaps the most utilized mammalian cells for recombinant protein production. A typical example is the manufacture of recombinant tissue-type plasminogen activator protein (tPA), a glycosylated serine protease used to treat cardiac and stroke patients because of its ability to prevent or break up blood clots. The glycosylation pattern of tPA is critical to its effectiveness, and neither unmodified yeast nor *E. coli* is an acceptable fermentation system. The FDA recently determined that distinct glycosylation patterns indicate significant differences that can serve as the

basis of regulation. More such drugs will likely prove to be similarly sensitive—and similarly the subject of regulatory scrutiny. CHO glycosylation is close to the human pattern, although it produces *N*-glycans terminating in *N*-glycolylneuraminic acid.

Mouse cells. Of course, among mammalian cells, the first and classic mouse-derived cell lines are the hybridomas used for antibody production. They are derived by fusing the spleen cells of an immunized animal with mouse cancer cells. Although still popular, hybridomas have often been replaced for convenience and yield considerations with recombinant antibodies produced in other cell lines, including *E. coli* and the yeasts. Other, nonhybrid, murine cell lines have been used for recombinant protein production.

Insect cells. Insect cell fermentation systems are produced using a baculovirus vector to introduce the recombinant protein. These cell types also produce nonhuman patterns of carbohydrate side chains, high in mannose, though shorter than those in yeast—potentially having similar half-life problems.

There is little likelihood that the need for cells as bioreactors will disappear in the near future.

The medium needs the massage

Just choosing the correct cell system does not guarantee efficient protein production. Yield can vary widely even under an optimal growth environment (which may be difficult to monitor and control). The original recombinant vector construct and the location of insertion of the gene of choice, as well as promoter considerations, can all play a part.

Many researchers have worked to develop means of optimizing these parameters in more than a trial-and-error fashion based on monitoring bulk cell growth and protein production. One such study done recently at the National University of Singapore (*www.nus.edu.sg*) used green fluorescent protein/fluorescent-activated cell sorting (GFP/FACS)-based screening with homologous recombination to generate high-yielding subclones. The GFP/FACS system also proved efficient at optimizing a serum-free medium for growth of these cells (*3*).

A matter of definition

Because recombinant protein cell systems are biological, they have unique and sometimes seemingly onerous problems involved in their use and purification compared with the "simple" chemistries of small-drug synthesis. These difficulties include ensuring safety through cGMPs (current Good Manufacturing Practices). In addition, issues of media appropriateness and safety, as well as cell standardization, are profound in recombinant protein production.

Originally, animal cell cultures were grown in the presence of clarified blood serum or with added animal proteins that provided a nutritional component deemed necessary for efficient cell growth. But using animal components such as human or bovine serum, or even purified natural proteins, in the growth media added the real risk of contamination (*4*). Researchers feared contamination from animal viruses, mycoplasmas, and even prions. This problem is compounded by the necessity of monitoring the cell lines themselves for infection or contamination, either from the original source stock, from human handling, or from improperly cleaned containers and piping in the reactor process.

For this reason, numerous companies, including Sigma-Aldrich (*www.sigmaaldrich.com*) and Gibco-BRL (a subsidiary of Invitrogen, *www.invitrogen.com*) have developed both defined media and defined cell lines that can give the reproducibility and response required by manufacturers and researchers alike (*5*).

Not only are defined media and fermentation conditions critical for cleanliness purposes, they can also be necessary for appropriate glycosylation of recombinant proteins, even in cell systems capable of correct processing. To make matters more complex, improper nutrition, pH, or shear stress can lead to incomplete or faulty glycosylation despite the presence of the appropriate enzymes. In addition, cells grown under bioreactor conditions can be highly susceptible to apoptosis, requiring stringent control of nutrient and oxygen availability.

To HeLa and back

Cell cultures, being living systems, have unique problems. In particular, living contaminants can piggyback or even take over cell systems covertly, confounding data and destroying product in a way that might not be noticeable until it's too late. Like after publication. The original and most traumatic example of this problem was the mass contamination of cell cultures revealed in 1999. Rod Macleod, head of the DSMZ, a German cell culture collection, showed that of 252 cancer cell lines, 18% were mislabeled or overgrown by other cell types, such as HeLa cells. The revelation sent shock waves through cell culture researchers and the cell culture industry (*7*). And according to the American Type Culture Collection (ATCC, *www.atcc.org*), the problem continues, with two-thirds of journals failing to cite sources of biological materials or demanding that they be deposited in places of record. However, even that might not be sufficient, since, in addition, "Many of the cultures ATCC receives for deposit are misidentified or contaminated."

Fermentation for pharma

Today, many companies are producing recombinant proteins for research and therapy using fermentation—from small flasks to huge bioreactors.

Much effort is expended on developing the best fermentation scheme, generally dividing the methods into two main classes based on whether the cells move or are stationary (*6*). Mobile cell systems include classic cell suspension systems such as "stirred tank reactors", which evolved from the laboratory spinner flask.

Stationary fermenter systems include the classic roller bottle systems (in this case, the cells adhere to the inner walls of the flasks) and cells plated out on Mongo plates. A more recent

development is microcarrier bead systems. The beads provide porous surfaces of different dimensions (depending on commercial source) that serve as a three-dimensional substrate for cells to adhere to while the beads are agitated in media or, if stationary, have media circulated around them.

Additionally, woven mats or hollow-fiber bioreactors have been developed that maintain the cells in stationary adherence on the surface of the fibers, with the channels available for the passage of nutrients and secretion of the desired protein products.

For manufacturing purposes, cell lines are classified and maintained with certain defined status categories: the master cell bank (MCB) is prepared from the initially transformed and cloned cell line. The MCB exists as distributed aliquots stored under defined conditions; these aliquots act as the "seed stock" for the working cell bank (WCB), which can be used to form production-level quantities of cells or as the start of a new MCB. Under GMP protocols, MCBs and the WCBs derived from MCBs are tested for various potential problems, from contamination to construct or product degradation, with the most stringent testing reserved for the MCB. Contract research organizations such as Charles River Laboratories (*www.criver.com*) have developed to provide cell banking for the critical MCBs and WCBs, com-

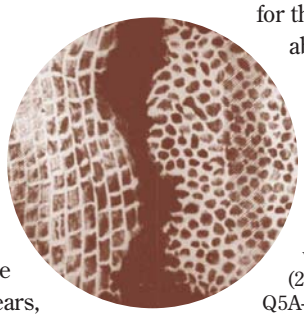
Selected providers of cell culture equipment and biologicals

Supply area	Company	Website
Culture equipment	BD Biosciences	www.bdbiosciences.com
	Corning	www.corning.com/lifesciences
	Cytogration	www.cytogration.com
	Greiner Bio-One	www.greinerbioone.com
	Kimble/Kontes	www.kimble-kontes.com
	New Brunswick Scientific	www.nbsc.com
	Nalge Nunc	www.nalgenunc.com
	Sarstedt	www.sarstedt.com
	Vivascience	www.vivascience.com
	Wave Biotech	www.wavebiotech.com
	Westfalia Separator	www.westfalia-separator.com
	Wheaton Science Products	www.wheatonsci.com
Media	American Tissue Type Collection	www.attc.org
	AMRESCO	www.amresco-inc.com
	BD Diagnostic Systems	www.bd.com/ds
	BioSource International	www.biosource.com
	Gibco-BRL	www.invitrogen.com
	Harlan BioProducts	www.harlan.com
	ICN Biomedicals	www.icnbiomed.com
	JRH Biosciences	www.jrhbio.com
	Molecula Research Laboratories	www.molecula.com
	Qbiogene	www.qbiogene.com
	Serologicals Corp.	www.serologicals.com
	Sigma-Aldrich	www.sigma-aldrich.com
Cell lines	Analytical Biological Services	www.absbioreagents.com
	Active Motif	www.activemotif.com
	American Tissue Type Collection	www.attc.org
	Cascade Biologics	www.cascadebio.com
	Charles River Laboratories	www.criver.com
	New England Biolabs	www.neb.com
	Promega	www.promega.com

For a more complete list of providers, visit *www.labguideonline.com.*

plete with cGMP analytical services such as sterility and mycoplasma contamination testing.

Problems and promises



There is little likelihood that the need for cells as bioreactors will disappear in the near future. The complex chemistries of protein production and secondary modifications, while perhaps theoretically doable in test tubes or solid-phase synthesis, are easier and far more economical in living cells. But there is some question about whether vat fermentation is the optimal method of utilizing those cells. In recent years, many people have raised fears that, as more biopharmaceuticals are developed, the fermentation capacity of the pharmaceutical industry will rapidly be used up, creating a significant bottleneck.

Many are therefore gambling that these long-term problems of overloaded manufacturing capacity coupled to the significant costs of fermentation culture, both in production and regulatory monitoring, may lead away from biofermenter cell culture and back to the purification of products from whole plants and animals, this time made transgenic for the genes of interest to produce desired proteins in milk or seeds. This is the impetus behind the burgeoning field of gene "farming".

But despite the promise of therapeutic proteins produced "on the hoof" or "on the cob" in transgenic animals and plants, it is likely that bioreactor fermentation of biologics is here to stay—at least for the immediate future. Fermentation is a defined, scalable, and much-studied and "comfortable" technology, highly adaptable, constantly improving. Current successes will likely continue, promoting replication and imitation for the foreseeable future.

References

(1) Zopf, G.; Vergis, G. *Pharmaceutical Visions*, Spring 2002; www.neose.com/pdf/Pages_10-14.pdf.
(2) Viruses in cell culture lines; www.fda.gov/cder/guidance/Q5A-fnl.PDF.
(3) web.mit.edu/sma/events/symposium/2003_symposium/papers/mebcs/MEBCS012.pdf.
(4) Hamilton, S. R.; et al. *Science* **2003,** *301,*1244–1246.
(5) www.the-scientist.com/yr1996/apr/tools_960401.html.
(6) www.the-scientist.com/yr2003/oct/lcprofile_031006.html.
(7) Coghlan, A. *New Scientist,* Sept 20, 2003, pp 8–9.

Mark S. Lesney is a senior associate editor of *Modern Drug Discovery*. Send your comments or questions about this article to mdd@acs.org or to the Editorial Office address on page 3. ■

KEY TERMS: automation, cell biology, medicinal chemistry, process, regulations, technique

Request more at AdInfoNow.org