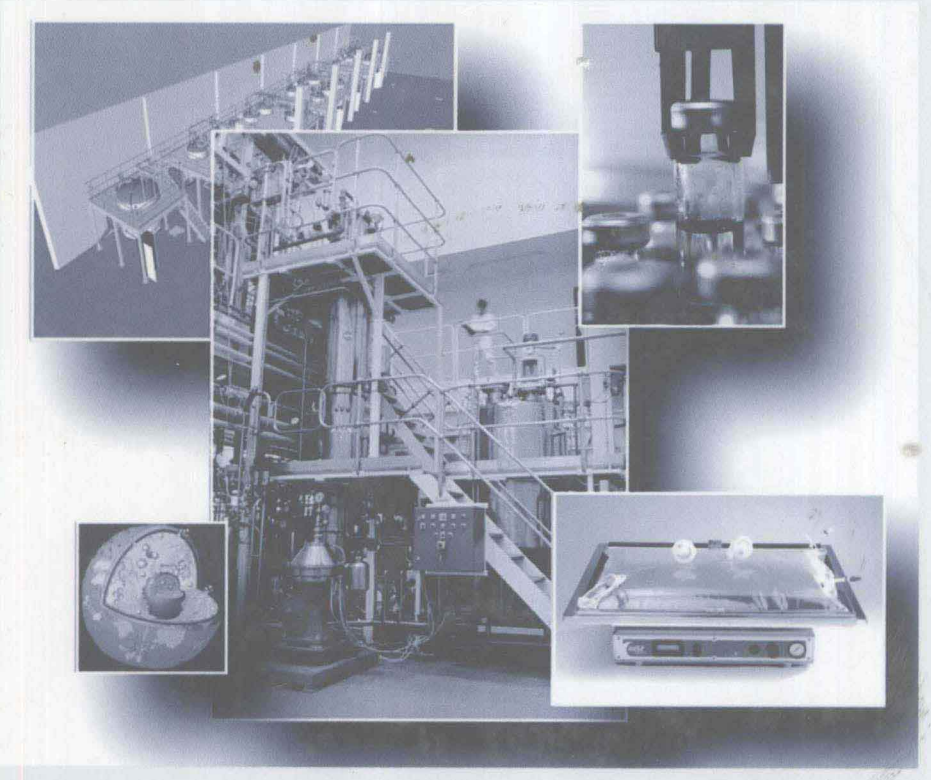
CELL CULTURE TECHNOLOGY FOR PHARMACEUTICAL AND CELL-BASED THERAPIES



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CELL CULTURE TECHNOLOGY FOR PHARMACEUTICAL AND CELL-BASED THERAPIES

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Preface

In the past quarter century, we have seen protein therapeutics greatly expand the horizon of health care. Many life-threatening diseases or chronic dysfunctions—once beyond the reach of medicine—are now readily treatable. Much of the impact of protein therapeutics has come about through the successful use of a new production vehicle—mammalian cells. Although the emergence of cell culture cultivation technology is relatively recent, the demand for technological advances to meet medical needs has induced a rapid industrial transformation. Cell culture processes have become an established method of creating new medicines in a short decade.

Numerous books, encyclopedias, and conference proceedings have captured important aspects of cell culture processing over the years, helping to disseminate knowledge of this technology. As we have watched the reach of protein therapeutics continue to grow and many new professionals continue to enter careers related to this technology, we have come to the realization that a comprehensive, single-volume treatise on cell culture technology would be a worthwhile addition. We envisioned the book to cover cell culture bioprocessing from A to Z, starting from cell line development to the finished product. It encompasses both fundamental principles and practical aspects of the technology to serve as a resource for information for seasoned professionals, and as comprehensive reading for those new to any specific area of cell culture technology. This book is designed to last for many years to come.

Covering a wide range of topics essential to cell culture technology in a single volume is no easy task. We are fortunate to have the participation of many esteemed experts as contributors. Their dedication, commitment, and hard work transformed our ideal into reality. Chapter 1, an overview of cell culture technology, reviews the state-of-the-art technology and provides an historical perspective. The following chapters on cell line development, medium, and process take the reader through a journey of product development. Chapter 2 focuses on recombinant DNA technology and cell line development. A detailed discussion on medium development is presented in Chapter 3. Chapter 4 outlines the current understanding of cell metabolism for optimizing the performance of cell culture systems. An extensive review on protein glycosylation is presented in Chapter 5. Chapter 6 gives an overview of cell culture bioreactors. In this chapter and Chapter 7, a number of reactor operational topics, including aeration, mixing, and hydrodynamics in bioreactors is discussed. Process instrumentation and process control are important for production of cell culture-derived products, and these topics are covered in Chapter 8. This

discussion is followed by Chapter 9, which covers the utilization of cell culture kinetics and modeling. Cell culture bioreactors operate in different modes, and this book covers both fed-batch (Chapter 10) and high-density perfusion (Chapter 11) operation. Both chapters are comprehensive in their coverage and they provide extensive guidelines for the cultivation of mammalian cells for the production of recombinant proteins.

Cell separation and product capture in cell culture technology are the topics of Chapter 12. This is followed by detailed coverage of downstream processing (Chapter 13) and formulation (Chapter 14). The next two chapters focus on commercialization of cell culture processes. Chapter 15 deals with process and equipment validation, and Chapter 16 provides extensive guidelines for a commercial facility design. Two somewhat unconventional methods for protein production using cell culture technology are presented in Chapters 17 and 18, which cover transient expression and large-scale propagation of insect cells, respectively. The final two chapters of this book are dedicated to stem cells and their application for cell-based therapies.

We thank all the contributing authors for their dedication and diligence in bringing this project to fruition. Our families and many friends have been extremely supportive during the editing of this book. In addition, we would like to recognize Amy Fayette, Denny Kraichely, Subinay Ganguly, David Epstein, and Kristen Cosgrove for their help in proofreading and Anita Lekhwani for the initiation and support of this project.

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Cell Culture Technology—An Overview

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INTRODUCTION

The last three decades have witnessed a major development in cell culture technology. The field developed substantially and became an essential part of biotechnology (1). During its evolution, cell culture technology successfully integrated various disciplines, including cell biology, genetic engineering, protein chemistry, genomics, and chemical engineering. Cell culture technology is now the established method of producing a number of important proteins, especially those that are large, complex, and glycosylated (2).

Cell culture technology–derived products are currently used as medicines to prevent and treat serious diseases such as cancer, viral infections, heredity deficiencies, and a variety of chronic diseases. The products are proven to be safe, effective, and economical. The capacity requirements for these products and the market they generated exceed the initial estimates. Some of the cell culture–derived products have a demand of 500 kg/year and generate $1–2 billion in revenue.

Cell culture technology went through a significant evolution from its origin to its commercialization. Cell culture techniques were originated as study tools to investigate cell and tissue behavior and function in vitro (3,4). Utilization of cell cultures for therapeutic purposes started with the use of cells for vaccine production. Cultured cells were successfully used as hosts to grow viruses, opening the field of large-scale vaccine manufacturing.

The next big step for cell culture technology was the acceptance of continuous cell lines by regulatory agencies. Continuous cell lines can grow indefinitely, have less stringent growth requirements, and, most importantly, they can be cultured in suspension. Elimination of solid substrate in suspension culture allows scaling up by volume and allows the cells to grow in bioreactors using well-established methods similar to those used for microbial systems.

Genetic engineering and the use of recombinant DNA technology made it possible to produce a vast number of products in cell culture. A number of vectors are now used in a variety of cell lines to produce native and modified human proteins. Engineering the machinery of the cells also allowed the modification of protein products for stability, efficacy, and biological activity. A parallel development in

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cell culture technology resulted in tissue engineering, where cells are produced as products for tissue replacement and gene therapy.

Advancement and development of cell culture technology required an interdisciplinary approach. As a result of close collaboration and tight integration between cell biology and biochemical engineering, cell lines with excellent productivities can now be cultivated in large-scale bioreactors and at very high cell densities. Issues related to shear sensitivity, aeration, and mixing in bioreactors are largely resolved. The processes can be scaled up successfully and cells can be cultivated consistently in 20,000 L bioreactors. Advances in medium development and cell retention technologies resulted in very high cell densities in the bioreactors. In addition to enhancing productivity in the bioreactors, the cost of the cell culture process was diminished significantly by the elimination of serum and other high-cost proteins from the medium. Now, chemically defined medium is a reality. This progress contributes significantly to the safety and reliability of raw materials in the production of biologicals. Finally, the technology now utilizes efficient separation, purification, and virus inactivation methods that result in highly purified, efficacious, and safe products.

This chapter overviews the development and evolution of cell culture technology from a historical perspective, and reviews the state of the art of cell culture technology. There are several products from cell culture technology approved for treatment of diseases; a brief description of these products is also presented. Finally, a look into the future of the biotechnology field and an assessment of current products in development are provided.

A BRIEF HISTORY OF CELL CULTURE TECHNOLOGY

Methods for growth and maintenance of primary cells in vitro opened the doors for cell culture technology. Early studies with embryos and fibroblasts provided tools for studying cell behavior and function in vitro. These studies also provided a tool for developing cell culture media. The cells used in those days had a limited life span unless they were transformed or were originated from tumors. The production of biologicals from cell culture evolved gradually over the last three decades.

Early Days of Cell Culture Technology—First Products

Utilization of cultured cells for the production of viral vaccines was the first application of cell culture technology. Viruses for vaccine production need living cells to propagate. Embryonic chicken in eggs traditionally was used for vaccine production. Due to increased demand, alternative methods were sought and cell culture technology was the answer. The production of polio vaccine using cells grown in culture started in 1954 (4). The cells were primary monkey kidney cells grown on surfaces (attachment dependent cells). A similar development took place for the vaccine against foot-and-mouth disease for veterinary use. Large-scale vaccine production for FMD virus was a major activity 36 years ago. Increased demand and process economics required more effective and scalable processes. Baby hamster kidney (BHK) cells were adopted for this process and FMD vaccine could then be produced at a 5000 L scale in suspension bioreactors. The medium used for this production was Eagle's medium with 5% adult bovine serum. Virus production continued to utilize cell culture technology, and vaccines such as measles, rabies, mumps, rubella, and

varicella are produced in large quantities. In addition to these human vaccines several veterinary vaccines are manufactured by cell culture (5).

Production of biologicals for medical applications also underwent a significant evolution. Interferon (IFN) alpha was the first cell culture derived product used as a drug. IFNs were traditionally made from human white blood cells from blood donors. The production of IFN was very limited, as it was dependent on donor blood cells and was present at very low concentrations. Work at Wellcome Research Laboratories was instrumental in this area. Initially, researchers at Wellcome wanted to use BHK cells as they could be expanded in suspension and these cells have already been approved for vaccine production. However, IFN-alpha was to be used as a medicine and issues were raised for the BHK cell line. BHK cells contain C-type virus particles and they have oncogenic potential. Nawalwa lymphoblastoid cells, on the other hand, had a better biosafety profile and could be cultivated as easily as BHK cells. The production of IFN moved cell culture technology to the 10,000 L scale and the process is being used successfully for IFN production.

Progress in Cell Line Acceptability

As mentioned before, cells used for early vaccine production were primary cells such as monkey kidney cells and chicken embryo. These cells are attachment-dependent cells requiring a surface on which to grow. In addition the primary cells have stringent growth requirements (growth factors, serum) and their expansion requires a lot of manipulation (trypsinization). These requirements inhibit the use of attachment-dependent cells for large-scale production. The shift from attachment cells to suspension cells was a major advancement for cell culture technology. The use of BHK cells for veterinary vaccines came from the need to meet the demand for FMD vaccine. BHK cells are continuous cell lines and can grow in suspension. Although the BHK cells were accepted for veterinary vaccines, these cells could not be approved for human vaccines. BHK cells were considered unsafe because they contain tumorigenic agents and viruslike particles. As a result, regulatory agencies (WHO, FDA) evaluated other options and, in the end, they approved the use of human diploid cells such as WI-38 and MRC-5 (6). These cells are "normal" cells, virus-free, and contain no carcinogenic substrate. Although the biosafety profile of these cells was acceptable, these cells were still a challenge in large-scale production. They have a limited life span and are attachments-dependent cells.

The concerns of regulatory agencies about the use of transformed cells or cells derived from cancer tissue were addressed by tight monitoring and control of cell lines. In the 1980s, agencies accepted Namalwa cells for the production of IFNs based on characterization data of the master cell bank. These cells did not have any contaminants and the product did not have detectable infective agents. Even though these cells had Epstein-Barr (EB) virus, it was not a serious drawback. The product from these cells could be purified in a process that effectively removes the majority of cell-derived proteins and DNA (6).

Continuous cell lines such as BHK, Chinese hamster ovary (CHO), myeloma cells (SP2/0, NS0), and human embryonic kidney (HEK) cells were accepted gradually for use in cell culture technology. These cells can be grown in suspension and are easy to scale up. Since the cells are continuous cell lines, their growth requirements are manageable. In fact, these cells can grow in chemically defined media without serum or protein supplements. In addition, these cells can be modified genetically to produce recombinant proteins. In 1987, tissue plasminogen activator (tPA) was

approved from recombinant CHO cells. In the last decade, tissue engineering was used to generate tissues for replacement therapy and the cells themselves were approved as products for tissue replacements. The latest development in tissue engineering is the use of embryonic stem cells. These cells can differentiate to different tissues that can be implanted or used as tissue replacement.

Progress in Large-Scale Cell Culture Technology

While small-scale cell culture provided sufficient products for investigational and diagnostic purposes, the demand for vaccines and therapeutic products required large-scale production. Some of the cell culture technology-based products such as monoclonal antibodies need to be manufactured at 1000 kg/year. This can only be done in large bioreactors (up to 20,000 L) by efficient processes that produce several kilograms a day (1,7).

Early cell lines used for vaccines were attachment-dependent cells. These cells required medium supplemented with serum and surfaces on which to grow. The expansion and propagation of cells required trypsinization. Scale-up of these systems was achieved by adding more surfaces into the bioreactor system. Roller bottles, T-flasks, and disk propagators are still used to scale up these cultures. A modernized version of these systems is the cell cube, which bears a similarity to a multichamber T-flask. Compared to suspension systems, attachment-dependent cell systems are more difficult to scale up because the surface-to-volume ratio gets smaller at larger scales.

The development of microcarrier cultures was a major breakthrough for attachment-dependent cell systems (8). The microcarriers can be suspended in stirred tanks similar to those used for microbial systems. Modifications to the impellers and aeration systems ensured the success of the bioreactor process. The cells, when inoculated into microcarrier-containing bioreactors, attach and grow on microspheres. The cells can be fed by fresh medium and the product can be removed from the bioreactor continuously. Microcarriers are easier to scale up; one can simply put more microcarriers in larger bioreactors. These systems are now used for vaccine production as well as for protein production at scales of 10,000 L or higher. An improvement in the microcarrier culture was the use of macroporous carriers in the bioreactors. These macroporous carriers allow cells to grow inside as well as outside, thus achieving higher densities by the extra room provided.

The acceptance of continuous cell lines made it possible to grow the cells in suspension in large scale bioreactors. Continuous cell lines are mostly suspension cells that can be agitated and aerated in bioreactors. However, culturing cells in bioreactors was not straightforward. The absence of cell membranes and the stringent growth requirement of cells required both the cultivation procedures and bioreactor operating conditions to be customized. Optimal growth conditions in the bioreactor were achieved by implementation of the results from intensive studies on shear, agitation, aeration, and medium optimization. Today, cells can be cultivated at large-scale bioreactors (up to 20,000 L), at high densities (up to 50 million cells/mL), for many months of operation (9).

High-Efficiency Cell Culture Processes

The efficiency of cell culture processes is measured by the productivity of the cultures, i.e., the amount made per volume per day. This quantity, the volumetric

productivity, is the product of the cell density achieved and the productivity of each cell (specific productivity). Volumetric productivity determines the amount made in a certain production period that can be measured as accumulated product concentration in the bioreactor.

Novel transfection, amplification, and selection methods resulted in high-producing cell clones with high specific productivities. Specific production rate is a measure of how much product is secreted from the cell per unit of time. For monoclonal antibodies, the specific production rate is commonly expressed in picograms per cell per day. For commercial production of antibodies, a minimal specific productivity of 10–20 pg/cell/day is expected. Advances in cell line development and optimization of culture conditions can result in specific productivities as high as 40–100 pg/cell/day.

In addition to specific productivity, the volumetric productivity is directly proportional to the cell density in the bioreactor. Cell density attainable in the bioreactor depends on several factors such as cell growth and death rates, composition of the medium, and aeration capacity (9). In early large-scale bioreactor development studies, the aeration was a major limitation for achieving high cell density. Over the years effective aeration strategies were developed and most of the issues with sparging have been resolved. An optimized design of the sparger (gas flow rate, diameter, etc.) and some media additives (F68, antifoam) can effectively minimize the impact of sparging. Today, the cell density in the bioreactor is mostly dictated by the media composition and media exchange rate.

Mammalian cells require a highly complicated medium and a delicate formulation is required to maximize cell growth. If the aeration is not limiting, achievable cell density in the bioreactor is controlled by: (a) the nutrient levels in the medium, and (b) the medium exchange rate (9). Nutrient levels in the medium can be increased by extensive medium optimization. Media development and optimization is an art and a science. Many variables are involved in cell metabolism and the components of the medium interact with each other. Early media formulations provided cell densities of 1–2 million cells/mL. Today, a cell density of 10 million cells/mL is achievable in a batch culture. This is an order of magnitude improvement, accomplished in the last two decades. In perfusion cultures, the cell density can be even higher due to high medium exchange rates resulting in cell densities of 20–50 million cells/mL (9).

Media Development

Process economics and implementation of cost-effective processes benefited not only by increasing the productivity, but also by reducing the manufacturing cost. Medium development contributed greatly to the process economics by allowing cells to grow to high densities and by decreasing the cost of the medium components.

In the beginning, cell culture medium contained animal products such as serum, albumin, and growth factors. For attachment-dependent cells, other components were also added to stimulate attachment and to form extracellular matrices. These components added to the cost of production and complicated the material sourcing and material release processes. Over the years, serum and other animal products were eliminated from the medium and most continuous cell lines can now be grown in serum- and protein-free media formulations. The use of simple media made it possible to produce kilogram amounts of protein per year economically from volumes close to 100,000 L.

Serum-free media development was followed by the complete elimination of animal proteins from the medium. Initially, albumin and other proteins have been used in serum-free media to substitute for some of the serum components. However, concerns about viruses and animal disease such as bovine spongiform encephalopathy (BSE) required the development of animal product-free (APF) media formulations. Early APF media relied heavily on the use of plant hydrolysates such as soy peptone and yeast extracts for optimal results. Thanks to extensive media development efforts, these components could also be eliminated from the medium with minimal loss in productivity. These efforts resulted in chemically defined media (CDM) formulations. Chemically defined medium is now utilized in many biotechnology processes as the medium of choice because every component could be traced and consistently manufactured.

PRODUCTS FROM CELL CULTURE TECHNOLOGY

Viral Vaccines

Vaccination for the prevention of infectious diseases has been an effective strategy for many years. Polio vaccine was the first cell culture technology–based vaccine and was produced in cultured monkey kidney cells (7). The cell-based vaccine technology evolved in the last four decades; the production of vaccines now utilizes primary cells, human diploid cells, and continuous or even recombinant cell lines. Vaccines against hepatitis B, measles and mumps, rubella, rabies, and FMD had been very effective in preventing life-threatening diseases. New vaccines target human immunodeficiency virus (HIV), herpes simplex virus, respiratory syncytial virus, cytomegal virus (CMV), and influenza, and continue to utilize cell culture technology for production. The latest developments in vaccine development include genetically engineered vaccines and DNA vaccines that will open new frontiers in this field. Development of new vaccines for HIV and cancer is very exciting and these efforts should come to successful conclusion in the next decade.

Cytokines (Interferons and Interleukins)

Cytokines are soluble mediators or glycoproteins helping cells communicate and function as part of the immunological, hematological, and neurological systems (10). Interferons and interleukins are cytokines with enormous therapeutic potential. Alpha interferon (Wellferon®) was developed as the first cell culture-derived biological for treatment of cancer (4). This was achieved in 8000 L bioreactors using Nawalwa cells at the Wellcome facility. It was followed by the production of several other interferons and interleukins. Due to the small size and relatively simple molecular structure of cytokines, production by simpler cell systems (microbial or yeast) seemed to be adequate. However, mammalian cells express more native cytokines, and cell culture technology is the method of choice for alpha, beta, and gamma interferons. In addition, interleukins 2 to 4, 6, 11, and 12 are also produced by cell culture technology (7).

Hematopoietic Growth Factors

The formation and differentiation of hematopoietic cells to give rise to mature blood cells require a series of growth factors. These growth factors are mainly single-chain

polypeptides and can be produced by microbial systems. However, cell culture technology can offer advantages when the molecule is complicated by glycosylation and when the native form of the molecule is required for therapy.

Erythropoietin (EPO) is a hormone that controls the maturation of red blood cells and it is used for clinical applications in anemia. Recombinant EPO was genetically engineered in CHO cells and the product was launched in 1989 under the names of Epogen® (Amgen), Procrit® (Johnson and Johnson), and Eprex® (Johnson and Johnson). EPO was launched in 1990 in Europe and Japan under the names of Epogin® and Recormon®.

Growth Hormones

Although conventionally expressed in *Escherichia coli,* human growth hormone can be produced efficiently using cell culture technology. Seostim® and Saizen® are produced by C127 cells and marketed by Serono S.A.

Monoclonal Antibodies

Antibodies were hailed as "magic bullets" for targeting and neutralizing their antigens as therapeutic agents. Initial application of antibodies involved in vivo and in vitro small-scale production for diagnostic kits. It took almost two decades since then for the field to mature and produce antibodies that are safe and effective therapeutics. Today, antibodies constitute more than 25% of total biotechnology production (11,12).

The first antibody was produced by murine ascites. OKT3 antibody was approved in 1987 for the treatment of transplant rejection. Since then both the molecular structure of the antibodies and their production methods evolved significantly. The mouse antibodies were replaced first by chimeric, then by humanized, and recently by fully human antibodies. This allowed the gradual elimination of immunigenicity of antibodies. Antibodies are now produced in hundreds of kilograms using stirred tank bioreactors.

Table 1 presents the antibodies approved to date. Antibodies are used for HIV, cancer, allergic diseases, arthritis, renal prophylaxis, septic shock, transplantation, asthma, CMV, and anti-idiotype vaccines. In addition to those presented in Table 1, there are a large number of antibodies in clinical trials.

Recombinant Thrombolytic Agents

Recombinant tissue plasminogen activator (tPA) was the first product from cell culture (7). Genentech obtained the approval for tPA production from CHO cells in 1987 (Table 2). tPA is produced in a large-scale (>10,000 L) cell culture process. Although the current tPA is highly efficacious, there are several attempts to improve the pharmacokinetics of the molecule. In addition, there is a new generation of recombinant thrombolytic agents under development.

Recombinant tPA from Genentech is marketed as Activase® and it is used for acute myocardial infarction (approved in 1987), acute massive pulmonary embolism (approved in 1990), acute myocardial infarction accelerated infusion (approved in 1995), and ischemic stroke within 3–5 hr of symptom onset (approved in 1996).

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