Appl Microbiol Biotechnol (2005) 68: 283–291
DOI 10.1007/s00253-005-1980-8

MINI-REVIEW

Michael Butler

Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals

Received: 11 February 2005 / Revised: 23 March 2005 / Accepted: 31 March 2005 / Published online: 16 April 2005

Abstract There has been a rapid increase in the number and demand for approved biopharmaceuticals produced from animal cell culture processes over the last few years. In part, this has been due to the efficacy of several humanized monoclonal antibodies that are required at large doses for therapeutic use. There have also been several identifiable advances in animal cell technology that has enabled efficient biomanufacture of these products. Gene vector systems allow high specific protein expression and some minimize the undesirable process of gene silencing that may occur in prolonged culture. Characterization of cellular metabolism and physiology has enabled the design of fed-batch and perfusion bioreactor processes that has allowed a significant improvement in product yield, some of which are now approaching 5 g/L. Many of these processes are now being designed in serum-free and animal-component-free media to ensure that products are not contaminated with the adventitious agents found in bovine serum. There are several areas that can be identified that could lead to further improvement in cell culture systems. This includes the down-regulation of apoptosis to enable prolonged cell survival under potentially adverse conditions. The characterization of the critical parameters of glycosylation should enable process control to reduce the heterogeneity of glycoforms so that production processes are consistent. Further improvement may also be made by the identification of glycoforms with enhanced biological activity to enhance clinical efficacy. The ability to produce the ever-increasing number of biopharmaceuticals by animal cell culture is dependent on sufficient bioreactor capacity in the industry. A recent shortfall in available worldwide culture capacity has encouraged commercial activity in contract manufacturing operations. However, some analysts indicate that this still may not be enough and that future manufacturing demand may exceed production capacity as the number of approved biotherapeutics increases.

M. Butler (✉)
Department of Microbiology,
University of Manitoba,
Buller Building,
Winnipeg, Manitoba, Canada, R3T 2N2
e-mail: butler@cc.umanitoba.ca
Tel.: +1-204-4746543
Fax: +1-204-4747603

Introduction

Although animal cell cultures have been important at a laboratory scale for most of the last 100 years, it was the initial need for human viral vaccines in the 1950s (particularly for poliomyelitis) that accelerated the design of large-scale bioprocesses for mammalian cells (Kretzmer 2002). These processes required the use of anchorage-dependent cells and the modern version of this viral vaccine technology currently employs microcarrier support systems that can be used in pseudosuspension cultures designed in stirred tank bioreactors.

However, more recently the enhanced interest in mammalian cell culture bioprocesses is associated with recombinant protein technology developed in the 1970s and 1980s. The first human therapeutic protein to be licensed from this technology in 1982 was recombinant insulin (Humulin from Genentech) but the relative structural simplicity of this molecule allowed its large-scale production to be developed in *Escherichia coli*, which is fast growing and robust compared to mammalian cells. It was soon realised that the subsequent targets for recombinant therapeutics were more complex and required the post-translational metabolic machinery only available in eukaryotic cells. At the present time there are up to 30 licensed biopharmaceuticals produced from mammalian cell bioprocesses (Walsh 2003; Pavlou 2003, Molowa and Mazanet 2003). These are defined as recombinant proteins, monoclonal antibodies and nucleic acid-based products. Since 1996, the chimeric and humanized monoclonal antibodies have dominated this group with such blockbuster products as Rituxan, Remicade, Synagis and Herceptin (Brekke and Sandie 2003; Pavlou 2004). A chimeric antibody (e.g. Rituxan) consists of a molecular construct in which the mouse variable region is linked to the human constant region. A further step to humanizing an antibody can be made by replacement of the

murine framework region, leaving only the complementarity determining regions (CDRs) that are of murine origin. These hybrid construct molecules are far less immunogenic than their murine counterparts and have serum half-lives of up to 20 days.

In this review, key factors for the recent achievements in the production of biopharmaceuticals from animal cell culture processes are discussed.

Cell line transfection and selection

The ability to produce and select for a high-producing animal cell line is key to the initial stages of the development of a cell culture bioprocess (Wurm 2004; Andersen and Krummen 2002). Chinese hamster ovary (CHO) cells have become the standard mammalian host cells used in the production of recombinant proteins, although the mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) or human-retina-derived (PER-C6) cells are alternatives. All these cell lines have been adapted to grow in suspension culture and are well suited for scale-up in stirred tank bioreactors. The advantage of CHO and NSO cells is that there are well-characterised platform technologies that allow for transfection, amplification and selection of high-producer clones. Transfection of cells with the target gene along with an amplifiable gene such as dihydrofolate reductase (DHFR) or glutamine synthetase (GS) has offered effective platforms for expression of the required proteins. In these systems, selective pressure is applied to the cell culture with an inhibitor of the DHFR or GS enzymes that causes an increase in the number of copies of the transfected genes including the target gene.

The DHFR system is routinely used with CHO cells deficient in the DHFR activity ($DHFR^-$). The target gene is delivered to the cells along with the DHFR marker gene, usually on the same plasmid vector (Gasser et al. 1982; Lucas et al. 1996). The expression vector normally contains a strong viral promoter to drive transcription of the recombinant gene and this is delivered into the cells by one of a number of possible non-viral transfer techniques. These include calcium phosphate, electroporation, lipofection or polymer-mediated gene transfer. The transfected cells are selected in media requiring the activity of DHFR for nucleotide synthesis and cell growth. Exposure of the cells to several rounds of gradually increased concentrations of the DHFR enzyme inhibitor, methotrexate, promotes amplification of the DHFR and the co-transfected target gene. Methotrexate treatment enhances specific protein production following an increased gene copy number, which can be up to several hundred in selected cells.

The glutamine synthetase expression system is an alternative that works as a dominant selectable marker, which is an advantage because this does not require the use of specific mutant cells (Bebbington et al. 1992). CHO cells that contain endogenous enzyme activity can be used although NS0 cells are preferred because of the absence of GS. This means that a lower activity of an enzyme inhibitor can be used for selection and amplification in NS0. GS enzyme allows the synthesis of glutamine intracellularly and so the transfected cells are selected in a glutamine-free media. The added advantage of this is that the cell cultures produce less ammonia, which is a potentially toxic metabolic by-product of mammalian cells that affects protein glycosylation and may inhibit cell growth. Gene amplification in this system is mediated by methionine sulphoximine (MSX), which is required at concentrations of 10–100 μM to provide clones with amplified genes and sufficiently high specific productivities. Typically copy numbers of only four to ten genes per cell are found in these cells but they give as high expression levels as the cells from the DHFR system. The advantage of this is that the GS high-producer clones can be produced in around 3 months, which is half the time it takes for the selection of DHFR clones.

High yields of recombinant proteins can also be produced from a human cell line, notably PER.C6, which was created by immortalizing healthy human embryonic retina cells with the E1 gene of adenovirus (Jones et al. 2003). This cell line has been well characterized and has been shown to be able to produce high levels of recombinant protein with relatively low gene copy numbers and without the need for amplification protocols. The added value of these cells is that they ensure the recombinant proteins produced receive a human profile of glycosylation.

Screening for a high-producer clone can be a lengthy process that depends upon assaying the secreted proteins to determine productivities of all candidate clones. High-throughput selection systems have been devised based on rapid assays or the use of flow cytometry to identify clones that have an appropriate product marker on the cell surface (Borth et al. 2001; Carroll and Al-Rubeai 2004). A cell clone with a specific productivity of up to 10 pg $cell^{-1}$ day^{-1} can be produced fairly routinely for recombinant protein production. However, higher specific productivity (up to 90 pg $cell^{-1}$ day^{-1}) may be possible with improvements in vector technology and further understanding of the parameters that control protein expression in the cell (Wurm 2004).

Stability of gene expression

The stability of selected clones over long-term culture is a critical parameter for commercial production (Kim et al. 1998). The application of selective pressure such as methotrexate in the case of the DHFR selection system causes gene amplification but a proportion of these genes are unstable and removal of the selective agent, as is necessary in production cultures, results in a gradual loss of the gene copy number. Fann et al. (2000) reported the stepwise adaptation of tissue plasminogen activator-producing CHO cell lines to 5 μM of methotrexate, which resulted in a maximum specific recombinant protein production of 43 pg $cell^{-1}$ day^{-1}, but on removal of the methotrexate the maximum productivity decreased to 12 pg $cell^{-1}$ day^{-1} within 40 days. This decrease in productivity could be correlated

with a reduction of gene copy number for individual clones. Barnes et al. (2004) studied the stability of antibody expression from NS0 cells amplified with the GS system. They reported that there was a loss of mRNA for the recombinant protein over long-term culture but this was only reflected in a decrease in protein expression if the mRNA was below a threshold level. This indicates that selection for clones for high levels of recombinant mRNA may be useful as a predictor of stable protein production. Above a saturation level of mRNA it is argued that the limitation to protein expression resides in the translational/ secretory machinery of the cell.

Production of a high-producer clone is dependent on the integration of the expression vector in the host cell genome. The site of integration has a major effect on the transcription rate of the recombinant gene and progressive gene silencing can occur over successive culture passages after clonal isolation. This is thought to be associated with the spread of heterochromatin structure, which is condensed and transcriptionally silent. Most expression systems cause random transgene integration into the host cell and this leads to positional effects that cause variable expression and stability. However, the genetic control elements that are responsible for establishing a transcriptionally active transgene are not fully understood. Vectors in which the genes are flanked with insulators, boundary elements or ubiquitous chromatin opening elements may promote stable expression by insulating the transgene from positional effects of the chromatin. These elements that can be incorporated into expression vectors include matrix (or scaffold) attachment regions (MAR or SAR) that allow open chromatin structure to be maintained. This can allow higher efficiency of expression of the integrated genes (Kim et al. 2004). Gene regulatory elements associated with ubiquitously expressed housekeeping genes have been recently isolated (Antoniou et al. 2003). These regulatory elements appear to confer a dominant chromatin opening function and give rise to an ability to resist transgene silencing. These ubiquitous chromatin opening elements (UCOE) have also been incorporated into transgene vectors to prevent gene silencing and give consistent, stable and high-level gene expression irrespective of the chromosomal integration site (Haines et al. 2004).

Culture modes

A producer cell clone may be grown in batch cultures to above 10^6 cells/mL over 3–4 days to allow synthesis and product secretion. The limits for growth and production are related to the accumulation of metabolic by-products, such as ammonia and lactate, or the depletion of nutrients, such as glucose or glutamine (Butler and Jenkins 1989). The growth of cells can be extended if these limitations are addressed through perfusion culture in which the constant supply of nutrients and the removal of media can lead to cell densities of at least 10^7 cells/mL (Butler et al. 1983). These principles have been extended to fed-batch cultures, which have been shown to be operationally simple, reliable and flexible for multi-purpose facilities (Bibila and Robinson 1995, Cruz et al. 2000; Xie and Wang 1997). The most successful strategies involve feeding concentrates of nutrients based upon the predicted requirements of the cells for growth and production. This can involve slow feeding of low concentrations of key nutrients. The maintenance of low concentration set points of the major carbon substrates enables a more efficient primary metabolism with leads to lower rates of production of metabolic by-products, such as ammonia and lactate. As a result the cells remain in a productive state over extended time frames. The strategic use of fed-batch cultures has enabled considerable enhancement of yields from these processes. This is often combined with a biphasic strategy of production in which cell proliferation is allowed in the first phase so that high cell densities accumulate, followed by a phase in which cell division is arrested to allow cells to attain a high specific productivity. In this type of strategy, growth can be arrested by a decrease in culture temperature (Fox et al. 2004; Yoon et al. 2003). By directly supplying cells with a balanced nutrient feed, a fed-batch culture can now be expected to yield upwards of 2 g/L of recombinant protein, which is probably at least tenfold higher than the maximum that could be expected by a simple batch culture in standard culture medium.

Animal cell cultures are normally grown in stainless steel, stirred tank bioreactors that are designed with impellers that minimize shear forces (Kretzmer 2002). Producer cells can be made to be sufficiently robust in this environment if they are provided with suitable growth media and gas sparging is carefully controlled. The capacity of commercial bioreactors for animal cells has gradually increased over the past two decades, with capacities now reported up to 20,000 L from some of the larger biopharmaceutical companies. Airlift bioreactors have also been applied to large-scale animal cells and these have been shown to be efficient for protein production.

Perfusion cultures are more demanding to set up at a large scale but they have the potential advantage of allowing a continuous stream of product over several weeks or even months (Mercille et al. 2000). A further advantage is the rapid removal of any potentially labile products from the culture environment. An effective cell separator will allow the protein-containing media to be fed directly into a chromatography column suitable for extraction and downstream processing (Shirgaonkar et al. 2004; Castilho and Medronho 2002; Wen et al. 2000). A further advantage of this mode of culture is that the bioreactor may be up to ten times smaller for the production of the same quantity of product (Ryll et al. 2000).

This area of bioprocess design will become of even greater importance as some of the first-generation blockbuster drugs (e.g. erythropoietin, human growth hormone and α-interferon) start being produced as generics (Walsh 2003). Eleven biopharmaceuticals with combined annual sales of $13.5 billion lose patent protection in 2006 (Walsh 2003). The challenge then will be to produce bioequivalents in efficient low-cost bioprocesses.

There are several challenging areas of bioprocess development that are required to be addressed to ensure the future success of animal cell culture processes. These in-

clude serum-free media, apoptosis, glycomics and the capacity crunch.

Serum-free media

Bovine serum was used as a supplement of cell culture media for several decades. It is a rich source of hormones, growth factors and trace elements that promote rapid cell growth and also its high albumin content ensures that the cells are well protected from potentially adverse conditions such as pH fluctuations or shear forces. However, the composition of serum is variable and undefined, which leads to inconsistent growth and productivity. Early attempts to develop serum-free substitutes incorporated such components as insulin, transferrin, albumin and cholesterol. However, the mad cow crisis in the beef industry alerted a concern for the use of animal serum and any other animal-derived components in the production of biotherapeutics. This has now led to a strong demand for cell culture formulations that are free of all animal components. The challenge that this demand poses is to be able to identify effective substitutes for all the growth-promoting factors that are present in serum. It turns out that producer cell lines are quite fastidious in their growth requirements and that such requirements vary considerably from one cell line to another. Therefore, it has not been possible to design a single serum-free formulation to act as a serum substitute suitable for the growth of all cell lines. In fact even different clones of CHO cells may require different formulations for optimal growth. This has given rise to a strong drive for the development of serum-free and animal-component-free formulations that are tailored to the needs of specific producer cell lines.

There are several strategies that can be used to design these formulations. Combinations of standard basal media may be tested to determine those that result in good cell growth and productivity at minimal serum levels. In some cases metabolic analysis may help in media design. For example, NS0 myeloma cells lack a functional pathway for cholesterol synthesis and so cholesterol is required as a lipoprotein supplement in the medium (Gorfien et al. 2000). Protein hydrolysates from non-animal sources have been found to provide good growth promotion in some culture systems (Sung et al. 2004). Analysis of the depletion of media components may lead to the identification of specific nutrients that may be required at higher supplement levels or for inclusion in feeding regimes. Statistical approaches can be used, such as the Plackett–Burman experimental design, so that mixtures of components may be tested simultaneously in matrix experiments of growth and productivity (Castro et al. 1992). Another original approach is the identification by microarray analysis of specific receptors expressed during cell growth, so that corresponding ligands may be incorporated into the medium (Donahue 2004). These approaches are presently being used by various specialized media companies to customise animal-component-free formulations for the production of the plethora of recombinant proteins that are being introduced into the market.

Apoptosis

Cell cultures are often terminated because of cell death that may be caused by one of several factors including nutrient depletion, metabolic by-product accumulation, excessive shear forces or hypoxia. Cell death may be by necrosis caused by extreme conditions resulting in physical damage to the cells. Alternatively and more commonly, cell death in a bioreactor occurs by apoptosis, which is a form of programmed cell death regulated through a cellular cascade of activities in response to one of the factors mentioned above (Arden and Betenbaugh 2004). Characteristic changes include chromatin shrinkage followed by membrane blebbing and the formation of apoptotic bodies. The DNA of the cell is fragmented and this can be the basis of an assay to quantify apoptosis in a cell population.

It is of considerable value to be able to prevent or inhibit apoptosis in culture in order to extend the time of high cell viability and prolong protein production. There are two strategies that can be used for this. The cellular environment can be manipulated through media supplementation or the intracellular environment can be modified by genetic engineering.

Nutrient feeding can provide protection and this is normally used as the first preventive measure to control the cellular environment to delay apoptosis. Serum is known to contain unidentified anti-apoptotic factors that can offer protection (Zanghi et al. 1999). However, the serum-free formulations that are required for production processes make the cells more vulnerable to apoptosis. Some supplements such as suramin (Zanghi et al. 2000) or insulin growth factor (Sunstrom et al. 2000) may provide independent anti-apoptotic protection in serum-free cultures. There are also other specific caspase inhibitors available to suppress apoptosis (Tinto et al. 2002) but their expense in large-scale cultures is likely to be prohibitive.

Genetic strategies involve the transfection of anti-apoptotic genes such as *bcl-2* or *bcl-x*$_2$ into a host cell. The expression of the corresponding proteins inhibits the release of pro-apoptotic molecules from the mitochondria and may prolong the viability of the cell. This strategy has been shown to work for several cell lines, which have shown higher viabilities and improved robustness under conditions that would normally be expected to cause apoptosis (Tey et al. 2000; Mastrangelo et al. 2000; Kim and Lee 2002).

Glycosylation

Animal cells are used for biomanufacture because of their capabilities of adding carbohydrates (glycans) to synthesised proteins (Butler 2004). These are produced as pools of different glycoforms with varying glycan structures attached to a single peptide backbone with a known amino acid sequence. The basic protein structures can be controlled and directed by the expression of appropriate genetic sequences. However, controlling the pool of glycan structures (glycomics) that occupy a recombinant protein is still difficult. Variations may be found in the site occupancy (mac-

roheterogeneity) or in the structure of attached glycans (microheterogeneity).

For the production of a recombinant protein as a biotherapeutic it is essential to ensure that a consistent glycosylation profile is maintained between batches (Restelli and Butler 2002). However, this may not be so easy to control given that the extent of glycosylation may decrease over time in a batch culture (Curling et al. 1990). This is likely to be due to the depletion of nutrients, particularly glucose or glutamine, which have been shown to limit the glycosylation process (Hayter et al. 1992; Nyberg et al. 1999). Fed-batch strategies should also be designed to ensure that the concentrations of these key nutrients do not decrease to a critical level that could compromise protein glycosylation (Xie and Wang 1997). These lower levels were found to be <0.1 mM glutamine and <0.7 mM glucose for the production of γ-interferon from CHO cells (Chee et al. 2005). In one report it is suggested that site occupancy could vary with the growth state of cells and correlates with the fraction of cells in the G_0/G_1 phase of the cell cycle (Andersen et al. 2000). This suggests a mechanism by which glycosylation efficiency improves at a reduced rate of protein translation.

Culture parameters such as nutrient content, pH, temperature, oxygen or ammonia, may have a significant effect on the distribution of glycan structures found on the resulting recombinant protein (microheterogeneity). This of course is of major concern in trying to produce consistent biopharmaceuticals. Decreased sialylation or altered patterns of glycan branching occur when the ammonia level accumulates in culture (Andersen and Goochee 1994; Zanghi et al. 1998; Yang and Butler 2000). Non-optimal pH conditions (<6.9 and >8.2) have also been shown to alter the pattern of glycosylation (Rothman et al. 1989; Borys et al. 1993). Reduced terminal galactosylation has been shown in the glycans of immunoglobulin (IgG) produced under low oxygen conditions (Kunkel et al. 1998). Nabi and Dennis (1998) observed an increase in the polylactosamine content of a protein produced at lower temperatures and attributed this to changes in the transit time through the Golgi.

The pattern of protein glycosylation is dependent on the expression of various glycosyltransferase enzymes that are present in the Golgi of the cell. Differences in the relative activity of these enzymes among species can account for significant variations in structure. In one systematic study of glycan structures of IgG produced from cells of 13 different species significant variation was found in the proportion of terminal galactose, core fucose and bisecting GlcNAc (Raju et al. 2000). The structure of sialic acid may also vary, with *N*-glycolyl-neuraminic acid (NGNA) found in goat, sheep and cows rather than the *N*-acetyl-neuraminic acid (NANA) found in humans. NGNA is the predominant sialic acid in mice, but CHO-produced glycoproteins have predominantly NANA, although a small proportion (up to 15%) of NGNA can occur (Baker et al. 2001). These differences in glycan structure are important tential immunogenicity of these structures in humans. Mouse cells express the enzyme α1,3 galactosyltransferase, which generates Galα1,3-Galβ1,4-GlcNAc residues that are highly immunogenic in humans (Jenkins et al. 1996). Fortunately, this enzyme appears to be inactive in CHO and BHK cells, which are the most commonly used cell lines for the production of recombinant proteins. However, both CHO and BHK show differences in their potential for glycosylation compared to human cells. The sialyl transferase enzyme (α2,6 ST) that normally provides an α2,6 linkage for terminal sialic acid in glycoproteins produced in humans is absent in the hamster and thus CHO and BHK cells produce exclusively α2,3 terminal sialic acid residues. Furthermore, the absence of a functional α1,3 fucosyltransferase in CHO cells prevents the addition of peripheral fucose residues and the absence of *N*-acetylglucosaminyltransferase III (Gn TIII) prevents the addition of bisecting GlcNAc to glycan structures (Jenkins and Curling 1994). However, these differences in glycosylation potential between CHO and human cells do not appear to result in glycoproteins that are immunogenic. Natural human erythropoietin (EPO) consists of a mixture of sialylated forms: 60% are 2,3 linked and 40% are 2,6 linked. Because of the restricted sialylation capacity of CHO cells, the commercially available EPO is sialylated entirely via the α2,3 linkages. Nevertheless, recombinant EPO produced from CHO cells has proven to be a highly effective therapeutic agent with no evidence of an adverse physiological effect due to the structural differences in terminal sialylation.

The production of specific protein glycoforms may allow the possibility of even more efficacious drugs (Shriver et al. 2004). Functional glycomics is an expanding area of science that attempts to understand the physiological function of specific carbohydrate groups. This approach established the importance of the sialylation of EPO with the discovery that the removal of sialic acid groups from the glycans resulted in a significantly reduced half-life in the blood stream (Erbayraktar et al. 2003). Protein engineering has allowed the creation of a modified EPO with two extra glycan attachment sites and with the potential to incorporate eight extra sialic acid groups per molecule. This has led to a new-generation EPO called darbepoetin, which has a three times higher drug half-life (Egrie et al. 2003). This strategy of enhancing the half-life of a biotherapeutic has also been successful for other recombinant proteins such as follicle-stimulating hormone (Perlman et al. 2003) and thyroid-stimulating hormone (Thotakura et al. 1991).

Structural changes of glycans can also be brought about by metabolic engineering of the host cell line. This includes gene knockout of already expressed glycosyltransferases or the insertion of novel activities (Weikert et al. 1999). The presence of a bisecting *N*-acetylglucosamine (Umana et al. 1999; Davies et al. 2001) or the absence of fucose (Shields et al. 2002; Shinkawa et al. 2003; Okazaki et al. 2004) in the conserved glycan of an IgG antibody has been shown to enhance attachment to Fc receptors and result in an increase in antibody-dependent, cell-mediated cytotoxicity (ADCC). This has been of value in the design of antibody therapeutics. For example, recent work with Herceptin, which is a novel humanized antibody approved

for the treatment of breast cancer, has shown that a glycoform with no fucose has a 53 times higher binding capacity to an Fc receptor that triggers its therapeutic activity (Shinkawa et al. 2003). This enhancement of ADCC allows the antibody to be effective at lower doses. Afucosylated antibodies can be produced from cells in which the gene for fucosyl transferase has been removed by gene knockout technology.

Complete glycosylation of recombinant proteins is usually associated with maximisation of galactosylation and sialylation. Often these two processes are incomplete and this gives rise to considerable glycan structural variation. CHO cells can be engineered with a combination of human β1,4-galactosyltransferase and α2,3-sialyltransferase to ensure high activities of these enzymes. The recombinant proteins produced by these cells exhibited greater homogeneity compared to controls and increased terminal sialic acid residues (Weikert et al. 1999). An alternative approach involves glycoengineering of the proteins in vitro (Raju et al. 2001). Preparations of these terminal transferase enzymes can be immobilized so that glycoproteins can be galactosylated and sialylated in the presence of appropriate galactose and sialic acid donors.

The capacity crunch

With an increase in the number and demand for recombinant biopharmaceuticals, there is a requirement for greater biomanufacturing capacity. This created a major problem in 2001 when the demand for Enbrel, a recombinant fusion protein commercialized by Immunex for the treatment of rheumatoid arthritis, exceeded expectations. However, there was insufficient large-scale culture manufacturing capacity to meet this clinical demand, even by contract manufacturers available at that time. Although the problem of Enbrel was eventually solved, the episode highlighted a general problem that the pipeline of biotherapeutic products is expanding more rapidly than the world capacity for cell culture production (Mallik et al. 2002). It is estimated that the present world capacity for cell culture production stands at 475,000 L, of which 75% is controlled by biopharmaceutical companies and the remainder by contract manufacturers. Fig. 1 shows the increase in the number of biopharmaceuticals approved for production by mammalian cell bioprocesses over 20 years with the actual and expected demand in kilograms. The sharp increase in 2000 is largely due to the number of approved therapeutic antibodies that are required at relatively high clinical doses. Since 2001 there has been a substantial increase in biomanufacturing capacity to meet this demand, but some estimates suggest that there is still a potential shortfall with manufacturing demand continuing to exceed the production capacity (Molowa and Mazanet 2003). The estimates of the shortfall vary and depend on a number of difficult predictions (Thiel 2004).

One of the reasons for this extra demand for biomanufacturing capacity is the dose requirement for the novel therapeutic humanized monoclonal antibodies that are now being commercialized. The requirement for hundreds of kilograms per annum far exceeds other recombinant therapeutics such as erythropoietin, which is more potent at smaller doses. The extra demand for production is being met by the construction of increased bioreactor capacity by some biopharmaceutical companies such as Biogen, Lonza Biologics and Genentech. However, the requirement for large-capacity bioreactors may be offset by an increased productivity of cell culture systems, some of which may be capable of producing up to 5 g/L and is up to 100 times the productivity that would have been expected a few years ago. Clearly, a tenfold enhancement of cell line productivity reduces the volumetric capacity required of the bioreactor for manufacture by an equivalent factor.

A rider to this problem of the capacity crunch is the personnel crunch, with a shortage of highly qualified per-

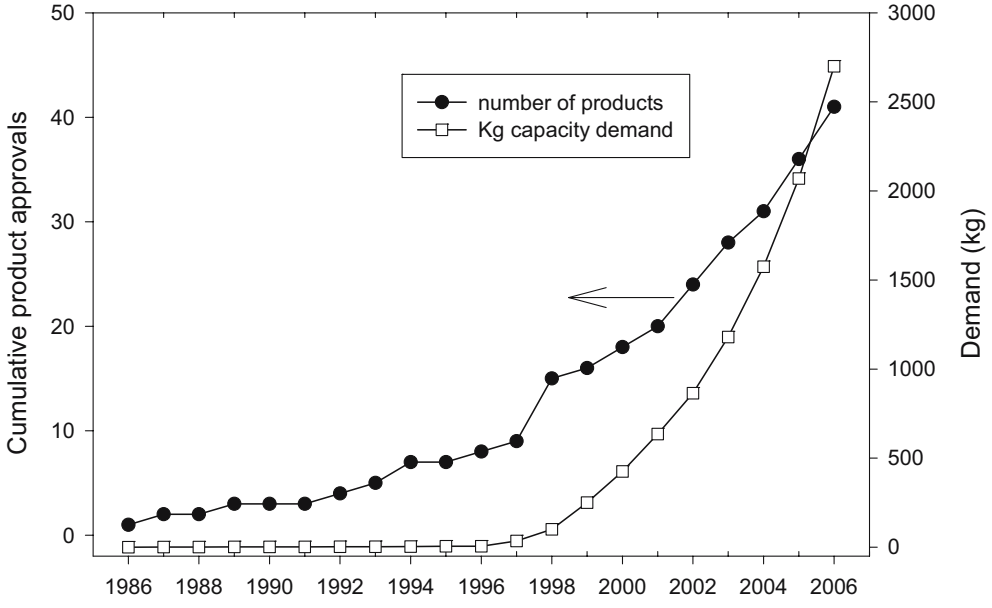

Fig. 1 The demand for mammalian cell culture products (data taken from Molowa and Mazanet 2003)

sonnel available to manage the impending demand for production of the new series of cell culture products. It is clear that the number of graduates or Ph.D.s with expertise in the key areas of this technology is declining at a time when the demand for such expertise is likely to be expanding (Mallik et al. 2002). It is hoped that this problem is recognised so that the full potential of animal cell technology for biopharmaceutical production can be achieved.

Acknowledgements The author would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for financial support in animal cell technology.

References

Andersen DC, Bridges T, Gawlitzek M, Hoy C (2000) Multiple cell culture factors can affect the glycosylation of Asn-184 in CHO-produced tissue-type plasminogen activator. Biotechnol Bioeng 70:25–31

Andersen DC, Goochee CF (1994) The effect of cell-culture conditions on the oligosaccharide structures of secreted glycoproteins. Curr Opin Biotechnol 5:546–549

Andersen DC, Krummen L (2002) Recombinant protein expression for therapeutic applications. Curr Opin Biotechnol 13:117–123

Antoniou M, Harland L, Mustoe T, Williams S, Holdstock J, Yague E, Mulcahy T, Griffiths M, Edwards S, Ioannou PA, Mountain A, Crombie R (2003) Transgenes encompassing dual-promoter CpG islands from the human TBP and HNRPA2B1 loci are resistant to heterochromatin-mediated silencing. Genomics 82:269–279

Arden N, Betenbaugh MJ (2004) Life and death in mammalian cell culture: strategies for apoptosis inhibition. Trends Biotechnol 22:174–180

Baker KN, Rendall MH, Hills AE, Hoare M, Freedman RB, James DC (2001) Metabolic control of recombinant protein N-glycan processing in NS0 and CHO cells. Biotechnol Bioeng 73:188–202

Barnes LM, Bentley CM, Dickson AJ (2004) Molecular definition of predictive indicators of stable protein expression in recombinant NS0 myeloma cells. Biotechnol Bioeng 85:115–121

Bebbington CR, Renner G, Thomson S, King D, Abrams D, Yarranton GT (1992) High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. Biotechnology (N Y) 10: 169–175

Bibila TA, Robinson DK (1995) In pursuit of the optimal fed-batch process for monoclonal antibody production. Biotechnol Prog 11:1–13

Borth N, Zeyda M, Kunert R, Katinger H (2001) Efficient selection of high-producing subclones during gene amplification of recombinant Chinese hamster ovary cells by flow cytometry and cell sorting. Biotechnol Bioeng 71:266–273. Erratum in Biotechnol Bioeng (2002) 77:118

Borys MC, Linzer DI, Papoutsakis ET (1993) Culture pH affects expression rates and glycosylation of recombinant mouse placental lactogen proteins by Chinese hamster ovary (CHO) cells. Biotechnology (N Y) 11:720–724

Brekke OH, Sandlie I (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. Nat Rev Drug Discov 2:52–62

Butler M (2004) Animal cell culture and technology, 2nd edn. Bios Scientific, Oxford

Butler M, Jenkins H (1989) Nutritional aspects of growth of animal cells in culture. J Biotechnol 12:97–110

Butler M, Imamura T, Thomas J, Thilly WG (1983) High yields from microcarrier cultures by medium perfusion. J Cell Sci 61:351–363

Carroll S, Al-Rubeai M (2004) The selection of high-producing cell lines using flow cytometry and cell sorting. Expert Opin Biol Ther 4:1821–1829

Castilho LR, Medronho RA (2002) Cell retention devices for suspended-cell perfusion cultures. Adv Biochem Eng Biotechnol 74:129–169

Castro PM, Hayter PM, Ison AP, Bull AT (1992) Application of a statistical design to the optimization of culture medium for recombinant interferon-gamma production by Chinese hamster ovary cells. Appl Microbiol Biotechnol 38:84–90

Chee Furng Wong D, Tin Kam Wong K, Tang Goh L, Kiat Heng C, Gek Sim Yap M (2005) Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. Biotechnol Bioeng 89:164–177

Cruz HJ, Moreira JL, Carrondo MJ (2000) Metabolically optimised BHK cell fed-batch cultures. J Biotechnol 80:109–118

Curling EM, Hayter PM, Baines AJ, Bull AT, Gull K, Strange PG, Jenkins N (1990) Recombinant human interferon-gamma. Differences in glycosylation and proteolytic processing lead to heterogeneity in batch culture. Biochem J 272:333–337

Davies J, Jiang L, Pan LZ, LaBarre MJ, Anderson D, Reff M (2001) Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII. Biotechnol Bioeng 74:288–294

Donahue L (2004) Molecular approaches to cell culture medium development. In: IBC International Conference on Transmissible Spongiform Encephalopathies. IBC Life Sciences, Washington

Egrie JC, Dwyer E, Browne JK, Hitz A, Lykos MA (2003) Darbepoetin alfa has a longer circulating half-life and greater in vivo potency than recombinant human erythropoietin. Exp Hematol 31:290–299

Erbayraktar S, Grasso G, Sfacteria A, Xie QW, Coleman T, Kreilgaard M, Torup L, Sager T, Erbayraktar Z, Gokmen N, Yilmaz O, Ghezzi P, Villa P, Fratelli M, Casagrande S, Leist M, Helboe L, Gerwein J, Christensen S, Geist MA, Pedersen LO, Cerami-Hand C, Wuerth JP, Cerami A, Brines M (2003) Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo. Proc Natl Acad Sci U S A 100: 6741–6746

Fann CH, Guirgis F, Chen G, Lao MS, Piret JM (2000) Limitations to the amplification and stability of human tissue-type plasminogen activator expression by Chinese hamster ovary cells. Biotechnol Bioeng 69:204–212

Fox SR, Patel UA, Yap MG, Wang DI (2004) Maximizing interferon-gamma production by Chinese hamster ovary cells through temperature shift optimization: experimental and modeling. Biotechnol Bioeng 85:177–184

Gasser CS, Simonsen CC, Schilling JW, Schimke RT (1982) Expression of abbreviated mouse dihydrofolate reductase genes in cultured hamster cells. Proc Natl Acad Sci U S A 79:6522–6526

Gorfien S, Paul B, Walowitz J, Keem R, Biddle W, Jayme D (2000) Growth of NS0 cells in protein-free, chemically defined medium. Biotechnol Prog 16:682–687

Haines AWS, Wedgwood J, Cliffe S, Simpson D, Mountain A, Irvine A (2004) Rapid antibody production from stable pools of mammalian cells and isolation of high expressing clones using UCOEs. In: Cell culture and upstream processing. IBC Life Sciences, Berlin

Hayter PMCE, Baines AJ, Jenkins N, Salmon I, Strange PG, Tong JM, Bull AT (1992) Glucose-limited chemostat culture of Chinese hamster ovary cells producing recombinant human interferon-γ. Biotechnol Bioeng 39:327–335

Jenkins N, Curling EM (1994) Glycosylation of recombinant proteins: problems and prospects. Enzyme Microb Technol 16: 354–364

Jenkins N, Parekh RB, James DC (1996) Getting the glycosylation right: implications for the biotechnology industry. Nat Biotechnol 14:975–981

Jones D, Kroos N, Anema R, van Montfort B, Vooys A, van der Kraats S, van der Helm E, Smits S, Schouten J, Brouwer K, Lagerwerf F, van Berkel P, Opstelten DJ, Logtenberg T, Bout A (2003) High-level expression of recombinant IgG in the human cell line per.c6. Biotechnol Prog 19:163–168

Kim JM, Kim JS, Park DH, Kang HS, Yoon J, Baek K, Yoon Y (2004) Improved recombinant gene expression in CHO cells using matrix attachment regions. J Biotechnol 107:95–105

Kim NS, Lee GM (2002) Response of recombinant Chinese hamster ovary cells to hyperosmotic pressure: effect of Bcl-2 overexpression. J Biotechnol 95:237–248

Kim NS, Kim SJ, Lee GM (1998) Clonal variability within dihydrofolate reductase-mediated gene amplified Chinese hamster ovary cells: stability in the absence of selective pressure. Biotechnol Bioeng 60:679–688

Kretzmer G (2002) Industrial processes with animal cells. Appl Microbiol Biotechnol 59:135–142

Kunkel JP, Jan DC, Jamieson JC, Butler M (1998) Dissolved oxygen concentration in serum-free continuous culture affects N-linked glycosylation of a monoclonal antibody. J Biotechnol 62:55–71

Lucas BK, Gierre LM, DeMarco RA, Shen A, Chrisholm V, Crowley, CW (1996) High-level production of recombinant proteins in CHO cells using a dicistronic DHFR intron expression vector. Nucleic Acids Res 24:55–71

Mallik A, Pinkus GS, Sheffer S (2002.) Biopharma's capacity crunch. McKinsey Q 9

Mastrangelo AJ, Hardwick JM, Zou S, Betenbaugh MJ (2000) Part II. Overexpression of bcl-2 family members enhances survival of mammalian cells in response to various culture insults. Biotechnol Bioeng 67:555–564

Mercille S, Johnson M, Lanthier S, Kamen AA, Massie B (2000) Understanding factors that limit the productivity of suspension-based perfusion cultures operated at high medium renewal rates. Biotechnol Bioeng 67:435–450

Molowa DT, Mazanet R (2003) The state of biopharmaceutical manufacturing. Biotechnol Annu Rev 9:285–302

Nabi IR, Dennis JW (1998) The extent of polylactosamine glycosylation of MDCK LAMP-2 is determined by its Golgi residence time. Glycobiology 8:947–953

Nyberg GB, Balcarcel RR, Follstad BD, Stephanopoulos G, Wang DI (1999) Metabolic effects on recombinant interferon-gamma glycosylation in continuous culture of Chinese hamster ovary cells. Biotechnol Bioeng 62:336–347

Okazaki A, Shoji-Hosaka E, Nakamura K, Wakitani M, Uchida K, Kakita S, Tsumoto K, Kumagai I, Shitara K (2004) Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcgammaRIIIa. J Mol Biol 336:1239–1249

Pavlou AK (2003) Marketspace: trends in biotherapeutics. J Commer Biotechnol 9:358–363

Pavlou AK (2004) The immunotherapies markets, 2003–2008. J Commer Biotechnol 10:273–278

Perlman S, van den Hazel B, Christiansen J, Gram-Nielsen S, Jeppesen CB, Andersen KV, Halkier T, Okkels S, Schambye HT (2003) Glycosylation of an N-terminal extension prolongs the half-life and increases the in vivo activity of follicle stimulating hormone. J Clin Endocrinol Metab 88:3227–3235

Raju TS, Briggs JB, Borge SM, Jones AJ (2000) Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. Glycobiology 10:477–486

Raju TS, Briggs JB, Chamow SM, Winkler ME, Jones AJ (2001) Glycoengineering of therapeutic glycoproteins: in vitro galactosylation and sialylation of glycoproteins with terminal N-acetylglucosamine and galactose residues. Biochemistry 40: 8868–8876

Restelli V, Butler M (2002) The effect of cell culture parameters on protein glycosylation. In: Al-Rubeai M (ed) Glycosylation, vol 3. Kluwer, Dordrecht, pp 61–92

Rothman RJ, Warren L, Vliegenthart JF, Hard KJ (1989) Clonal analysis of the glycosylation of immunoglobulin G secreted by murine hybridomas. Biochemistry 28:1377–1384

Ryll T, Dutina G, Reyes A, Gunson J, Krummen L, Etcheverry T (2000) Performance of small-scale CHO perfusion cultures using an acoustic cell filtration device for cell retention: characterization of separation efficiency and impact of perfusion on product quality. Biotechnol Bioeng 69:440–449

Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity. J Biol Chem 277:26733–26740

Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, Uchida K, Anazawa H, Satoh M, Yamasaki M, Hanai N, Shitara K (2003) The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. J Biol Chem 278:3466–3473

Shirgaonkar IZ, Lanthier S, Kamen A (2004) Acoustic cell filter: a proven cell retention technology for perfusion of animal cell cultures. Biotechnol Adv 22:433–444

Shriver Z, Raguram S, Sasisekharan R (2004) Glycomics: a pathway to a class of new and improved therapeutics. Nat Rev Drug Discov 3:863–873

Sung YH, Lim SW, Chung JY, Lee GM (2004) Yeast hydrolysate as a low-cost additive to serum-free medium for the production of human thrombopoietin in suspension cultures of Chinese hamster ovary cells. Appl Microbiol Biotechnol 63:527–536

Sunstrom NA, Gay RD, Wong DC, Kitchen NA, DeBoer L, Gray PP (2000) Insulin-like growth factor-I and transferrin mediate growth and survival of Chinese hamster ovary cells. Biotechnol Prog 16:698–702

Tey BT, Singh RP, Piredda L, Piacentini M, Al-Rubeai M (2000) Influence of bcl-2 on cell death during the cultivation of a Chinese hamster ovary cell line expressing a chimeric antibody. Biotechnol Bioeng 68:31–43

Thiel KA (2004) Biomanufacturing, from bust to boom...to bubble? Nat Biotechnol 22:1365–1372

Thotakura NR, Desai RK, Bates LG, Cole ES, Pratt BM, Weintraub BD (1991) Biological activity and metabolic clearance of a recombinant human thyrotropin produced in Chinese hamster ovary cells. Endocrinology 128:341–348

Tinto A, Gabernet C, Vives J, Prats E, Cairo JJ, Cornudella L, Godia F (2002) The protection of hybridoma cells from apoptosis by caspase inhibition allows culture recovery when exposed to non-inducing conditions. J Biotechnol 95:205–214

Umana P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE (1999) Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. Nat Biotechnol 17:176–180

Walsh G (2003) Biopharmaceuticals benchmarks—2003. Nat Biotechnol 21:865–887

Weikert S, Papac D, Briggs J, Cowfer D, Tom S, Gawlitzek M, Lofgren J, Mehta S, Chisholm V, Modi N, Eppler S, Carroll K, Chamow S, Peers D, Berman P, Krummen L (1999) Engineering Chinese hamster ovary cells to maximize sialic acid content of recombinant glycoproteins. Nat Biotechnol 17:1116–1121

Wen ZY, Teng XW, Chen F (2000) A novel perfusion system for animal cell cultures by two step sequential sedimentation. J Biotechnol 79:1–11

Wurm FM (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22:1393–1398

Xie L, Wang DI (1997) Integrated approaches to the design of media and feeding strategies for fed-batch cultures of animal cells. Trends Biotechnol 15:109–113

Yang M, Butler M (2000) Effects of ammonia on CHO cell growth, erythropoietin production, and glycosylation. Biotechnol Bioeng 68:370–380

Yoon SK, Kim SH, Lee GM (2003) Effect of low culture temperature on specific productivity and transcription level of anti-4-1BB antibody in recombinant Chinese hamster ovary cells. Biotechnol Prog 19:1383–1386

Zanghi JA, Fussenegger M, Bailey JE (1999) Serum protects protein-free competent Chinese hamster ovary cells against apoptosis induced by nutrient deprivation in batch culture. Biotechnol Bioeng 64:108–119

Zanghi JA, Mendoza TP, Knop RH, Miller WM (1998) Ammonia inhibits neural cell adhesion molecule polysialylation in Chinese hamster ovary and small cell lung cancer cells. J Cell Physiol 177:248–263

Zanghi JA, Renner WA, Bailey JE, Fussenegger M (2000) The growth factor inhibitor suramin reduces apoptosis and cell aggregation in protein-free CHO cell batch cultures. Biotechnol Prog 16:319–325