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Production technologies for monoclonal antibodies and their fragments

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In recent years, monoclonal antibodies have emerged as an increasingly important class of human therapeutics. A variety of forms of antibodies, including fragments such as Fabs, Fab′$_2$s and single-chain Fvs, are also being evaluated for a range of different purposes. A variety of expression systems and improvements within these systems have been developed to address these growing and diverse needs.

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Current Opinion in Biotechnology 2004, **15**:456–462

This review comes from a themed issue on
Biochemical engineering
Edited by Manuel Carrondo and John G Aunins

Available online 25th August 2004

0958-1669/$ – see front matter
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DOI 10.1016/j.copbio.2004.08.002

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
CHO	Chinese hamster ovary
DHFR	dihydrofolate reductase
IgG	immunoglobulin G
PEG	polyethylene glycol

Introduction

Several recent successful clinical results have led to an increased interest in approaches for expressing various forms of monoclonal antibodies. Large doses are often required for therapeutic purposes, in some cases exceeding a gram per patient per year. This need has driven the development of a variety of production systems to make these molecules efficiently and cost-effectively. Truncated forms of antibodies, including Fab, Fab′$_2$ and single-chain Fv forms, have also been used for a variety of clinical applications. Depending on the specific use, different forms and different production hosts might be desirable and several recent reviews have specifically addressed issues surrounding production in microbial systems [1,2•] and transgenic plants [3], as well as general production issues [4]. Given the rapid pace of progress and large amount of active work in this field, this review will necessarily be incomplete and will focus on a subset of the most recently reported advances and developments in expression technologies for antibodies and antibody fragments.

Antibody fragments and microbial production

Antibody fragments such as Fabs or Fab′$_2$s can be the therapeutic of choice for some applications where Fc-mediated effector functions are either not required or are deleterious. The smaller antibody fragments exhibit a shorter circulating half-life than full-length immunoglobulin G (IgG) molecules, but their smaller size can also make them more suitable for applications such as tumor penetration and imaging [4].

These antibody fragments can be generated from mammalian-produced full-length antibodies by treating the IgGs with pepsin or papain. However, *Escherichia coli* remains the production system of choice for antibody fragments used in therapeutic applications for several reasons. Firstly, *E. coli* production can offer a rapid means to progress from antibody selection to good manufacturing practice (GMP) production of antibodies, due to the ease and speed of making productive cell lines compared to eukaryotic cell lines [1]. Secondly, as will be discussed below, high production levels of antibody fragments are usually attainable when *E. coli* is used as the production organism. Currently, there are several Fabs or Fab′s in clinical trials (Genentech http://www.gene.com and Celltech http://www.celltech.com) produced using *E. coli*.

A variety of approaches have been adopted by different groups to improve the production levels of antibody fragments using the *E. coli* system. Expression levels of up to 2 g/L have been reported [5]. The approaches taken include optimizing the expression of light and heavy chains to obtain increased titers of a Fab′ [6], host-cell engineering to prevent proteolysis of the light chain [5], and co-expressing chaperones to improve protein folding [7] or disulfide bond formation [8].

Although the short circulating half-life of antibody fragments is suitable for some applications such as tumor imaging, it can also be a limitation for a wider range of therapeutic uses. Chapman showed that site-specific attachment of either a single or a double 25 kDa or a single 40 kDa polyethylene glycol (PEG) moiety to an Fab′ could increase the circulating half-life by up to 80% of that seen for the IgG without a loss in antigen binding [9]. Leong *et al.* showed that the circulating half-life of an Fab′ could be modulated by the choice of size and

ALXN2043
IPR2023-00999

ALXN2043
IPR2019-00739

structure of the PEG moiety attached [10]. A recent review by Chapman further discusses the increases in circulating half-life that can be obtained by attaching PEG moieties to antibodies [11]. Dennis *et al.* describe an albumin-binding peptide that they fused to the C terminus of a Fab light chain. Using this peptide they were able to demonstrate a 26- to 37-fold increase in circulating half-life in either mice or rabbits [12••]. The development of such techniques to increase the circulating half-life can extend the therapeutic usefulness of antibody fragments and allows for less frequent patient dosing.

There has also been a recent report of producing full-length antibodies in *E. coli* [13••]. Up until this report, the dogma had been that it was too difficult to make Fc-containing antibodies in *E. coli*. The full-length antibodies produced in *E. coli* are aglycosylated and lack the ability to bind to the various Fc_γ receptors, but they do retain binding to the neonatal Fc receptor (FcRn) and, thus, have a long circulating half-life. Although many therapeutic uses of antibodies are thought to require the effector functions mediated through Fc_γ receptors, there are also therapeutic indications such as the binding of a soluble antigen that do not require effector functions. Aglycosylated antibodies can be a suitable choice for these types of indications.

Fungal production

There have been recent reports describing the use of *Aspergillus niger* for the production of monoclonal antibodies or antibody fragments. Fungal production of antibodies could offer potential advantages over *E. coli*, if production titers can be boosted in the fungal systems. Antibodies and fragments produced in the *E. coli* system are usually retained in the *E. coli* periplasm, necessitating some type of cell lysis and recovery of the antibody of interest from a complex protein milieu. Proteins made in fungal systems are generally secreted to the culture supernatant, a potential advantage for product purification. Filamentous fungi such as *Aspergillus* are capable of secreting high levels of homologous proteins, but these high levels have yet to be obtained with heterologous proteins [14].

Ward *et al.* expressed full-length IgGs in *A. niger* using an N-terminal fusion to glucoamylase for both light and heavy chains. They relied on the endogenous KexB protease in *A. niger* to cleave off the fusion during the secretion of the antibody [15•]. The authors found inefficient cleavage of glucoamylase from the antibody, but were able to partially remedy the problem with the addition of glycine residues between the glucoamylase and either the light chain or heavy chain sequence. The resulting antibody was found to have glycine residues at the N terminus of the two chains. Expression levels of up to 900 mg/L are reported.

An outstanding question concerning antibody production in fungal systems is whether the differences in glycosylation compared with mammalian systems will cause immune responses or lead to a lack of effector functions in therapeutic uses. The antibodies made in *A. niger* were found to contain a mixture of glycosylated and aglycosylated heavy chains, but did demonstrate a long circulating half-life and antibody-dependent cellular cytotoxicity (ADCC).

An interesting new approach to modifying the glycans attached to recombinant proteins made in fungal systems was recently described by Hamilton *et al.* [16••]. They used a combinatorial library to optimize the cloning of various glycotransferases to humanize the glycosylation pathway of *Pichia pastoris*. If this approach can be combined with high-level expression of antibodies (up to 1.2 g/L has been reported [17]), it could compete with existing mammalian culture systems for the production of antibodies that retain effector functions and have human-like glycosylation. In another recent study, monomeric scFv production in *P. pastoris* was also demonstrated and optimized [18].

Plant production

Research has continued into using plants as a production platform for antibodies and other therapeutic proteins. A recent review [3] discusses the current state of progress and the outstanding issues that need to be resolved in order for a plant-derived antibody for parental delivery to be approved for marketing. The potential advantages for the plant system that are often cited are capital cost savings, the ability to either increase or decrease production output depending on market needs, and the lack of mammalian pathogens in plants. Some of the current hurdles include a long initial lead time for production, regulatory uncertainties, and questions about the suitability of plant glycans for human therapeutics. One approach has been to mutate the N-linked glycosylation site found in the Fc of monoclonal antibodies and to thereby produce an aglycosylated antibody. Another approach taken has been to try and modify the glycosylation enzymes in plants to more closely resemble those in humans. Bakker *et al.* co-expressed human β1,4-galactosyltranferase and an antibody in tobacco plants and obtained a galactosylation profile resembling that found in mammalian antibodies [19].

Mammalian cell production

While specific types of antibodies and antibody fragments are amenable to production in microbial or other systems, mammalian cells remain the dominant system for the production of the majority of full-length therapeutic antibodies. Recombinant Chinese hamster ovary (CHO) and NS0 cells remain the most prominent cell lines of choice, although numerous other cell lines have been explored as well. For example, the human cell line

PER.C6 has recently been demonstrated to produce significant levels of recombinant products including antibodies in serum-free, suspension cultures [20,21].

Cell line development

The generation of stable cell lines that produce high levels of product is certainly one of the most critical elements required for the development of an efficient cell culture process. The use of dihydrofolate reductase (DHFR) as a marker for selection and amplification using methotrexate represents the most common system for generating cells with high expression in CHO cells. In a recent modification of this standard approach, Bianchi and McGrew divided DHFR into two fragments that could re-associate, aided by a leucine zipper, into an active molecule [22•]. The two pieces could then be inserted into the expression vector adjacent to light and heavy chains, respectively, to increase the probability that only amplification events that amplified both the heavy and light chain segments would be selected. Several groups have also identified flanking elements to include in vectors for the purpose of increasing the development of stable, high-producing clones, such as the recently reported example of expression augmenting sequence elements (EASE) [23] (reviewed in [24–26]).

The most commonly used approaches for transfection with random integration also lead to a wide diversity of expression levels (e.g. see [27]). Recent approaches to screen through this diversity in a high-throughput manner include approaches based on fluorescence-activated cell sorting (FACS) using a labeled anti-Fc antibody [28], a metallothionein green fluorescent protein (GFP) fusion [29] or a surface affinity matrix [30].

Cellular engineering

A recent trend has been the development of additional genetic manipulations to antibody-producing cell lines to confer properties enabling improved bioreactor performance. Specifically, manipulations have been directed primarily at controlling cell growth, preventing cell death or influencing folding and glycosylation.

Over the past decade, a variety of approaches have been developed and evaluated to enable growth control with the goal of decoupling growth and recombinant protein production (reviewed in [25]). A recent example is the use of inducible expression of p27 to induce growth arrest in a serum-free recombinant CHO culture [31]. Another study correlated the increased productivity observed in p27-arrested CHO cultures with increased cell energy usage [32]. Bi *et al.* also recently reported that specific antibody expression was increased approximately fourfold in an NS0 culture arrested using p21 overexpression [33]. Non-genetic approaches for growth control with sustained protein production, which hold the potential advantage of being easier to implement for existing cell culture processes, have also been reported in CHO systems through the use of galactose in place of glucose as a carbon source [34] or the use of nucleosides such as adenosine [35]. Finally, an approach using FACS-sorting of GFP-expressing cells was used to isolate protein-producing CHO cells in a growth-arrested state [36].

Apoptosis can be the major mechanism for cellular death in fed-batch cell culture. Consequently, several different approaches have been recently evaluated to prolong culture viability and, potentially, increase product titers. Members of the Bcl family, specifically *bcl-2* and *bcl-x_L*, have represented the most common elements for engineering apoptosis resistance (see [37]), although recent studies have also demonstrated effects using Aven [38], XIAP and CrmA [39] and approaches targeting Caspase-3 at the RNA level have been reported [40]. Another study showed a modest benefit of hsp70 overexpression on NS0 apoptosis-resistance and on the productivity of hybridoma cells generated from these hosts [41]. Following earlier work in which Bcl-2 expression was shown to improve antibody and recombinant protein expression in the presence of butyrate in CHO cultures, an apotosis-resistant, DHFR- and Bcl-2 overexpressing host was recently created by Lee and Lee [42]. In one recent study, the relative merits of Bcl-2 and Bcl-x_L were evaluated using a serum-free CHO-DG44 line producing a soluble intercellular adhesion molecule. In this analysis, Bcl-x_L was observed to have more potent apoptosis-resistance effects, although benefits on total recombinant protein expression were only observed after amplifying the Bcl-x_L expression to relatively high levels [43]. In response to potential concerns about the long-term effects of Bcl-x_L overexpression, another group recently developed an inducible system, using the metallothionein promoter, for Bcl-x_L expression and apoptosis-resistance during the production phase of hybridoma cultures [44]. Finally, the combination of growth control and anti-apoptosis effects via genetic engineering was recently evaluated in perfusion NS0 cultures using combined p21 and Bcl-2 overexpression and enabled antibody specific productivities approaching 50 pg/cell/day [45•].

In addition to growth control and apoptosis, limitations in the secretory pathway represent a third target for cellular engineering approaches. For example, one recent study evaluated the levels of three potentially important secretory pathway proteins, heavy chain binding protein (BiP), glucose regulated protein 94 (GRP94) and protein disulfide isomerase (PDI), in hybridomas grown in serum-containing and serum-free medium and found a correlation between GRP94 and PDI levels and monoclonal antibody production rates [46]. In another study, calnexin and calreticulin overexpression were found to nearly double the specific productivity of thrombopoietin in recombinant CHO cultures [47].

A variety of approaches have been demonstrated for engineering the glycosylation pathway to manipulate the characteristics of antibodies mediated by oligosaccharides (reviewed by [24,48,49]). Recent studies have demonstrated that the absence of fucose can dramatically increase ADCC [50•,51]. As previously described for plant cultures, an alternative approach is to engineer the antibody sequence to manipulate effector functions (reviewed by [52]). In another recent study, Fc sequence changes were identified to increase serum half-life as well [53].

Process conditions

As monoclonal antibody production processes are increasingly scaled to larger fermentor volumes to meet increasing commercial demands, traditional scale-up issues may present new challenges. For example, a recent study using NS0 cultures explored the effects of pH perturbations, which can occur in large fermentors as a consequence of base addition for pH control [54]. Depending on the fermentor configuration, elevated carbon dioxide levels can also become an issue at larger scales. Carbon dioxide could have direct effects on cell metabolism or could affect culture performance by more general mechanisms related to the increased osmolality resulting from increased carbon dioxide levels. To distinguish between these potential mechanisms, carbon dioxide and osmolality were independently manipulated in hybridoma cultures and revealed an increase in productivity with increased osmolality, while carbon dioxide alone inhibited productivity [55]. Additionally, galactosylation of a hybridoma-produced IgG2a decreased with elevated osmolality, while it increased with elevated carbon dioxide levels (and controlled osmolality) [56]. Certain amino acids were also shown to restore the hybridoma growth rate, although only glycine betaine was found to restore specific productivity at high carbon dioxide levels [57]. In a separate study, antibody productivity was observed to increase by 50% with increased osmolality, which was attributed to improved antibody processing and assembly in the secretory pathway [58]. In another hybridoma example, an IgG3 was observed to have increased galactosylation at higher pH (pH 7.4 versus 7.2 and 6.9) and an increased ratio of *N*-acetylneuraminic acid to *N*-glycolylneuraminic acid [59].

A series of four antibody-producing CHO lines, differing in gene copy number, were also evaluated under hyperosmotic conditions. Antibody productivity was observed to increase most significantly in the lines with the lowest gene dosages (and corresponding mRNA levels) [60]. A proteomics approach was used to investigate the mechanism underlying the beneficial aspects of hyperosmolality on antibody specific productivity in CHO culture. The study identified an upregulation of certain glycolytic enzymes that could lead to increased metabolic energy in these cultures [61].

The effects of temperature have also been evaluated in recent studies. One recent analysis of antibody production in a CHO cell line showed no change in specific productivity at reduced temperature, which contrasts with other reports including a study using the same CHO host and a cytomegalovirus promoter with a different protein [62,63]. In another system, specific Fab production by a CHO line was seen to increase at reduced temperatures [64]. These results demonstrate that the effects of temperature might be cell-line specific. Given that the optimal temperature for growth and productivity can differ for a given cell line, a modeling approach was recently reported to aid identification of the optimal profile for a given process [65].

Complex raw materials such as hydrolysates are still commonly used in cell-culture processes, although the mechanisms for the benefits of these additives are not well understood and may vary between cell lines. A recent study in which certain di- to pentapeptides were added to the medium showed increased monoclonal antibody production in hybridoma cultures using specific peptides, supporting at least one possible type of beneficial component in the hydrolysates [66]. In another recent study, a hollow-fiber microbioreactor was used to distinguish between the effects of fresh medium on hybridoma specific productivity and conditioned medium in increasing cell density [67].

Conclusions

As the clinical applications and needs for recombinant antibodies and antibody fragments have increased in recent years, the need for improvements in expression technologies to support these demands has correspondingly increased. Much recent work has led to significant improvements in the development of multiple expression platforms across the range of microbial, fungal, plant and mammalian systems. Improvements in the microbial production of antibodies and fragments have resulted from host-cell engineering to give increased productivity. There is also a trend towards producing antibodies or fragments with increased circulating half-life. This can be done either post-translationally, by adding PEG moieties, or during translation by adding an albumin-binding peptide or by expressing full-length IgG. There is a growing interest with both fungal and plant systems to address the issues surrounding non-mammalian glycosylation, either through glycosylation engineering or through the mutagenesis of glycosylation sites. For the case of mammalian systems, a growing trend has been towards manipulation of specific cellular pathways to improve performance, both regarding protein titers and antibody characteristics. The development of these approaches has also led to an increased understanding of the fundamental cellular phenomena controlling cell growth, death and productivity in these systems.

Acknowledgements

The authors would like to thank Brad Snedecor and Bill Bennett for their critical review of the manuscript and suggestions.

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