ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Glycosylation of an Immunoglobulin Produced From a Murine Hybridoma Cell Line: The Effect of Culture Mode and the Anti-Apoptotic Gene, *bcl-2*

F.A.A. Majid,[1] M. Butler,[2] M. Al-Rubeai[1,3]

[1]Department of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

[2]Department of Microbiology, University of Manitoba, Winnipeg, Manitoba, R3T 2N2 Canada

[3]School of Chemical & Bioprocess Engineering, University College Dublin, Belfield, Dublin 4, Ireland; telephone: +353-1-7161862; fax: +353-1-7161177; e-mail: m.al-rubeai@ucd.ie

Received 8 May 2006; accepted 1 September 2006

Published online 29 September 2006 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.21207

ABSTRACT: The impact of *bcl-2* over-expression on the glycosylation pattern of an antibody produced by a *bcl-2* transfected hybridoma cell line (TB/C3.bcl-2) was investigated in suspension batch, continuous and high cell density culture (Flat hollow fibre, Tecnomouse system). In all culture modes *bcl-2* over-expression resulted in higher cell viability. Analysis of the glycans from the IgG of batch cultures showed that >95% of the structures were neutral core fucosylated asialo biantennary oligosaccharides with variable terminal galactosylation (G0f, G1f and G2f) consistent with previous analysis of glycans from the conserved site at Asn-297 of the IgG protein. The galactosylation index (GI) was determined as an indicator of the glycan profile ($=(G2+0.5^{*}\ G1)/(G0+G1+G2)$). GI values in control cultures were comparable to *bcl-2* cultures during exponential growth (0.53) but declined toward the end of the culture when there was a loss in cell viability. Low dilution rates in chemostat culture were associated with reduced galactosylation of the IgG glycans in both cell lines. However, at the higher dilution rates the GI for IgG was consistently higher in the TB/C3.bcl-2 cultures. In the hollow fibre bioreactor the galactosylation of the IgG glycans was considerably lower than in suspension batch or continuous cultures with GI values averaging 0.38. Similar low galactosylation values have been found previously for high density cell cultures and these are consistent with the low values obtained when the dissolved oxygen level is maintained at a low value (10%) in controlled suspension cultures of hybridomas.

Biotechnol. Bioeng. 2007;97: 156–169.

KEYWORDS: apoptosis; bcl-2; cell culture; IgG; glycosylation; galactosylation; hybridoma

Introduction

The maximisation of productivity in mammalian cell cultures has been a vital goal for economically viable manufacture of biotherapeutics (Butler, 2005; Warnock and Al-Rubeai, 2006; Wurm, 2004). An important aspect of this is the ability to maintain cells at a high concentration and viability for an extended period of time in culture. However, there are a variety of environmental signals including the depletion of nutrients or the accumulation of metabolic by-products that accompanies the stationary phase of culture that often leads to apoptosis and a rapid decline in cell viability.

Apoptosis has been found to be a major mechanism of cell death in bioreactors of hybridoma (Al-Rubeai et al., 1990; Simpson et al., 1997; Singh et al., 1994) as well as in CHO cell lines (Goswami et al., 1999; Tey et al., 2000a). In contrast to necrosis, apoptotic death is a regulated and controlled process determined in a pre-programmed manner and plays a vital role in tissue regulation. Apoptosis in cultured cells is characterised by condensation, margination and fragmentation of chromatin, reduction in cellular volume and radical changes in the cellular morphology which includes the modification of cytoskeleton (Al-Rubeai, 1998). The cells eventually fragment into apoptotic bodies that contain cytoplasmic organelles and, usually, nuclear fragments. Morphologically, necrosis, apoptosis, secondary necrosis and viable cells can be easily identified using dual staining of unfixed cells with acridine orange and propidium iodide (PI).

Correspondence to: M. Al-Rubeai

Contract grant sponsor: Universiti Technologi Malaysia

Apoptosis has been shown to result in a decrease in cell viability during the death phase of mammalian cell cultures (Singh et al., 1994, Mercille and Massie, 1994a,b) and subsequently results in accumulation of proteases and cell debris which may effect product integrity and complicate down stream processing. Rapid progress has been made in the identification of the factors that trigger apoptosis in bioreactors and the development of strategies, which prevent this form of death (Singh et al., 1996). The main inducers of apoptosis in culture have been found to be glutamine limitation, glucose limitation, and ammonia toxicity (Mercille and Massie, 1994a; Simpson et al., 1997; Singh et al., 1994), oxygen limitation (Mercille and Massie, 1994b; Simpson et al., 1997), excess oxygen (Simpson et al., 1997), shear stress, sub-optimal pH and hyperosmolarity (Perani et al., 1998) and amino acid deprivation (Simpson et al., 1998; Singh et al., 1996).

Transfection with the anti-apoptosis gene, *bcl-2* has proved to be an effective strategy for the suppression of apoptosis in the bioreactor environment (Simpson et al., 1997, 1999; Al-Rubeai and Singh, 1998; Arden and Betenbaugh, 2004). The transfected cell lines have been successfully adapted to suspension culture and serum free media without prior lengthy adaptation. Mammalian cells transfected with the *bcl-2* gene have been shown to exhibit improved robustness in various environmental culture conditions known to induce apoptosis (Bierau et al., 1998; Fassnacht et al., 1998, 1999; Perani et al., 1998; Simpson et al., 1998, 1999). Moreover, the suppression of apoptosis was also found to improve culture productivity (Fassnacht et al., 1998; Simpson et al., 1997). In addition, the prevention of cell death minimised the release of DNA and proteases into the culture medium, which would be expected to increase product stability and aid recovery in unit operations.

The inhibition of apoptosis by *bcl-2* has been shown to affect protein glycosylation in some studies. Olsen et al. (1996) showed that influenza virus-induced apoptosis could be blocked by the presence of *bcl-2* in MDCK cells in culture. Viral replication was less efficient in the MDCK/bcl-2 cells and this was partially attributed to a decreased level of glycosylation in the viral hemagglutinin. Marquina et al. (2004) studied the over-expression of *bcl-2* in transgenic mice. They showed that the autoimmune syndrome developed in the mice was characterised by the production of IgA with reduced sialylation and galactosylation. Many studies have shown the positive impact of *bcl-2* on cell culture performance for the production of recombinant proteins (Al-Rubeai, 1998; Arden and Betenbaugh, 2004). However, there has been little reported on the impact of the suppression of apoptosis on the post-translational modification of recombinant glycoproteins. The integrity and consistency of these proteins are of vital concern to the regulatory authorities, especially when the products are intended for human therapeutic applications. Thus, the main objective of this study was to investigate the impact of bcl-2-induced suppression of apoptosis on the consistency of the protein glycosylation.

Glycosylation is one of the most important post-translational modifications that are known to affect the structure and biological properties of glycoproteins (Jefferis, 2005). Immunoglobulin (IgG) contains 2–3% carbohydrate by mass depending upon the species. IgG contains 2.3 N-linked oligosaccharide chains per molecule in mice (Mizuochi et al., 1987) and 2.8 in humans (Parekh et al., 1985). Two of these represent the conserved glycosylation sites in the Fc portion of all IgGs at Asn-297. The remainder may be found in the hypervariable regions of the Fab of some IgGs. The Fab glycans which are present in only a small proportion of all IgG antibodies are characterised by a high incidence of sialylated structures. The conserved Fc glycan at Asn-297 is a biantennary structure with variable galactosylation but limited sialylation. The position of the Fc glycans between the C_H2 domains of the heavy chains may limit the accessibility of processing enzymes and this is the likely cause of limited galactosylation. The terminal groups of these Fc glycans vary between digalacto- (G2), monogalacto- (G1) and agalacto- (G0) structures and the distribution of these glycoforms can be characteristic of disease states. The normal human IgG content of galactose is 1.15 mol gal/glycan (GI = 0.57). Low galactosylation of IgG may have a critical role in the pathology of autoimmune disorders such as rheumatoid arthritis, lupus and erythematosus (Kuroda et al., 2001; Kuroki et al., 2002) and can occur through aging (Shikata et al., 1998). Functional differences have been recognised between these glycoforms. Agalactosylated IgG fails to activate complement (Nose and Wigzell, 1983), is more liable to proteolytic attack (Leatherbarrow and Dwek, 1983) and is not recognised by cells expressing FcγRI and FcγRII receptors (Walker et al., 1989).

It is well known that the pattern of glycoforms that arise in recombinant proteins including monoclonal antibodies can be affected by the cell line and culture conditions during production (Andersen and Goochee, 1994; Jefferis et al., 1990; Jenkins and Curling, 1994; Restelli and Butler, 2002). Nahgrang et al. (1999) found that the glycosylation pattern of recombinant human anti-rhesus D IgG1 varied significantly when produced in SP2/0, CHO and BEK-293 cell lines. Sheeley et al. (1997) compared the glycosylation of a recombinant humanised murine monoclonal immunoglobulin (CAMPATH) expressed in two different cell lines: a murine myeloma (NS0) and CHO cells. The glycosylation expressed in CHO cells was consistent with the one found in native IgG while the antibody expressed in NS0 cells included potentially hypergalactosylated immunogenic oligosaccharide structures. These molecules contain the Gal α(1–3) Gal terminal residues which are immunogenic in humans. Hayter et al. (1992) found that glycosylation patterns of interferon-γ are dependent on the age of the cell culture while monoclonal IgG produced in ascites, hollow fibre bioreactors and static culture was shown to be differently glycosylated (Cabrera et al., 2005; Lund et al., 1993; Patel et al., 1992). Robinson et al. (1994) characterised a recombinant IgG1 produced by mouse NS0 cell line during

a serum-free, fed batch process. They observed that the antigen-binding characteristics of the antibody remained constant throughout the process but the carbohydrate composition changed.

Because of these multiple factors that could affect protein glycosylation in a condition where the rate of cell death is altered it is now important to assess the impact of *bcl-2* over-expression on the glycosylation pattern of a secreted glycoprotein. As a consequence the present study investigated the effect of *bcl-2*-mediated suppression of apoptosis upon the glycosylation of the secreted monoclonal antibody (IgG1) in a murine hybridoma (TB/C3.bcl-2).

Materials and Methods

Cell Lines

The TB/C3 cell line is an NS1-derived murine hybridoma, which produces immunoglobulin G subclass 1 (IgG1) monoclonal antibody specific to the hapten Cγ2 domain at the Fc region of human IgG. The cell line was transfected with bcl-2 carrier (TB/C3.bcl-2) or control plasmids (TB/C3.pEF) as described in Simpson et al. (1997).

Medium

Cells were grown and maintained in RPMI 1640 supplemented with 5% ultra-low Ig foetal calf serum (Gibco-BRL, Paisley, UK). The serum was treated with a proprietary chromatographic procedure to remove gamma-globulin to a concentration less than 5 μg/mL. This amounted to a maximum concentration of 250 ng/mL in the complete medium, which was unlikely to interfere with the analysis of the hybridoma-secreted IgG.

Cell Maintenance

The cell lines were maintained in cultures in T-flasks or in 50–100 mL Duran bottles at 37°C in RPMI 1640 medium supplemented with 5% FCS. The cells were routinely sub-cultured after 3 days from mid exponential growth phase.

Batch Cultures

Cells were resuspended in 250 mL medium to give an initial viable cell density of 2×10^5 cells/mL in 500 mL Duran bottles that were stirred magnetically at 75 rpm. Samples were taken at 24 h intervals for routine cell counting and cell fraction analysis. Supernatants (100 mL) were collected during the growth phase (48 h post-inoculation) and during the death phase (120 h post-inoculation) for glycosylation analysis.

Continuous Cultures

Magnetically stirred culture flasks (Schott, Germany) were modified to provide two inlets for medium and 5% CO_2/air and a single outlet for waste/exhaust gas. Medium was fed continuously into the flasks using calibrated peristaltic pumps. Outlets were arranged such that the culture working volume remained at 250 mL for both cell lines. Two cultures were run in parallel in a 37°C water bath and fed from a common 5 L reservoir of medium stored on ice. The medium feeding rate was maintained over the passage of approximately 10 culture volumes at each dilution rate. Culture supernatants (typically 5 mL) were taken via the reactor outlet on a daily basis when the vessel volume reached its steady state at each dilution rate. The initial dilution rate was 0.8 day^{-1} and this was reduced step-wise to 0.2 day^{-1}. Culture samples were taken after at least two volume changes of media following each change in dilution rate. Steady state was assumed under each condition when the cell concentration was constant over at least five daily samples.

Tecnomouse High Density Culture System

A viable cell suspension (5 mL at 1×10^7 cells/mL in complete medium) was loaded into an OxyCell^R hollow fibre cassette aseptically, one for each cell line. Temperature was controlled at 37°C and the cultures were aerated with 5% CO_2/air. RPMI 1640 medium (without serum) was circulated through the cassette at a flow rate of 100 mL/h from a 2 L-reservoir bottle (kept on ice). Supernatants were harvested from the extracapillary space at weekly intervals and assayed for IgG. Glucose concentrations were determined using Reflolux II^R system (Boehringer Mannheim GMBH, Munich, Germany). The circulating medium was replaced at regular intervals so that the glucose concentration remained above 3 mM.

Cell Counting and Viability

Cells were counted in samples from suspension cultures by the trypan blue exclusion method (Patterson, 1979). Samples were mixed with an equal volume of 0.1% (w/v) trypan blue solution and loaded into an improved Neubauer haemocytometer counting chamber.

Apoptotic Cell Counting

Apoptotic scoring was carried out as described in Simpson et al. (1997). Cells in suspension were mixed with an equal volume of staining solution containing 10 μg/mL acridine Y orange (AO) and 10 μg/mL PI. The number of viable (V), apoptotic (A), secondary necrotic (SN) and necrotic (N) cells was determined using rhodium coated improved Neubauer haemocytometer under fluorescent microscopy using blue light at 488 nm. The cells were classified by colour

DOI 10.1002/bit

and chromatin morphology and expressed as a percentage of the total cells (V + A + SN + N).

Purification of IgG

IgG was purified from each supernatant sample by Protein G-Sepharose 4 Fast Flow affinity chromatography as described by the manufacturer's manual (Pharmacia Biotech, Uppsala, Sweden). Purified protein was detected in eluted fractions by the Coomassie protein assay reagent (Pierce, Rockford, IL).

Assay for Immunoglobulin G

Antibodies secreted by the TB/C3 cells were determined by a standard sandwich-type enzyme-linked immunosorbent assay (ELISA) as previously described (Al-Rubeai and Emery, 1990). The plates (96-well) were coated with human IgG as the capture antibody. IgG in culture supernatants were detected by sheep anti-mouse IgG peroxidase conjugate, as the secondary antibody and OPD (*o*-phenylenediamine dihydrochloride) as substrate. The absorbance of each well was then measured at 492 nm using a plate reader (SLT Spectra, Salzburg, Austria). Sample IgG concentration was obtained by comparing of the absorbance value against that of the IgG standard curve.

Glycan Release From Immunoglobulin

The N-linked oligosaccharides were liberated from purified IgG enzymatically using N-glycosidase F enzyme, PNGase F as described in Routier et al. (1998). Two hundred microlitres of release buffer (40 mM K_2HP0_4, 10 mM Na-EDTA pH 7.4), was added to 0.5 mg lyophilised glycoprotein giving a concentration of 2.5 mg/mL sample. Samples were dissolved using gentle vortex mixing. PNGase F enzyme (1 unit, 5 μL) was added to the sample and gently mixed. The surface of sample-enzyme mixtures was then covered with 5 μL toluene and sealed with parafilm to avoid any evaporation during incubation. Samples were incubated for 72 h in a water bath at 37°C.

After the incubation period, the sample was placed on ice to stop further enzymatic reaction. Cold absolute ethanol (400 μL) was added to the sample and vortexed gently. The mixture was allowed to precipitate on ice for 20 min. The sample was centrifuged for 1 min at 6,500 rpm to separate precipitated proteins from oligosaccharides. The clear supernatant containing released oligosaccharides was gently aspirated and transferred to a clean 1.5 mL Eppendorf tube for glycan analysis. The precipitated protein was washed in cold absolute ethanol (200 μL) four times to ensure complete recovery of the glycans. The pool of supernatants containing the glycans (approximately 1.2 mL) was then lyophilised overnight prior to further analysis. The precipitated deglycosylated protein was reconstituted in 250 mL PBS (approximately 2 mg/mL) and kept at −20°C prior to analysis by SDS–PAGE.

Glycan Desalting

Desalting was carried out using a 2 mL P-2 Bio-Gel (BioRad, Hemel Hempstead, UK) size exclusion chromatography. Lyophilised oligosaccharide samples were reconstituted with 60 μL degassed deionised double distilled water containing 0.02% sodium azide. The column flow rate was adjusted using the clamp to get an effluent flow of 1 drop per 90 s under gravity. The column was initially equilibrated with three bed volumes of water. Once the sample solution was absorbed into the column bed under gravity, the column headspace was refilled with water and the eluent drops collected (approximately 4 drops/tube; 6 min) for 26 tubes. The tubes were then lyophilised overnight in a Savant S260 bench top vacuum drier. The pooled oligosaccharide fractions were reconstituted with 60 μL deionised double distilled water. The samples were then sterile filtered and transferred into an ampoule and analysed.

HPEAC-PAD for Glycan Analysis

The oligosaccharide fractions liberated from glycoprotein IgG were analysed by high pH anion exchange chromatography using pulsed amperometric detection (HPAEC-PAD; Dionex Corp., Sunnyvale, CA). The Dionex DX500 system was operated automatically by a pre-programmed operating module adapted from Hardy and Townsend (1994). The CarboPac^{TM} PA-100 (4 × 250) and CarboPac^{TM} PA-100 column guard were assembled to the system and the solution flow rate was set up at 1 mL/min. The system was run at 30°C. All the solutions were sparged with helium to displace dissolved CO_2. The oligosaccharide separation was performed using a 0–250 mM NaOAc (gradient) in the presence of 100 mM NaOH, over a period of 45 min. Between 12 and 23 mM NaOAc, neutral oligosaccharides were released while between 23 and 250 mM NaOAc, sialylated oligosaccharide structures were released. The separated oligosaccharides were detected by PAD with a gold electrode and triple-pulse amperometry (E1 = 0.05 V, tl = 480 ms; E2 = 0.75 V, t2 = 120 ms; E3 = 0.2 V, t3 = 60 ms).

Determination of the Neutral Glycans by RAD

The relative area distribution (RAD) of these three neutral structures represented more than 95% of the total oligosaccharides released while sialylated oligosaccharide was only around 5% of the total structures released from IgG. The neutral oligosaccharide peaks released from all the samples analysed were identified using purified standard oligosaccharides (Oxford Glycosciences, Oxford, UK), which were separated under the same chromatographic conditions.

Galactosylation Index

The predominant glycan structures identified in the immunoglobulin samples were agalactosylated, monogalactosylated and digalactosylated. In order to compare the degree of terminal galactosylation of the characterised structures, a galactosylation index (GI) was assigned to each glycan profile, determined from the relative areas of peaks as:

$$GI = \frac{G2 + 0.5 * G1}{G0 + G1 + G2}$$

where G0, G1 and G2 are the relative areas for the agalactosylated, monogalactosylated and digalactosylated glycans, respectively.

Monitoring *bcl-2* Gene Over-Expression

The level of *bcl-2* over-expression in the hybridoma, TB/C3.bcl-2 was monitored at regular intervals by immunostaining of the *bcl-2* protein using Western blotting (Simpson et al., 1997).

Rates of Production

The specific rate of production of IgG in batch culture was determined from the slope of a plot of the IgG concentration against the integral values of cell concentration-time (Renard et al., 1988).

Results

Growth and Production Characteristics in Suspension Batch Cultures

The investigation was initiated by growing both the bcl-2 transfected (TB/C3.bcl-2) and control plasmid transfected hybridomas (TB/C3.pEF) in suspension batch culture for 120 h. The viable cell density of both cultures is presented in Figure 1. The viable cell density of the *bcl-2* transfected culture increased from its initial seeding concentration of around 2×10^5 cells/mL to approximately 1.0×10^6 cell/mL after 48 h and to a maximum concentration of 1.1×10^6 cells/mL at 72 h. The most significant difference between the two cultures was in the rate of the decline phase. For the TB/C3.bcl-2 cells, viable density declined gradually after 72 h and finally dropped to around 7×10^5 cells/mL after 120 h post-inoculation. For the control culture, the viable cell density reached a maximum of around 11×10^5 cells/mL after 48 h post-inoculation. The viable cell density then declined rapidly during the next 48 h and finally reached zero at 120 h.

Cell Fraction Scoring

Fluorescence microscopic analysis of cells was performed by double staining with a mixture of PI and acridine orange as described in the Materials and Methods. The percentage of viable, apoptotic, SN and necrotic cells in TB/C3.bcl-2 and control, TB/C3.pEF cultures are presented in Figure 2a and b, respectively. During the first 48 h of the Bc1-2 culture, all the cells were viable. After 120 h post-inoculation, the viable cell fraction decreased to 83%. The loss in viability coincided with a slight increase in the necrotic cell fraction after 72 h post-inoculation. The apoptotic and SN cell fraction remained negligible for the duration of the culture. The viability of the control cell line, TB/C3.pEF remained high up to 48 h post-inoculation. However, a sharp decrease in the viable cell fraction was observed after 48 h which coincided with an increase in the fraction of apoptotic cells and a sharp increase in the SN cell fraction (Fig. 2b). The SN morphology is associated with late stage apoptosis as previously described (Singh et al., 1994). The differences between the two cell lines show the effect of bcl-2 in maintaining a high cell viability over an extended period of culture (TB/C3.bcl-2).

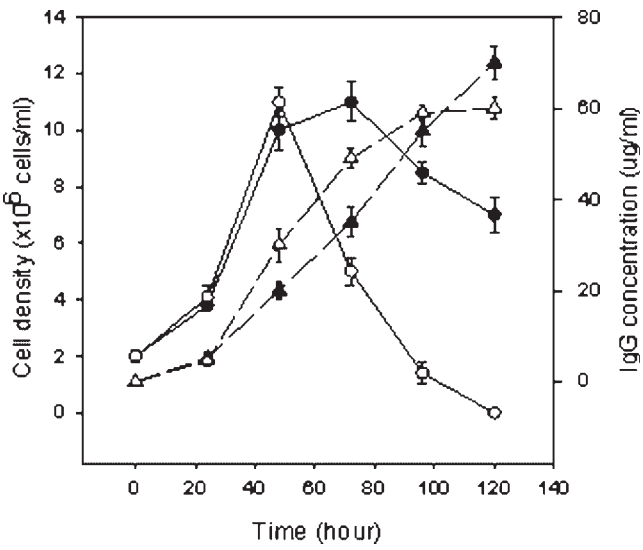

Figure 1. Growth in suspension batch cultures of TB/C3.pEF (○) and TB/C3.bcl-2 (●) cells. Cells were inoculated into 250 mL RPMI 1640 medium with 5% FCS to give an initial viable cell density of 2×10^5 cells/mL. Cultures were held in 500 mL Duran bottles that were stirred magnetically at 75 rpm. Viable cell concentrations were determined by the trypan blue exclusion assay for TB/C3.pEF (○) and TB/C3.bcl-2 (●) cells (continuous line). Daily samples were taken from TB/C3.pEF (△) and TB/C3.bcl-2 (▲) cultures and the IgG concentration of supernatants were determined by ELISA (broken line). Sample size, $n = 8$, error bars represent 95% confidence limits.

Immunoglobulin Production

The volumetric immunoglobulin (IgG1) production of both TB/C3.bcl-2 and control, TB/C3.pEF cultures are also presented in Figure 1. A similar amount of product was released by both cell lines from the beginning of each culture but the IgG concentration of the control culture was higher

DOI 10.1002/bit

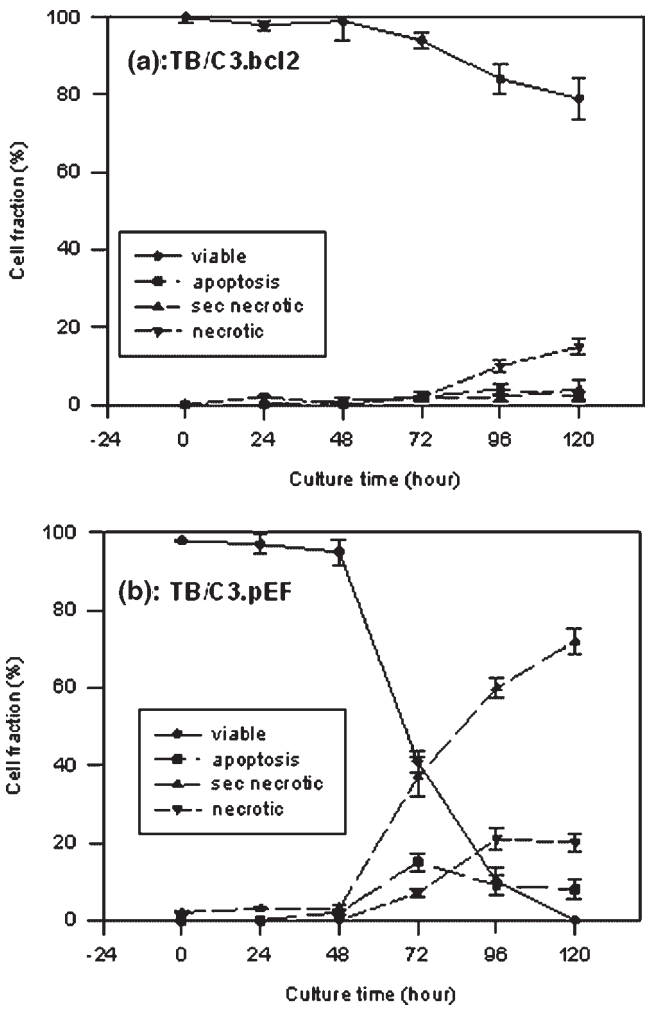

Figure 2. Change in cell fractions in suspension batch cultures. The % of viable (●), apoptotic (■), secondary necrotic (▲) and necrotic (▼) cells were determined by fluorescence microscopy for (**a**) TB/C3.bcl-2 cells and (**b**) TB/C3.pEF cells. Sample size $n = 100$, error bars represent 95% confidence limits.

between 48 and 96 h post-inoculation after which there appeared to be no further Ig production. It is be noted that the culture period between 48 to 96 h corresponded to the rapid loss of viability of these cells and it is probable that a significant amount of Ig was released during cell lysis that occurred during the necrotic phase shown in Figure 2. For the TB/C3.bcl-2 culture, after a 1 day lag there was a gradual increase in Ig throughout the culture up to 120 h. This corresponded to the high cell viability that was maintained with only a slight decrease to 85% after 120 h. Although the cultures were terminated at 120 h, it is likely that the IgG1 concentration would have continued to increase beyond the maximum of 70 mg/L that was reached. The increased volumetric productivity of the *bcl-2* transfected batch cultures during an extended death phase has previously been reported (Simpson et al., 1997).

The specific productivity (Q_P) of each culture was analysed from the integrated values (viability indices) obtained from the growth curve and regression analysis. This showed that in both cases the production of antibody was independent of the phase of culture but was directly related to the number of viable cells. Specific productivities (Q_P) were obtained from the slopes of these plots. The Q_P for the TB/C3.pEF was 28.5 ± 0.9 pg/cell per day whereas the Q_P for the TB/C3.bcl-2 was significantly lower at 18.3 ± 0.5 pg/cell per day.

The Glycosylation Profiles of Monoclonal IgG1 in Suspension Batch Cultures

The IgG isolated from culture samples was deglycosylated enzymatically. The liberation of glycans from the IgG samples was demonstrated by the mobility shift of bands for deglycosylated compared to glycosylated samples as shown by SDS–PAGE gels (data not shown). The positional shift of the bands observed in the gels following deglycosylation corresponded to a weight loss of around 2%, which is the expected mass of glycans attached to IgG (Deisenhofer, 1981).

Glycosylation analysis was carried out on samples of the monoclonal IgG1 produced at exponential (48 h) and decline (120 h) culture phases of both *bcl-2* transfected and control hybridoma cell cultures by HPAEC-PAD. The profiles of oligosaccharides liberated from IgG1 are presented in Figure 3. In all four chromatograms presented, there are three prominent neutral oligosaccharide peaks clearly separated when the sodium acetate concentration was gradually increased from 12 mM (at 5.1 min) to 23 mM (at 21.9 min). With reference to the standard oligosaccharides, these peaks were identified as G0f (asialo-agalacto-complex type biantennary oligosaccharide with core substituted with fucose), Glf (asialo-monogalacto-complex type biantennary oligosaccharide with core substituted with fucose) and G2f (asialo-digalacto-complex type biantennary oligosaccharide with core substituted with fucose), respectively. The RAD of these peaks was determined using the PeakNet^{TM} programme.

In *bcl-2* transfected samples, at maximum growth (48 h), the largest proportion of the glycans liberated were Glf structures, representing 45% of the total RAD of all the three oligosaccharides. The RAD of G0f and G2f structures were around 24 and 31%, respectively. However, at 120 h, both Glf and G2f structures (with RAD of around 44 and 40%, respectively) were the larger proportions with the RAD of G0f remaining the lowest, around 16% of the total RAD.

For the control samples at 48 h the largest proportion of oligosaccharides liberated was also Glf structures, representing around 43% of the total RAD. The RAD of G0f and G2f structures was around 26 and 31%, respectively. However, in contrast to the *bcl-2* transfected culture, for the late phase samples (120 h), there was an increase in G0f with a decrease in G2f. The other two structures, G0f and G2f were

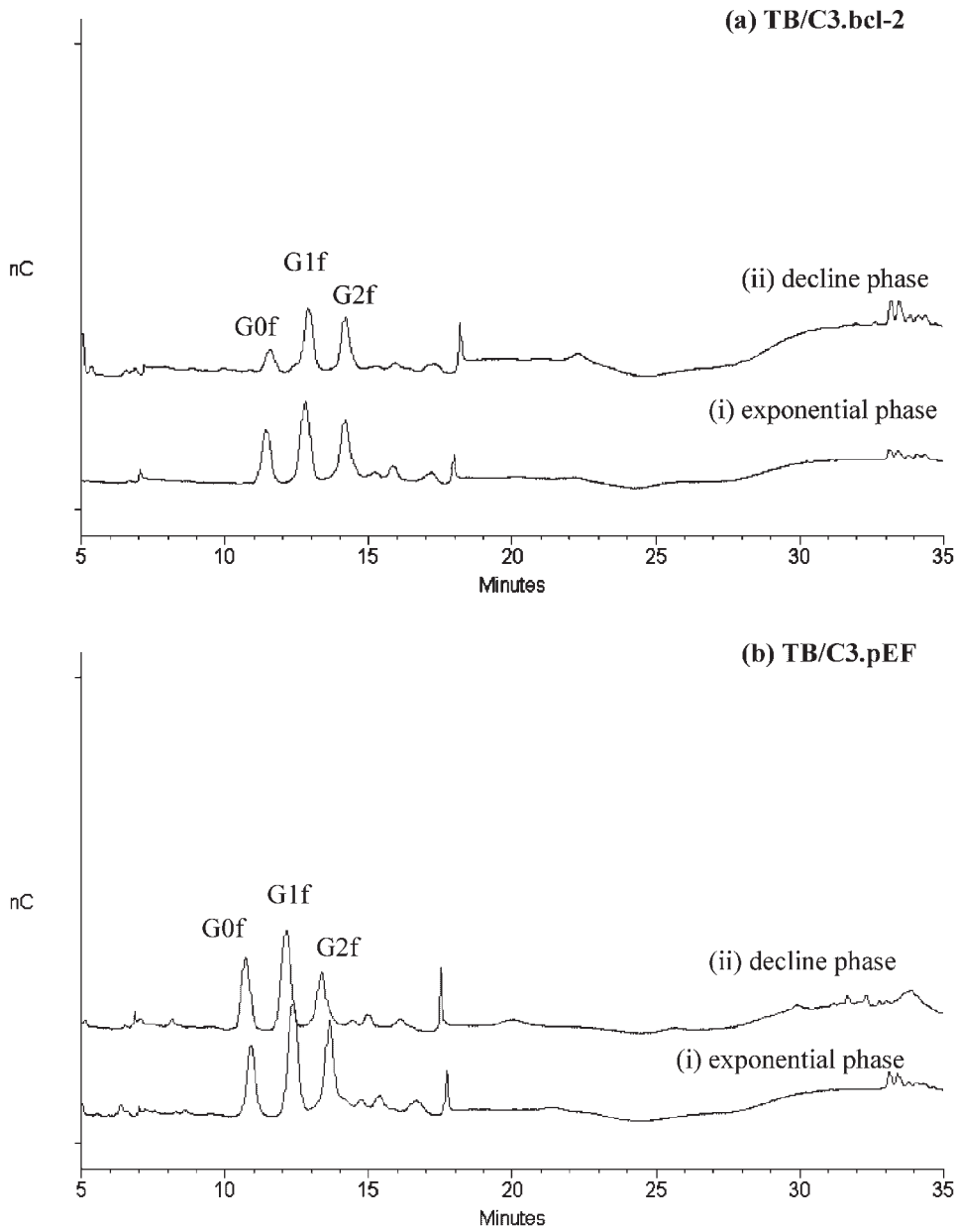

Figure 3. Typical glycan profiles of immunoglobulins extracted from suspension batch cultures. The chromatograms of oligosaccharides were obtained by HPAEC-PAD analysis of IgG samples of (**a**) TB/C3.bcl-2 and (**b**) TB/C3.pEF cultures. Samples were taken during (i) exponential and (ii) decline phases in suspension batch culture. Peaks 1, 2 and 3 were identified as G0f, G1f and G2f respectively with reference to standard oligosaccharides.

represented with RAD values of around 30 and 24%, respectively. The largest proportion was represented by the Glf structure with a RAD of around 46% of the total oligosaccharide RAD. It is interesting to note that Glf structure predominates in all the four samples. The small peaks at a retention time >15 min may correspond to a minimal proportion of sialylated glycan structures but these were not analysed.

The GI for each sample is shown in Table I as a measure of the extent of galactosylation from this analysis. The error associated with these values is normally below 3% (Kunkel et al., 1998). The samples from both cell lines taken at late-exponential phase (48 h) show no significant difference (0.53) whereas for the decline phase culture samples, the galactosylation of the bcl-2 samples (0.62) is significantly

Table I. Galactosylation indices of Ig glycans from suspension cultures.

Sample time	TB/C3.pEF	TB/C3.bcl-2
Exponential phase (48 h)	0.53	0.54
Decline phase (120 h)	0.47	0.62

DOI 10.1002/bit

higher than control (0.47). This result shows that in the decline phase (up to 120 h) the *bcl-2* transfected culture with a viability of around 85–90% produced a greater degree of galactosylation of the IgG glycans. This suggests that *bcl-2* over-expression prolonged cell viability leaving the cells intact and able to continue a relatively high level of galactosylation. The control cells had a significantly lower viability at that stage and released IgG with lower galactosylation. This difference in the observed level of terminal glycan galactosylation of Ig between the two cultures could be explained by loss of functional galactosylation in the control cultures during the decline phase or by an enhanced activity of glycosidases resulting from cell lysis.

Cell Growth and Production Characteristics in Continuous Cultures

Continuous chemostat cultures enabled the establishment of steady state conditions at various dilution rates. This system offers an excellent environment to investigate the impact of *bcl-2* over-expression on product glycosylation under optimal conditions over extended periods of time and at different dilution rates (Simpson et al., 1999).

The results of cell growth characteristics in relation to dilution rates of *bcl-2* transfected hybridoma and the control cell line are shown in Figure 4. The experiment was carried out in continuous mode from the highest dilution rate of 0.8 day^{-1} and gradually decreased to 0.2 day^{-1}. For this range of steady states the specific growth rates (μ) corresponded to 0.0083–0.033 h^{-1}. These values can be compared with the maximum specific growth rates (μ_{max}) observed in batch culture of 0.042 h^{-1} (TB/C3.pEF) and 0.039 h^{-1} (TB/C3.bcl-2).

At a dilution rate of 0.6 day^{-1} and above, the viability of both cell lines remained above 90%, with the viable cell concentration at 1.4×10^6 cells/mL. At 0.5 day^{-1} and below, there was a significant difference in viability and viable cell concentration between the two cell lines. For the TB/C3.bcl-2 cells at dilution rates of 0.5–0.3 day^{-1}, viability remained above 90% and the viable cell concentration remained above 1.4×10^6 cells/mL. In contrast there was a marked decrease in viability and a slight decrease in viable cell concentration from 0.5 to 0.3 day^{-1} in the control cell line culture. A marked decrease in viability and viable cell number was clearly evident in the TB/C3.bcl-2 cells from dilution rates of 0.3 to 0.2 day^{-1}. However, in comparison, the decrease in viability and viable cell concentration of the control cell line from 0.3 to 0.2 day^{-1} was much greater (below 40% and 6×10^5 cells/mL, respectively).

Using fluorescence microscopic analysis of nuclear morphology a decrease was shown in viability of *bcl-2* transfected cells that was coincidental with the increase in apoptotic and necrotic cells while the decrease in viability of control cell lines coincided with an increase in apoptotic cells only as shown in Figure 4.

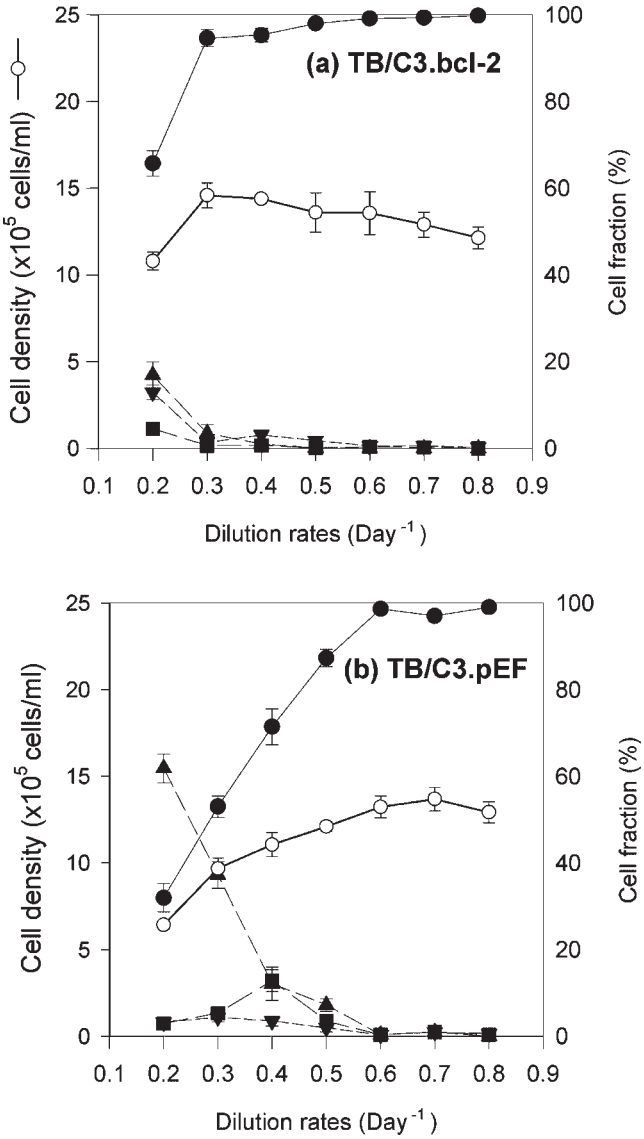

Figure 4. Growth characteristics in continuous cultures. Steady state continuous cultures (250 mL) were established for TB/C3.bcl-2 cells (**panel a**) and TB/C3.pEF cells (**panel b**) in magnetically stirred flasks. Medium was continuously fed into the flasks using calibrated peristaltic pumps from a common 5 L reservoir of medium stored on ice. The volume of each culture remained constant at 250 mL. The viable cell concentrations (●) were determined by the trypan blue exclusion assay from 5 mL samples obtained at regular intervals. The % of viable (○), apoptotic (■), secondary necrotic (▲) and necrotic (▼) in each of the cell populations was determined by fluorescence microscopy. Sample size, $n = 8$, error bars represent 95% confidence limits.

The Glycosylation of IgG Produced at Different Dilution Rates in Continuous Culture

The glycosylation profiles of IgG from *bcl-2* transfected and control cells were determined at each dilution rate. The three prominent peaks were separated in all the samples and were identified as neutral oligosaccharides, namely G0f, Glf and G2f with reference to standard oligosaccharide profiles. The RAD of these peaks was determined at each dilution rate. At

a dilution rate of 0.4 day^{-1} and above, samples analysed were dominated by the Glf structure, representing more then 50% of the total RAD while G0f and G2f remained around 25% each peak. At dilution rates of 0.3 day^{-1} and below, the RAD of Glf in all the samples analysed was reduced and the G0f was slightly increased. The RAD of G2f remained below 25% of the total peak RAD.

Similar patterns of RAD were observed at all dilution rates in the control cell line as were observed in the TB/C3.bcl-2 cultures. In these cultures, the glycan galactosylation of IgG from both cell lines increased with dilution rates (0.4–0.8 day^{-1}) in comparison to lower dilution rates (0.2–0.3 day^{-1}).

A plot of the GI of glycans isolated from Ig from cultures at varying dilution rates is shown in Figure 5. The range of values extended from 0.36 to 0.59 with the minimum value occurring at a dilution rate of 0.3 day^{-1} and the maximum at 0.7 day^{-1}. It is noted that the GI value increases steadily for both cell lines in cultures between these two dilution rates. This suggests that the extent of galactosylation increases with the specific growth rate of the cells. An alternative explanation is that the galactose is removed by a higher activity of extracellular glycosidases in the slower growing cultures, where the residence time of secreted IgG is higher.

Overall there appears to be a consistently higher index of galactosylation for Ig produced from the TB/C3.bcl-2 cells compared to the control TB/C3.pEF cells ($P = 0.01$ by paired *t*-test).

The Effect of bcl-2 Over-Expression on the IgG Glycosylation Profiles From Cells in High Cell Density Cultures

The influence of *bcl-2* over-expression on antibody fidelity in the Tecnomouse Hollow Fibre system (Integra Biosciences, Chur, Switzerland) was carried out in this study. This system is a continuous perfusion system where cells and products are retained in a flat hollow fibre cassette with a continuous supply of medium and a continuous out flow of spent medium. Five millilitres of cell suspension (5×10^6 cells/mL) of each cell line (TB/C3.bcl-2 and TB/C3.pEF) was inoculated into the extracapillary spacing of two separate hollow fibre cassettes as described in Materials and Methods. The cell growth was monitored by measurement of glucose consumption. To ensure maximum nutrient supply, 2 L of medium was circulated through the cassette at 100 mL/h. The glucose level was maintained above 2.5 mg/mL throughout the duration of the culture. Five microlitres of the supernatant from the extracapillary space (i.e. the product harvest) was withdrawn weekly and replaced with an equal volume of fresh medium. The dead cells from the culture were counted by the trypan blue exclusion method and remained below 10^4 cells/mL at every harvest for the entire duration of the culture.

Productivity From High Density Cultures

Both cell lines were grown under optimal culture conditions for 6 weeks. The supernatant from each culture was harvested on a weekly basis and the IgG concentration determined over the course of the culture. The IgG concentrations from the TB/C3.bcl-2 and control TB/C3.pEF cultures are presented in Figure 6. There was a

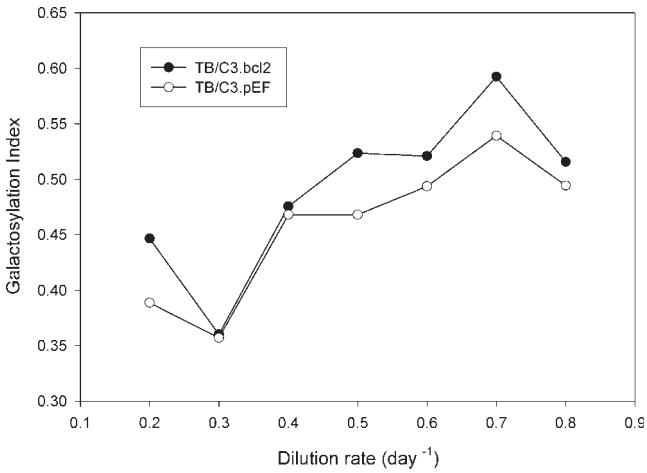

Figure 5. Galactosylation indices of glycans released from IgG samples secreted by TB/C3.pEF (○) and TB/C3.bcl-2 (●) cells in cultures maintained at various dilution rates. The culture conditions are described in the legend for Figure 4.

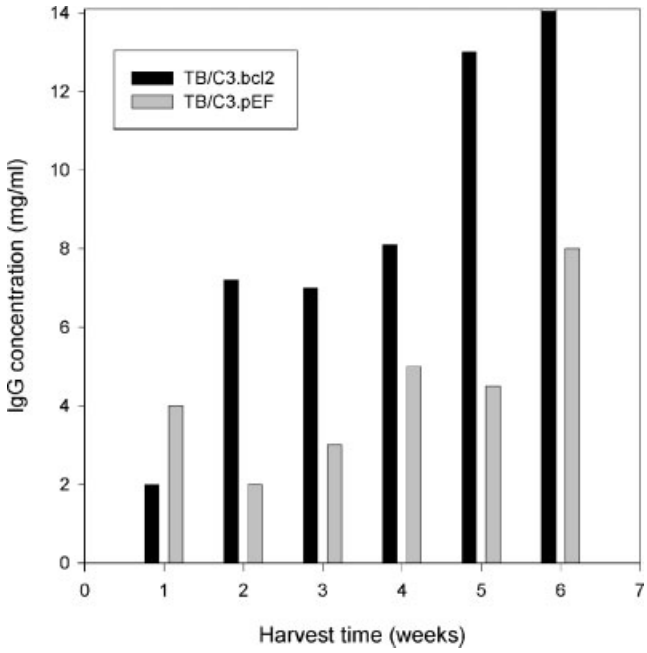

Figure 6. The volumetric production of immunoglobulin (IgG) from TB/C3.pEF and TB/C3.bcl-2 cells in high density cultures (Tecnomouse system). Samples harvested during weeks 1–6 were analysed for IgG by ELISA as reported in Fassnacht et al. (1999).

DOI 10.1002/bit

marked difference in the production of monoclonal IgG from *bcl-2* transfected to control culture through out the 6 weeks culture duration. The monoclonal mouse IgG production increased from around 2 mg/mL in week 1 to around 14 mg/mL in week 6. In the control culture, the increase in IgG production was slower with a maximum concentration of around 8 mg/mL in week 6. Clearly, *bcl-2* over-expression had a substantial impact on the productivity of the cells in this high cell density production system. Using the IgG purified from the supernatants of the experiment described above, the impact of *bcl-2* transfection and the effect of culture duration in both cultures with respect to product glycosylation profiles were then investigated and the results are presented below.

The Glycosylation Profiles of IgG From High Density Cultures

The IgG samples from both *bcl-2* transfected and control hybridoma cultures harvested in weeks 1, 3 and 6 were chosen to study glycosylation profiles. The RAD of the three prominent glycan peaks is presented in Figure 7. For the *bcl-2* cultures, the percentage of G0f was slightly decreased from around 50% at week 1 to approximately 40% at week 6. While the galactosylated structure, Glf was slightly increased from approximately 35% at week 1 to approximately 45% at week 6. The galactosylated. G2f structure increased slightly from over time from 14 to 16%. In control cultures, the percentage of G0f structure was slightly lower compared to the *bcl-2* cultures at 37% in week 1 and decreased slightly to approximately 30% in week 6. The percentage of Glf structure was slightly increased from 45% in week 1 to 55% in week 6, while the G2f structure remained constant at 15% between week 1 and week 6.

The GI was determined for each sample analysed (Table II). The results show an increased galactosylation for samples from the TB/C3.bcl-2 cultures from week 1 to week 6, whereas for the control cultures there was only a slight increase from week 1 to week 3. The GI values were slightly but significantly lower for the IgG from the TB/C3.bcl-2 cultures compared to the controls ($P < 0.05$). However, it is to be noted that these GI values are all significantly lower than those obtained from suspension batch cultures.

Discussion

The impact of *bcl-2* over-expression on growth and productivity of industrially important mammalian cell lines has attracted considerable attention since the mid 1990s. There is a positive impact of this protein on cell robustness and prolonged cell survival under environment stress in culture (Goswami et al., 1999; Itoh et al., 1995; Simpson et al., 1997; Singh et al., 1996; Tey et al., 2000a,b).

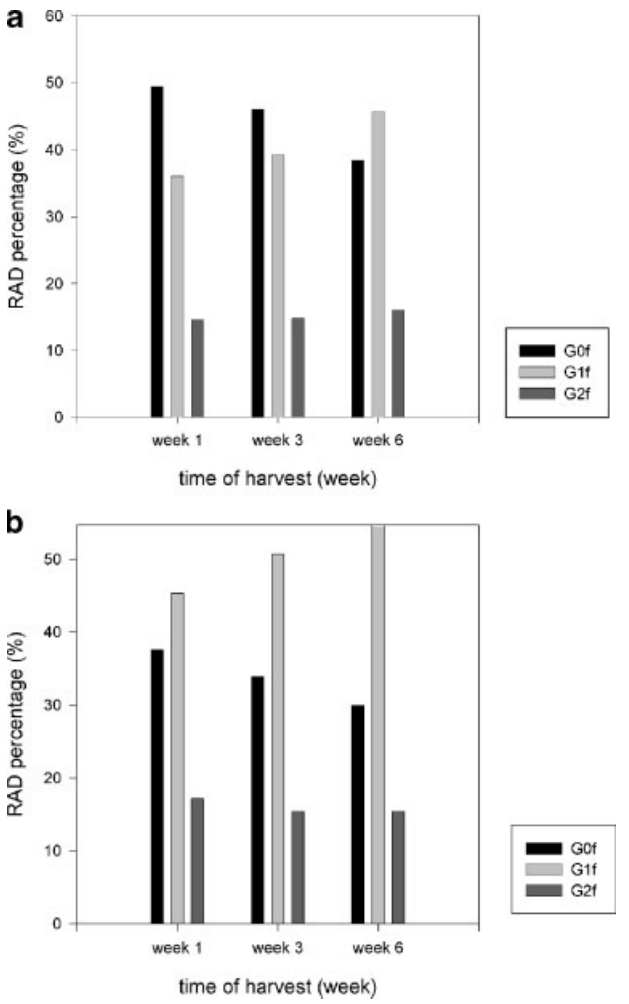

Figure 7. The relative distribution of glycans of IgG extracted from high density cultures. Values were determined from the relative peak area distributions obtained by HPAEC-PAD analysis of glycans from (**a**) TB/C3.bcl-2 and (**b**) TB/C3.pEF cultures at weeks 1, 3 and 6 as indicated.

In this study, the impact of *bcl-2* over-expression in a murine hybridoma on immmuoglobulin glycosylation was investigated. The growth profile of the bcl-2-transfected cell line shows that compared to a control a high viability of the cells was maintained for several days after attaining the maximum cell density. It is well recognised that B-lymphocyte hybridomas are particularly susceptible to apoptosis, manifested by the absence of a stationary phase and a rapid decline in cell viability following the peak of cell

Table II. Galactosylation indices (GI) for samples from the Tecnomouse.

Sample time	TB/C3.pEF	TB/C3.bcl-2
Week 1	0.398	0.326
Week 3	0.408	0.344
Week 6	0.408	0.388

density. From analysis of the cell populations in our study it is clear that *bcl-2* retards this effect by reducing the rate of apoptosis.

High protein production from a mammalian cell culture depends upon two important factors; a high cell specific productivity, as well as the attainment and maintenance of a high cell density. For the TB/C3 cells analysed in this study, the effect of *bcl-2* was to reduce the cell specific productivity. This resulted in lower concentrations of IgG during the exponential growth phase. However, the extended viability of the TB/C3.bcl-2 cells at peak cell density allowed IgG production to be continued for longer and at a point where production had stopped in the control cells. The net effect of these opposing effects was that the volumetric production of IgG was higher at the end of the TB/C3.bcl-2 cell culture.

Analysis of the glycan profiles of the IgG from these cultures by HPAEC-PAD showed three major peaks that are characteristic of the two conserved glycan structures at Asn-297, located in the interstitial space between the two heavy chains at the C_H2 domain (G0f, G1f and G2f). Different structural profiles are associated with changes in the relative peak areas that result from variable terminal galactosylation of the glycans. These three glycoforms amounted to 95% of the structures identified by the HPAEC system. Smaller peaks at higher retention times suggested the presence of a minimal amount of sialylated structures as previously identified with a similar separation (Kunkel et al., 1998). The G1f peak is likely to contain two isomers with the possibility of the single terminal galactose attached to either the α1–3 or the α1–6 antenna of the core mannose residue. Although 38 possible glycan structures have been identified from IgG isolated from 12 different mammalian species, the proportion found in mouse IgG is limited and predominantly represented by the biantennary core fucosylated asialo structures shown by HPAEC (Raju et al., 2000). Mouse IgG glycans are almost wholly fucosylated and contain no bisecting N-acetylglucosamine, that are present in human, rabbit and bovine IgG (Mizuochi et al., 1987). Minor incomplete structures have been identified, particularly under conditions of cellular stress (Serrato et al., 2004). However, the extent of terminal galactosylation of the biantennary structures on either heavy chain of IgG has been considered to be the main source of variability in mouse IgG (Masuda et al., 2000).

This variability can be quantified by the GI, which has theoretical limits of 0–1. Generally the variability is found in differences between the relative contents of G0f and G2f with the G1f remaining constant. An analysis of galactosylation of immunoglobulin from normal serum showed GI values of 0.62 for bovine IgG and 0.57 for human IgG (Kunkel et al., 1998). From this earlier work the effect of DO on the glycan structures from a murine hybridoma in culture showed an increase of GI from 0.32 to 0.56 as the DO level was increased from 10 to 100%. These values are comparable with the GI values obtained from glycan analysis of both TB/C3 cell lines during the exponential phase when cultured under identical conditions. However, there appeared to be a significant difference in the values during the decline phase; with a decrease in the value from TB/C3.pEF cultures and an increase in the TB/C3.bcl-2 culture. We might speculate that this difference could be related to the enhanced viability of the cells in the TB/C3.bcl-2 culture and the possibility of the release of glycosidases from the TB/C3.pEF cells.

The activities of fucosidases, β-galactosidases, β-hexosaminidase and sialidase have been shown to accumulate in the extracellular medium of CHO cells (Warner, 1999). Such glycosidases could also be secreted by hybridoma cells to cause some loss of terminal galactose. Alternatively, the efficiency of the galactosyl transferase enzyme necessary for galactosylation could be reduced in TB/C3.pEF cells at the point in the decline phase when the IgG samples were taken.

Reduced efficiency of glycosylation has been generally observed for other cell lines toward the end of a culture (Hooker et al., 1995). Limiting nutrients could result in a reduced efficiency of glycosylation due to limited sources for energy supply and nucleotide sugar synthesis. Nyberg et al. (1999) observed reduced glycosylation site occupancy of interferon-γ (IFN-γ) produced by CHO cell lines during a period of glutamine and glucose starvation. Another contributing factor could be the release of incompletely glycosylated molecules from transport vesicles during the transfer of these molecules between the Golgi apparatus when the cells burst.

Continuous chemostat culture is a valuable experimental tool for determining the effects of culture parameters during steady state growth conditions of cells. Here we show the loss of cell viability as dilution rates are reduced from 0.8 to 0.2 day^{-1}. This data was first reported by Simpson et al. (1999) and shows that *bcl-2* can protect the loss of viability. Analysis of the cell populations within this dilution range shows a significantly higher number of SN cells in the TB/C3.pEF culture compared to the TB/C3.bcl-2. This is an indication of late stage apoptosis.

At a dilution rate of 0.5 day^{-1} and higher, the antibody titre was similar in both cultures. Only at dilution rates of 0.3 and 0.4 day^{-1} was there a significant difference between cell lines with a significantly higher Mab titre in the TB/C3.pEF cultures (54–64 μg/mL). This divergence at these dilution rates could be explained by the fall in viability of the TB/C3.pEF cells and the possibility of the rapid release of antibody from SN cells. From the results presented in this article, analysis of the galactosylation of the glycans extracted from the IgG produced shows that the GI values increase significantly at higher dilution rates with values above 0.5 being characteristic of batch cultures and growth in bioreactors at higher oxygen levels (Kunkel et al., 1998).

Similar changes in glycosylation profile at low dilution rates were previously reported in continuous culture of CHO producing IFN-γ. Hayter et al. (1992) found that despite the changes in IFN-γ production rate and cell physiology, the profile of IFN-γ glycosylation was similar at all dilution rates except at the lowest growth rates where there was an increase in production of non-glycosylated IFN-γ. The increase in non-glycosylated IFN-γ was thought

DOI 10.1002/bit

to be due to glucose and glutamine limitation, which occur at low dilution rates (Nyberg et al., 1999). Glutamine and glucose are the major nutrients for cell growth and these are likely to be limiting at the lower dilution rates. Glutamine limitation appears to influence glycosylation by reducing amino sugar formation and hence UDP-GNAc (UDP-GIcNAc and UDP-GaINAc) concentration. (Nyberg et al., 1999). Other nucleotide sugars such as UDP-glucose, GDP-mannose and UDP-GlcNAc are derived from glucose-6-phosphate and fructose-6-phosphate and are also influenced by glucose limitation (Hayter et al., 1992).

Hayter et al. (1992) suggested an alteration of glycosyl transferase enzyme activities due to the onset of cell death to explain the reduced glycosylation at low dilution rates. However, this is an unlikely explanation in our study because low galactosylation of IgG was found in cultures of both cell lines at low dilution rates even though the viability of the TB/C3.bcl-2 was significantly higher than the control cells. The reduced galactosylation at low dilution rates (0.2 and 0.3 day^{-1}) could be related to the increase in culture residence time. This would cause the accumulation of metabolic by-products to higher concentrations. Ammonia in particular has been shown to have a significant effect on glycan microheterogeneity (Yang and Butler, 2000). Similarly any extracellularly released glycosidases could increase to higher levels causing an increased likelihood of glycan degradation.

At higher dilution rates (0.5–0.8 day^{-1}) and correspondingly higher cell growth rates the TB/C3.bcl-2 cells appeared to produce IgG with a slightly but significantly higher galactosylation. This is difficult to explain because the growth characteristics of the two cell lines appear to be the same within this dilution range. However, at least it shows that the use of bcl-2 to limit the rate of apoptosis in these cells does not adversely affect the microheterogeneity of glycosylation. These finding are important for strategic modification of cell culture systems for product optimisation.

The Tecnomouse culture system offers the possibility of process intensification by a continuous perfusion high cell density cultures over extended periods of time. This has the advantage of continuous production of antibodies over periods of several weeks. The design of the hollow fibre cassettes enables continuous feeding of fresh medium to the cells that are held in the extracapillary space from which the high molecular weight products like antibodies can also be harvested.

The hybridoma cell line was shown to grow successfully in the Tecnomouse culture system (Fassnacht et al., 1999). After the first week the IgG production from the TB/C3.bcl-2 culture was significantly and consistently higher than the TB/C3.pEF culture. After 6 weeks the IgG production from the *bcl-2* culture was twofold higher than the control culture. If it is assumed that the specific productivity of the TB/C3.bcl-2 cells was lower than the TB/C3.pEF cells as was shown in suspension culture, then the higher Mab titre must be related to a significantly higher cell density in the TB/C3.bcl-2 culture. This might be explained by the enhanced robustness of these cells and the ability to withstand adverse conditions that could cause loss of viability in the control cells.

Analysis of the glycans extracted from the IgG produced in the Tecnomouse system at three separate time points showed that the extent galactosylation as quantified by GI (mean = 0.38) was significantly and consistently lower than the values obtained in batch and continuous cultures. A possible explanation for this is that the cells could be exposed to adverse conditions that could arise through generation of the very high cell densities. High cellular demand could result in a nutrient and oxygen depletion as well as an unfavourable pH. There is a likelihood that gradients of nutrients, pH and oxygen developed within the mass of cells as they reach the tissue-like cell densities within the culture system.

A previous report has shown that a low DO can result in reduced galactosylation of IgG glycans with GI values similar to the ones reported here (Kunkel et al., 1998). Thus, we can speculate that the low GI values in the Tecnomouse system maybe a result of the oxygen gradients that undoubtedly arise within the hollow fibres as the cells reach high densities. The GI value for the TB/C3.bcl-2 culture was significantly lower (mean = 0.35) than that from the TB/C3.pEF culture (mean = 0.41). Although this appears to contradict the results from the other culture systems, this difference may be a reflection of the higher cell densities that would have been achieved in the TB/C3.bcl-2 culture compared to the control culture to maintain the high IgG production. The higher cell densities could produce the low DO levels known to adversely affect galactosylation.

Similar results were obtained by Cabrera et al. (2005) who studied the galactosylation of a hybridoma producing IgG in several different culture systems. They reported higher galactosylation of IgG produced from static culture flasks and spinner flasks with GI values consistently above 0.5 as reported for our cell lines in batch and continuous cultures. However, production of the same IgG in mouse ascites, a hollow fibre bioreactor or a membrane bioreactor produced significantly lower galactosylation. The GI level obtained from their hollow fibre system was 0.28 which was even lower than the value obtained in our Tecnomouse culture. The findings of Cabrera et al. (2005) support our hypothesis that the conditions prevailing in the high density cultures may cause an adverse environment for galactosylation of the immunoglobulins.

In conclusion, we found that the galactosylation of IgG produced in suspension batch cultures was equivalent to that found in polyclonal antibodies from animal sera or from bioreactors maintained with a DO set-point at or above 50% air saturation. Lower cellular growth rates in continuous culture reduced the level of galactosylation ($GI < 0.5$), although the IgG produced from *bcl-2* transfected cells had consistently higher GI values. This was partly related to the enhanced cell viability in these cultures. The high cell densities obtained in a hollow fibre culture system

resulted in high IgG production over an extended time period. However, this was associated with a significantly lower galactosylation of IgG compared to that obtained in stirred suspension cultures.

F.A.A.M is supported by a scholarship from the Universiti Technologi Malaysia. M. Al-R is supported by a principal investigator award from the Science Foundation Ireland.

References

Al-Rubeai M. 1998. Apoptosis and cell culture technology. Adv Biochem Eng/Biotech 59:225–249.

Al-Rubeai M, Emery AN. 1990. Mechanisms and kinetics of monoclonal antibody synthesis and secretion in synchronous and asynchronous hybridoma cell cultures. J Biotechnol 16:67–85.

Al-Rubeai M, Singh S. 1998. Apoptosis in cell culture. Curr Opin Biotechnol 9:152–156.

Al-Rubeai M, Mills D, Emery AN. 1990. Electron microscopy of hybridoma cells with special regard to monoclonal antibody production. Cytotechnology 4:13–28.

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

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

Bierau H, Perani A, Al-Rubeai M, Amery AN. 1998. A comparative of intensive cell culture bioreactors operating with hybridoma modified for inhibited apoptotic response. J Biotechnol 62:195–207.

Butler M. 2005. Animal cell cultures: Recent achievements and perspectives in the production of biopharmaceuticals. Appl Microbiol Biotechnol 68:283–291.

Cabrera G, Cremata JA, Valdes R, Garcia R, Gonzalez Y, Montesino R, Gomez H, Gonzalez M. 2005. Influence of culture conditions on the N-glycosylation of a monoclonal antibody specific for recombinant hepatitis B surface antigen. Biotechnol Appl Biochem 41:67–76.

Deisenhofer J. 1981. Crystallographic refinement and atomic models of human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9 and 2.8A resolution. Biochem 20:2361–2370.

Fassnacht D, Rossing S, Franek F, Al Rubeai M, Portner R. 1998. Effect of bcl-2 expression on hybridoma cell growth in serum supplemented, protein free and diluted media. Cytotechnol 26:219–225.

Fassnacht D, Rossing S, Singh RP, Al-Rubeai M, Portner R. 1999. Influence of bcl-2 on antibody productivity in high cell density perfusion cultures of hybridoma. Cytotechnol 30:95–105.

Goswami J, Sinskey AJ, Stephanopoulos GN, Wang DIC. 1999. Apoptosis in batch cultures of Chinese hamster ovary cells. Biotechnol Bioeng 62:632–640.

Hardy MR, Townsend RR. 1994. High-pH anion-exchange chromatography of glycoprotein-derived carbohydrates. Methods Enzymol 230:208–225.

Hayter PM, Curling EK, 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-gamma. Biotechnol Bioeng 39:327–335.

Hooker AD, Goldman MR, Markham NH, James DC, Ison AP, Bull AT, Salmon PG, Baines L, Jenkins N. 1995. N-Glycans of recombinant human Interferon-y changed during batch culture of Chinese hamster ovary cells. Biotechnol Bioeng 48:639–648.

Itoh Y, Euda H, Suzuki E. 1995. Over-expression of Bcl-2, apoptosis suppressing gene: Prolonged viable culture by hybridoma and enhanced antibody production. Biotechnol Bioeng 48:118–122.

Jefferis R. 2005. Glycosylation of recombinant antibody therapeutics. Biotechnol Prog 21:11–16.

Jefferis R, Lund J, Mizutani H, Nakagawa H, Kawazoe Y, Arata Y, Takahashi N. 1990. A comparative study of the N-linked oligosaccharide structures of human IgG subclass proteins. Biochem J 268:529–537.

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

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

Kuroda Y, Nakata M, Nose M, Kojima N, Mizuochi T. 2001. Abnormal IgG galactosylation and arthritis in MRL-Fas(lpr) or MRL-FasL(gld) mice are under the control of the MRL genetic background. FEBS Lett 507:210–214.

Kuroki A, Kuroda Y, Kikuchi S, Lajaunias F, Fulpius T, Pastore Y, Fossati-Jimack L, Reininger L, Toda T, Nakata M, Kojima N, Mizuochi T, Izui S. 2002. Level of galactosylation determines cryoglobulin activity of murine IgG3 monoclonal rheumatoid factor. Blood 99:2922–2928.

Leatherbarrow RJ, Dwek RA. 1983. The effect of aglycosylation on the binding of mouse IgG to staphylococcal protein A. FEBS Lett 164:227–230.

Lund J, Takahashi N, Nakagawa H, Goodall M, Bentley T, Hindley SA, Tyler R, Jefferis R. 1993. Control of IgG/Fc glycosylation: A comparison of oligosaccharides from chimeric human/mouse and mouse subclass immunoglobulin Gs. Mol Immunol 30:741–748.

Marquina R, Díez MA, López-Hoyos M, Buelta L, Kuroki A, Kikuchi S, Villegas J, Pihlgren M, Siegrist C, Arias M, Izui S, Merino J, Merino R. 2004. Inhibition of B cell death causes the development of an IgA nephropathy in (New Zealand White x C57BL/6)F_1-bcl-2 transgenic mice. J Immunol 172:7177–7185.

Masuda K, Yamaguchi Y, Kato K, Takahashi N, Shimada I, Arata Y. 2000. Pairing of oligosaccharides in the Fc region of immunoglobulin G. FEBS Lett 473(3):349–357.

Mercille S, Massie B. 1994a. Induction of apoptosis in nutrient limited cultures in hybridoma cells. Biotech Bioeng 44:1140–1154.

Mercille S, Massie B. 1994b. Induction of apoptosis in oxygen deprived cultures of hybridoma, cells. Cytotechnol 15:117–128.

Mizuochi T, Hamako J, Titani K. 1987. Structures of the sugar chains of mouse immunoglobulin G. Arch Biochem Biophys 257:387–394.

Nahgrang S, Kragten E, De-Jeans M, Bourgeois M, Dejardins D, Von-Stockar U, Marison IW. 1999. The effect of cell line, transfection procedure and reactor condition on the glycosylation of recombinant human anti-rhesus D IgG1. In: Bernard A, et al., editors. Animal Cell Technology: Product from cells, cell as products. Dordrecht: Kluwer Academic Publishers. p 259–261.

Nose M, Wigzell H. 1983. Biological significance of carbohydrate chains on monoclonal antibodies. Proc Natl Acad Sci USA 80:6632–6636.

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.

Olsen CW, Kehren JC, Dybdahl-Sissoko NR, Hinshaw VS. 1996. bcl-2 alters influenza virus yield, spread, and hemagglutinin glycosylation. J Virol 70(1):663–666.

Parekh RB, Dwek RA, Sutton BJ, Fernandes DL, Leung A, Stanworth D, Rademacher TW, Mizuochi T, Taniguchi T, Matsuta K, Takeuchi F, Nagano Y, Miyamoto T, Kobata A. 1985. Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. Nature 316(6027):452–457.

Patel TP, Parekh RB, Moellering BJ, Prior CP. 1992. Different culture methods lead to differences in glycosylation of a murine IgG monoclonal antibody. Biochem J 285:839–845.

Patterson MKJr.1979. Measurement of growth and viability of cells in culture. Methods Enzymol 58:141–152.

Perani A, Singh RP, Chauhan R, Al-Rubeai M. 1998. Variable functions of bcl-2 in mediating bioreactor stress induced apoptosis in hybridoma cells. Cytotechnol 28:117–188.

Raju TS, Briggs JB, Borge SM, Jones AJ. 2000. Species-specific variation in glycosylation of IgG: Evidence for the species-specific sialylation and

DOI 10.1002/bit

branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics. Glycobiology 10:477–486.
Renard JM, Spagnoli R, Mazier MF, Salles S, Mandine E. 1988. Evidence that monoclonal antibody production kinetics is related to the integral of the viable cells curve in batch system. Biotechnol Lett 10:91–96.
Restelli V, Butler M. 2002. The effect of cell culture parameters on protein glycosylation. In: Al-Rubeai M, editor. Cell Engineering Vol. 3: Glycosylation. Dordrecht: Kluwer publ. p 61–92.
Robinson DK, Seamans TC, Gould SL, DiStefano DJ, Chan CP, Lee DK, Bibila T, Glazomitsky K, Munshi S, Daugherty B. 1994. Optimization of a fed batch process for production of a recombinant antibody. Ann NY Acad Sci 745:285–296.
Routier FK, Hounsell EF, Rudd PM, Takahashi N, Bond A, Hay FC, Alavi A, Axford JS, Jefferis R. 1998. Quantification of the oligosaccharides of human serum IgG from patients with rheumatoid arthritis: A critical evaluation of different methods. J Immunol Methods 213:113–130.
Serrato JA, Palomares LA, Meneses-Acosta A, Ramirez OT. 2004. Heterogeneous conditions in dissolved oxygen affect N-glycosylation but not productivity of a monoclonal antibody in hybridoma cultures. Biotechnol Bioeng 88(2):176–188.
Sheeley DK, Merrill BM, Taylor LCE. 1997. Characterization of monoclonal antibody glycosylation: Comparison of expression systems and identification of terminal N-Linked Galactose. Ann Biochem 247:102–110.
Shikata K, Yasuda T, Takeuchi F, Konishi T, Nakata M, Mizuochi T. 1998. Structural changes in the oligosaccharide moiety of human IgG with aging. Glycoconj J 15:683–689.
Simpson NH, Milner AE, Al-Rubeai M. 1997. Prevention of hybridoma cell death by bcl-2 during sub-optimal culture conditions. Biotechnol Bioeng 54:1–16.
Simpson NK, Singh RP, Perani A, Goldenzon C, Al-Rubeai M. 1998. In hybridoma cultures, deprivation of any single amino acid leads to apoptotic death, which is suppressed by the expression of the bcl-2 gene. Biotechnol Bioeng 59:190–198.
Simpson NH, Singh RP, Emery AN, Al-Rubeai M. 1999. Bcl-2 overexpression reduces growth rate and prolongs GI phase in continuous chemostat cultures of hybridoma cells. Biotechnol Bioeng 64:2174–2186.
Singh RP, Al-Rubeai M, Gregory CD, Emery AN. 1994. Cell death in bioreactors. Role of apoptosis. Biotechnol Bioeng 44:720–726.
Singh RP, Emery AN, Al-Rubeai M. 1996. Enhancement of survivability of mammalian cells by over-expression of the apoptosis suppressor gene bcl-2. Biotech Bioeng 52:166–175.
Tey BT, Singh RP, Piredda L, Piacentini M, Al-Rubeai M. 2000a. Influence of bcl-2 over-expression on growth and productivity of a CHO cell line over-expressing a chimeric antibody. Biotechnol Bioeng 68:31–43.
Tey BT, Singh RP, Piredda L, Piacentini M, Al-Rubeai M. 2000b. Bcl-2 mediated suppression of apoptosis in myeloma, NSO cultures. J Biotechnol 79:147–159.
Walker MR, Lund J, Thompson KM, Jefferis R. 1989. Aglycosylation of human IgG1 and IgG3 monoclonal antibodies can eliminate recognition by human cells expressing FcγRI and/or FcγRII receptors. Biochem J 259:347–353.
Warner TG. 1999. Enhancing therapeutic glycoprotein production in Chinese hamster ovary cells by metabolic engineering endogenous gene control with anti-sense DNA and gene targeting. Glycobiology 9:841–850.
Warnock JN, Al-Rubeai M. 2006. Bioreactor systems for the production of biopharmaceuticals from animal cells. Biotechnol Appl Biochem 45:1–12.
Wurm FM. 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol 22:1393–1398.
Yang M, Butler M. 2000. The effect of ammonia on the glycosylation of human recombinant erythropoietin in culture. Biotech Prog 16:751–759.