The effects of G418 on the growth and metabolism of recombinant mammalian cell lines

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Abstract

It is widely reported that the growth of recombinant bacteria and yeast is adversely affected by increased metabolic load caused by the maintenance of plasmid copy number and recombinant protein expression. Reports suggest that recombinant mammalian systems are similarly affected by increased metabolic load. However, in comparison to bacterial systems relatively little information exists. It was the aim of this study to test the effects of recombinant gene expression on the growth and metabolism of two industrially important cell lines. A BHK and CHO cell line were stably transfected with the human gastric inhibitory peptide (h-GIP) and glucagon receptor respectively. Selection was by way of the neomycin resistance (*neo^r*) gene using G418. The growth and metabolism of both cell lines was affected by the presence of G418 in a manner indicative of increased metabolic load and which appeared to be caused by over-expression of the neomycin resistance protein. The two cell lines differed in their metabolic response to G418, which suggested that some cell lines or clones may be better able to tolerate a metabolic load than others. Growth under increased metabolic load was affected by medium composition with serum, insulin and glutamine concentration as influencing factors. Implications for the use of G418 are discussed.

Introduction

It is well documented that recombinant bacteria show lower growth rates than wild type strains due to an increased metabolic load or burden placed on the cells by maintenance of plasmid DNA and over-expression of recombinant protein (Seo and Bailey, 1985; Lee and Edlin, 1985; Betenbaugh et al., 1989; Bentley et al., 1990; Katayama and Nagata, 1990; Ryan and Parulekar, 1991; Kyslik et al., 1993; Glick, 1995; Cserjan-Puschmann et al., 1999). The same phenomenon has also been reported in *Saccharomyces* (Kennedy and Patching, 1997) and mammalian cells (Pendse et al., 1992; Gu et al., 1992, 1996; Kidane et al., 1997; Kim et al., 1998). However, in contrast to the bacteria, relatively little information exists for mammalian cells and data is often conflicting. Pendse et al. (1992) showed that a CHO cell line transfected with the amplifiable dihydrofolate reductase (*dhfr*)

gene, encoding methotrexate (MTX) resistance, linked to the co-expression of hepatitis B surface antigen (HbsAg), exhibited a marked decrease in growth rate as the expression of the two proteins was amplified. The maximum observed effect was a 63.5% reduction in growth rate at the highest amplification. Similarly, Kim et al. (1998) reported that the growth rate of a recombinant CHO cell line expressing chimeric antibody was inversely proportional to MTX concentration and expression level with a 40–45% decrease in the growth rate at the highest expression level. Similar work using the *dhfr* system, carried out by Gu et al. (1992, 1996) showed a 62% reduction in the growth rate of a recombinant CHO cell line when the *lacZ* gene was co-amplified with *dhfr* copy number. However, no effect on cell growth was observed when a non-amplified system using a neomycin (*neo^r*) resistance gene, encoding G418 resistance, was used with the same *lacZ* gene. It was therefore concluded that

Figure 1. Growth of (a) CHO 679 and (b) BHK 570 in T-flask on (i) PF-1, (ii) SF-1 and (iii) DMEM/F-12 + 5% FCS. Data shows growth in G418 free media (open circles) and media containing 1000 $\mu\text{g ml}^{-1}$ G418 (closed circles).

growth reduction, and hence increased metabolic load, occurred only as a result of co-amplification and over-expression. Furthermore, as higher *lacZ* expression occurred in the non-amplified G418 system, it was concluded that it was expression of the *dhfr* resistance protein, not *lacZ*, which contributed most to this metabolic load. Kidane et al. (1997) however, demonstrated that the growth of CHO cells was affected by systems using G418 as the selection agent and that the degree of this effect was dependent upon the level of expression of the recombinant protein. Two vectors were tested and the expression and growth rates compared to the parent, non-transfected cell line. The non-transfected cell line showed a doubling time of 26 h. Cells transfected with the vector resulting in the lowest expression (encoding the cytochrome b5 and neomycin resistance genes) exhibited a 36% increase in the doubling time. Cells transfected with a vector resulting in higher expression (encoding the same cytochrome b5 and neomycin genes but with a kozak consensus sequence) gave a 100% increase in expression and a further 12.5% increase in doubling time over the cells transfected with the lower expressing vector. Furthermore, the utilisation of glucose and glutamine was approximately 2 and 1.5 times higher, respectively, in the two transfected cell lines compared to the non-transfected cells. No difference in glucose or glutamine utilisation was observed between the two transfected cell lines. Metabolic rates as an indicator of metabolic load have also been used by Schroder and Friedl (1997) who reported a 3–4 times increase in the rate of glutamine utilisation for CHO clones expressing antithrombin III under control of the *dhfr* system, although no difference was observed in the growth rate. The increase in metabolic rates of the recombinant cells in these studies suggest either an increased metabolic flux into the biosynthesis of the recombinant proteins and/or an increased flux into energy production to counter the extra demands placed on the cell by the expression of the recombinant proteins.

It is clear that recombinant protein expression and gene copy number is an important factor in determining metabolic load in mammalian cells and that this has a considerable effect on growth and metabolism. However, there is clearly a demand for more information concerning the effects of increased metabolic load on mammalian systems in general and on the effects of G418 controlled expression in particular. It was therefore the aim of this study to determine if an increased metabolic load occurred in two commonly used, industrially important cell lines (CHO and BHK) stably

transfected with a gene of interest and maintained using G418 as the selective agent, to determine what affect this metabolic load had on the growth, metabolism and recombinant protein production of the cells and what methods could be used to reduce this load and give increased growth and production. It is known that under a metabolic load it is the cell's central metabolic and energy-intensive processes that are amongst the first to be affected. As well as the reports of Kidane et al. (1997) and Schroder and Friedl (1997), amongst bacteria the transformation of *Azotobacter vinelandii* and *Pseudomonas putida* with a plasmid encoding ampicillin and tetracycline resistance, reduces their ability to fix nitrogen and synthesise siderophores (Glick et al., 1989; Hong et al., 1991) and the ability of many genetically modified bacteria to compete and survive in the environment is reduced as a result of the metabolic load caused by their novel gene expression (De Leij et al., 1998). Metabolic data may give a more accurate determination of metabolic load than growth rates. Some cell lines may tolerate a metabolic load better than others, by being better able to divert metabolism to energy production or biosynthesis so that the growth rate was not seriously affected. Metabolic data has the added advantage that it may identify changes in metabolism that have implications in the production process, for example if there is an increase in the glucose utilisation rate. An accurate determination of increased metabolic load and the cells metabolic response may also allow the selection of cell lines or clones which can deal most efficiently with an increased metabolic load. The effect of metabolic load in bacteria appears to be independent of medium composition (Bentley et al., 1990). Strategies to overcome the problem are therefore based around altering plasmid copy number, the resistance marker or promoter strength (Glick, 1995; Cserjan-Puschmann et al., 1999). However, different approaches may be possible with mammalian systems. The effect of medium composition on mammalian cells is unknown and thus it may be possible to affect a cells response to metabolic load by medium development. Alternatively, if production of the resistance protein contributes significantly to the metabolic load, it may be possible to remove part or maybe all of the metabolic load by reducing or even eliminating (if expression is stable) the selective agent.

Figure 2. q_{glu} , q_{lac} , q_{amm} ($\mu\text{mol } 10^6 \text{ cells}^{-1} 24 \text{ h}^{-1}$), cell number ($10^6 \text{ cells ml}^{-1}$) and $Y_{lac,glu}$ of (a) CHO 679 and (b) BHK 570 grown in T-flask on (i) PF-1, (ii) SF-1 and (iii) DMEM/F-12 + 5% FCS. Right-hand column in each set represent media containing 1000 $\mu\text{g ml}^{-1}$ G418, left-hand column represents media without G418.

Figure 3. Glutamine synthetase activity in cultures of CHO 679 (circles) and BHK 570 (squares) on PF-1 with (closed symbols) and without (open symbols) $1000 \mu\text{g ml}^{-1}$ G418.

Materials and Methods

Cell lines, culture media and methods

The recombinant cell lines CHO 679, expressing the human glucagon receptor, and BHK 570, expressing the human gastric inhibitory peptide (h-GIP) receptor, were cloned after transfection of the wild type CHO-K1 and BHK-570 with the pcDNA3.1 vector (Invitrogen) containing the recombinant gene under control of the Cytomegalovirus (CMV) immediate early promoter and a SV40 polyadenylation site. Selection was by way of the neomycin (*neo^r*) gene encoding resistance to G418 (Life Technologies).

Both cell lines were maintained in three media, a serum containing medium which consisted of DMEM/F-12, 1:1 (Biowhittaker) plus 5% heat-inactivated North American foetal calf serum (Life Technologies), a proprietary serum-free medium, SF-1, which consisted of 1:1 mix of RPMI 1640 without glutamine (Biowhittaker) and DMEM with glutamine and 4.5 g l^{-1} glucose (Biowhittaker) plus concentrates of amino acids, fatty acids, vitamins, trace elements and 2.0 mg ml^{-1} recombinant human insulin (Novo Nordisk), and a proprietary protein-free medium, PF-1, which consisted of a 1:1 mix of RPMI 1640 without glutamine and DMEM/F-12 (Biowhittaker) in a 1:1 mix plus concentrates of amino acids, trace elements and vitamins. 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin (Biowhittaker) and 0.3% (w/v) pluronic F-68 (Sigma) were added to all media. The two recombinant cell lines were maintained continuously in

each medium, with and without $1000 \mu\text{g ml}^{-1}$ G418 (Life Technologies). Where other concentrations of G418 are used, cells were adapted to these concentrations before any experiments were carried out. Cells were considered adapted when the same doubling time was observed for 4 consecutive passages. Cells were passaged so that they were maintained in logarithmic growth. In serum containing media, cells were passaged by trypsinisation followed by re-suspension in fresh medium. In serum-free and protein-free media, cells grew in suspension in T-flask and so were decanted with the medium, centrifuged at 1000 rpm for 5 min and re-suspended in fresh medium. T-flask experiments were carried out in T-25's (Corning Costar) containing 5 ml medium. Spinner flask experiments were carried using 125 ml spinners (Techne) containing 60 ml medium. All data shown represents an average of at least four experiments. Standard deviation was plotted as error bars. If not shown, variation was less than 10%. Cells were adapted to suspension culture in spinner flask prior to any experiments. Cells were considered adapted when the doubling time in spinner was at least comparable to that obtained in T-flask and was reproducible for at least three generations. Cells were maintained in T-75's containing 16 ml medium.

Cells in T-flasks and spinners were incubated in a humidified incubator at 37°C and 5% CO_2 . Cell numbers were counted using a CASY automatic cell counter (Scharfe Systems). To ensure a single cell suspension prior to counting, 1 ml of cell suspension was mixed with 1 ml of Accumax (Innovative Cell Techno-

Figure 4. Effect of 0–1000 $\mu\text{g ml}^{-1}$ G418 on q_{glu} (closed triangles), q_{lac} (open squares), q_{amm} (closed squares) and cell number (open circles) of CHO 679 grown in T-flask.

logies) and incubated at 37 °C for 30 min. Cells were then re-suspended a number of times using a P1000 Gilson pipette. Viability was measured using Trypan Blue exclusion.

Preparation of cell extracts for glutamine synthetase assay

Cells were harvested, washed once in phosphate buffered saline (PBS) at 4 °C and re-suspended in 1 ml of extraction buffer at a concentration of 1.5×10^7 cells ml^{-1} . The extraction buffer (Sanfeliu and Stephanopoulos, 1999) consisted of 20 mM imidazole.HCl (pH 7.0), 150 mM NaCl, 0.02% (w/v) sodium azide, 1.0% Triton X-100, 0.2 mM EDTA, 100 $\mu\text{g ml}^{-1}$ PMSF and 1 $\mu\text{g ml}^{-1}$ aprotinin. Total protein was estimated by the Lowry method using a kit from Bio-Rad.

Analytical methods and assays

Glucose, lactate, NH_3 and lactate dehydrogenase (LDH) were measured using an Ektachem II analyser (Kodak). Glutamine was measured using a two-step assay method (Lund, 1974). The assay system consisted of 0.5 ml of 0.25 M sodium acetate, 0.1 ml of a 0.05 mg ml^{-1} solution of glutaminase (Sigma) and 0.1–0.4 ml of culture supernatant (depending on glutamine concentration). The reaction solution was made up to 1.0 ml with distilled water. A glutamine standard was run with 0.05 ml of 0.5 mM glutamine and 0.35 ml distilled water and a blank control with 0.4 ml distilled water. The reaction was run for 20 min

at 37 °C to ensure complete conversion of glutamine to glutamate. The glutaminase treated solution was then analysed for ammonia in the Kodak Ektachem II. Glutamine synthetase activity was measured using the glutamyltransferase assay (Sanfeliu and Stephanopoulos, 1999), based on the formation of glutamyl-hydroxamate from glutamine and hydroxylamine in the presence of catalytic amounts of ADP. The assay system consisted of 0.1 M imidazole.HCl (pH 7.2), 50 mM L-glutamine, 0.4 mM ADP, 0.2 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 62.5 mM hydroxylamine and 10 mM sodium arsenate. Reactions were initiated by the addition of 0.1 ml cell extract to 0.4 ml of the assay system and terminated by the addition of 0.5 ml ferric chloride solution (0.37 M FeCl_3 and 0.2 M tricarboxylic acid (TCA). Precipitated protein was removed by centrifugation at 9000 rpm for 12 min and the absorbance read at 535 nm against a reagent blank. Negative controls, which lacked ADP, arsenate and glutamine were run to ensure that the observed reaction was a true indication of glutamine synthetase activity. Receptor expression was determined by FACS analysis using a fluorescently-labelled, receptor-specific antibody. Results for each cell line were double-checked using an ELISA and specific, receptor-binding assay.

Results

Effects of G418 on cell growth and metabolism in T-flask

The recombinant CHO 679 and BHK 570 cell lines

Figure 5. Growth of (a) CHO 679 and (b) BHK 570 in T-flask on SF-1 with (closed symbols) and without (open symbols) $1000 \mu\text{g ml}^{-1}$ G418, and containing 2 mg l^{-1} (circles) and 10 mg l^{-1} (squares) human recombinant insulin.

were grown in T-flask on each of the three media, PF-1, SF-1 and DMEM/F-12 + 5% FCS, with and without $1000 \mu\text{g ml}^{-1}$ G418. Samples were taken every 24 h and cell number, glutamine, glucose, lactate, NH_3 and LDH measured. Figure 1 shows that the growth of both cell lines in SF-1 and PF-1 was inhibited in the presence of G418. The maximum cell density was reduced by 29 and 37% for CHO 679 and by 25 and 29% for BHK 570 in SF-1 and PF-1 containing G418. The effect of G418 was less marked in the serum-containing

medium, with only a 13% reduction in maximum cell density for CHO 679 and no discernible difference for BHK 570. The lower cell density in media containing G418 was not a result of increased cell death or lysis as viability, measured by trypan blue exclusion and cell lysis, measured by LDH release were not affected (data not shown).

Figure 2 shows the effects of G418 on the metabolism of the two cell lines. For CHO 679 grown in PF-1 and SF-1, glucose utilisation (q_{glu}) remained un-

affected while lactate production (q_{lac}) was reduced by 30% and 25% respectively. The yield of lactate from glucose ($Y_{\text{lac,glu}}$) was therefore reduced from 1.44 and 1.30 to 1.04 and 0.98. $Y_{\text{lac,glu}}$ is a measure of the flow of glucose through glycolysis either to lactate or through the TCA cycle or through other biosynthetic pathways such as pentose phosphate. A decrease in $Y_{\text{lac,glu}}$ suggested that a larger proportion of the transported glucose was being channelled away from lactate formation and into either biosynthetic pathways (for example for recombinant protein production) and/or into the TCA cycle for energy production. Either way, it provided evidence that the cells were under some kind of increased metabolic load caused by the presence of G418. In the serum-containing medium there was a very different response to G418, q_{glu} and q_{lac} increased by 30 and 46%, respectively, so that $Y_{\text{lac,glu}}$ increased slightly by 12% from 1.90 to 2.13.

For BHK 570, q_{glu} increased slightly by 15% in PF-1 and 11% in SF-1 while q_{lac} increased by 12 and 4%. This resulted in a largely unchanged $Y_{\text{lac,glu}}$ that suggested that BHK 570 was unable to significantly channel glucose away from lactate formation and into either the TCA cycle or biosynthetic pathways. A further significant alteration in cell metabolism was observed for both cell lines in the utilisation of glutamine and production of ammonia, which increased considerably in media containing G418. The specific rate of ammonia production (q_{amm}) for CHO 679 increased by 54, 39 and 38% in PF-1, SF-1 and DMEM/F-12 + 5% FCS, respectively, while q_{amm} for BHK 570 increased by 56.7, 68.3 and 39.7%, respectively, in the same three media. Almost identical increases in the specific rate of glutamine utilisation (q_{gln}) were observed (data not shown) so that the yield of ammonia from glutamine ($Y_{\text{amm,gln}}$) remained at 1.0 ± 0.1 in each medium, with and without G418. The presence of G418 did not affect the chemical breakdown of glutamine (data not shown). The increased q_{gln} and q_{amm} suggested an increased metabolic demand for glutamine either as a biosynthetic precursor and/or for energy production. Glutamine measurements (not shown) from the growth curves of CHO 679 in Figure 1 indicated that cessation of logarithmic growth coincided with glutamine exhaustion. Glutamine synthetase activity, an indicator of glutamine limitation (Christie and Butler, 1998; Sanfeliu and Stephanopoulos, 1999) of CHO 679 increased more rapidly in the presence of G418, reaching a maximum of $0.32 \text{ mmol h}^{-1} \text{ mg protein}^{-1}$ after 48 h compared to

72 h in medium without G418 (Figure 3). Glutamine synthetase activity in BHK 570 was largely unaffected by the presence of G418, probably because growth was additionally affected by high lactate concentration and resultant low culture pH. Culture pH dropped from 7.3 at inoculation to 6.4–6.7 after 48 h and 5.5–5.9 after 72 h (data not shown) and lactate concentration was 23–26 mM after 72 h. High lactate concentration and a culture pH below 6.8 can inhibit cell growth in BHK cells (Hassel et al., 1991).

It appeared that glutamine limitation, brought about by increased glutamine utilisation in the presence of G418, played an important role in determining cell density. This is probably not the only factor however as the doubling times of both cell lines were higher in media containing G418 when glutamine was not limiting, (ie: in the first 24 h). The doubling times of CHO 679 in PF-1 and SF-1 were 15–18% higher when G418 was present (21.3 and 19.9 h for PF-1 and SF-1 without G418 compared to 24.5 and 23.5 h with G418), while the doubling times for BHK 570 in the same media were 37% higher (22.7 and 22.1 for PF-1 and SF-1 without G418 compared to 31.0 and 30.3 h with G418).

Stability of gene expression with and without G418

To test the stability of receptor expression without selection, both cell lines were grown on media with and without G418 and routinely screened for expression by FACS analysis and receptor-binding assays. Expression in both cell lines was stable without selection over a six-month period (data not shown). This suggested that the observed effects on growth and metabolism caused by G418 in both cell lines was not due to the expression of the receptor proteins, as these were expressed equally in cells grown both with and without G418, but due therefore to the over-expression of the resistance protein. The stability of protein expression in these cell lines has important implications in the production process. The specific expression level was unchanged in cells grown with and without G418, although the volumetric production was higher without G418 due to the increased cell density. Significant improvements in production yields were therefore possible by removing the selection pressure.

Effect of G418 concentration on growth and metabolism of CHO 679

G418 at $1000 \mu\text{g ml}^{-1}$ was shown to have considerable influence on the growth and metabolism of both cell lines in the serum-free and protein-free media tested. To determine if this effect was dependent on G418 concentration, growth and metabolism of CHO 679 was tested in PF-1 with concentrations of G418 ranging from 0 to $1000 \mu\text{g ml}^{-1}$. Figure 4 shows that q_{glu} was unaffected by G418 concentration while q_{lac} and cell density decreased and q_{amm} increased with increasing G418 concentration. Increases in G418 concentration also resulted in increases in the *neo^r* mRNA number (data not shown), which suggested that the observed effects on growth and metabolism correlated with the copy number and therefore expression of the resistance protein *neo^r*.

Effect of glutamine on cell growth and metabolism

From the T-flask data, it appeared that glutamine limitation was an influential factor in determining maximum cell density. The most marked effect of G418 on cell growth was seen in PF-1, which was therefore selected to test the effects of glutamine concentration on growth. Growth was tested at 2, 4, 6 and 8 mM glutamine, in media with and without G418. Table 1a shows that in PF-1 medium without G418, an increase in glutamine concentration from 2 to 4 mM resulted in an increase in final cell number for CHO 679 of 37% from $1.64 \times 10^6 \text{ ml}^{-1}$ to $2.23 \times 10^6 \text{ ml}^{-1}$. Further increases in glutamine concentration resulted in no further increase in cell number. In PF-1 containing G418, the final cell number increased with glutamine concentration up to 6 mM, with a maximum cell number of $1.02 \times 10^6 \text{ ml}^{-1}$ at 2 mM glutamine rising by 63% to $1.66 \times 10^6 \text{ ml}^{-1}$ at 4 mM glutamine and by 88–98% to $1.92\text{--}2.02 \times 10^6 \text{ ml}^{-1}$ at 6 and 8 mM glutamine. Approximately equivalent final cell densities could therefore be achieved in media with and without G418 by using 4 and 6 mM glutamine respectively. The increase in cell density due to increased glutamine concentration was due to an extension of logarithmic growth not a decrease in the doubling times (data not shown). Table 1a also shows the effect of different concentrations of glutamine on the metabolism of CHO 679. Without G418, q_{glu} and q_{lac} dropped by 31% from 5.08 and $7.29 \mu\text{mol } 10^6 \text{ cells}^{-1} 24 \text{ h}^{-1}$ to 3.50 and $5.00 \mu\text{mol } 10^6 \text{ cells}^{-1} 24 \text{ h}^{-1}$, respectively, as glutamine concentration increased from 2–4 mM. The rates were then stable up

to 8 mM glutamine. The equal drop in q_{glu} and q_{lac} ensured that $Y_{\text{lac,glu}}$ remained largely unchanged over all glutamine concentrations. At the same time, q_{amm} increased by 23% from 1.13 to $1.39 \mu\text{mol } 10^6 \text{ cells}^{-1} 24 \text{ h}^{-1}$ between 2 and 4 mM glutamine and was then stable up to 8 mM glutamine. The decrease in q_{glu} and increase in q_{amm} between 2 and 4 mM glutamine suggested a switch to glutamine utilisation and that glutamine may be the preferred substrate. In the presence of G418, q_{glu} , q_{lac} and q_{amm} remained largely unaffected by glutamine concentration so that $Y_{\text{lac,glu}}$ was also unaffected and remained lower than in media without G418, over all glutamine concentrations. That q_{glu} did not decrease in media with G418 suggested that cells required a higher rate of metabolism in order to maintain the metabolic load. Table 1b shows that no significant improvement occurred in the final cell number for BHK 570 at any of the glutamine concentrations tested, with or without G418. However, as stated, the growth of this cell line may have been adversely affected by culture conditions. Culture pH (6.4–6.7 at 48 h and 5.5–5.9 at 72 h), lactate (23–26 mM) and ammonia (2.0–2.5 mM) were all at levels previously reported as inhibitory to BHK cells (Butler and Spier, 1984; Hassel et al., 1991). Table 1b also shows the effects of glutamine concentration on the metabolism of BHK 570. Without G418, q_{glu} , q_{lac} and q_{amm} remained largely unchanged at all glutamine concentrations with the result that the $Y_{\text{lac,glu}}$ was also largely unaffected. q_{glu} and q_{lac} were considerably higher in BHK 570 than CHO 679, which suggested that glycolysis is a more important pathway in this cell line and that the metabolism of glucose has more influence on the growth of the cell line than in CHO 679.

Effect of G418 on cell growth and metabolism in spinner flask

Both cell lines were grown in 125 ml spinners containing 60 ml SF-1 or PF-1. Each spinner was run until a steady state was reached, where cell number was stable, glucose and glutamine were not limiting and where lactate and ammonia were not at toxic levels. A cell bleed was operated to maintain cells in logarithmic growth and keep viability above 90% and q_{glu} , q_{lac} , q_{gln} and q_{amm} were calculated at steady state. Tables 2a and b show the effects of G418 on the growth and metabolism of CHO 679 and BHK 570 respectively. The data confirmed the results from the T-flask experiments with both cell lines showing in-

Table 1. Cell number, q_{glu} , q_{lac} , q_{amm} , $Y_{\text{lac,glu}}$ and $Y_{\text{amm,gln}}$ for (a) CHO 679 and (b) BHK 570 grown in T-flask on PF-1 with and without $1000 \mu\text{g ml}^{-1}$ G418

Glutamine conc.	Cell number	q_{glu}	q_{lac}	q_{amm}	$Y_{\text{lac, glu}}/Y_{\text{amm, gln}}$
(mM)	(10^6 ml^{-1})	($\mu\text{mol } 10^6 \text{ cells}^{-1} \text{ 24 h}^{-1}$)			
(a) CHO 679					
PF-1 – G418					
2	1.64	5.08	7.29	1.13	1.44/1.07
4	2.23	3.50	5.00	1.39	1.46/1.05
6	2.28	3.51	5.18	1.42	1.48/0.99
8	2.28	3.31	5.03	1.39	1.52/1.08
PF-1 + 1000 $\mu\text{g ml}^{-1}$					
2	1.02	5.09	5.44	1.71	1.07/1.05
4	1.66	4.80	5.13	1.71	1.07/1.10
6	2.02	4.69	5.08	1.72	1.08/1.02
8	1.92	4.68	5.32	1.81	1.14/1.06
(b) BHK 570					
PF-1 – G418					
2	0.91	7.04	12.33	1.25	1.76/1.02
4	1.00	6.76	11.82	1.26	1.75/1.07
6	0.79	6.86	11.52	1.35	1.68/0.97
8	0.82	7.66	12.46	1.29	1.63/1.02
PF-1 + 1000 $\mu\text{g ml}^{-1}$					
2	0.69	7.90	13.51	1.67	1.71/1.08
4	0.67	7.86	13.76	1.69	1.75/1.07
6	0.79	8.54	14.93	1.75	1.76/1.11
8	0.80	8.42	14.82	1.77	1.72/1.01

creased q_{amm} and q_{gln} , and CHO 679 a flux of glucose away from lactate in the presence of G418. $Y_{\text{lac,glu}}$ decreased by 17 and 24% from 1.84 and 1.63 to 1.40 and 1.35 in PF-1 and SF-1 respectively. q_{amm} and q_{gln} increased equally (q_{amm} by 64% and 39% in PF-1 and SF-1) while q_{glu} remained largely unaffected. This data allowed the development of media optimised to the metabolism of the cell line. For example, it can be seen from Table 2a that it is possible to obtain approximately $3 \times 10^6 \text{ cells ml}^{-1}$ in SF-1 with and without G418 and that, with a ratio of ammonia produced to glutamine utilised of 1:1, this cell density will require approximately 5.1 mM glutamine every 24 h in the presence of G418 and 3.6 mM every 24 h in media without G418.

Table 2b shows the response of BHK 570 cells to G418 in spinner. Through medium change it was possible to ensure that a build-up of waste products and drop in culture pH, which affected cell growth in T-flask, did not affect growth in spinner. As a result, lactate and ammonia concentration did not rise above 12 and 1.20 mM, respectively, while culture pH did not fall below 6.8 (data not shown). Under these conditions, the maximum cell density achieved in the presence of G418 was approximately half that achieved in media without G418, a far greater difference than seen in T-flask where culture pH, lactate and ammonia affected cell growth. The difference in cell density observed in spinner could therefore be attributed to the effects of G418. There was no difference in the metabolic response of BHK 570 to G418 between

Table 2. Cell number, q_{glu} , q_{lac} , q_{amm} , $Y_{\text{lac,glu}}$ and $Y_{\text{amm,gln}}$ for (a) CHO 679 and (b) BHK 570 grown in spinner flask on SF-1 and PF-1 with and without $1000 \mu\text{g ml}^{-1}$ G418

Medium	Cell number	q_{glu}	q_{lac}	q_{amm}	$Y_{\text{lac, glu}}/Y_{\text{amm, gln}}$
(mM)	(10^6 ml^{-1})	$(\mu\text{mol } 10^6 \text{ cells}^{-1} \text{ } 24 \text{ h}^{-1})$			
(a) CHO 679					
SF-1 – G418	3.25	3.10	5.00	1.21	1.63/1.04
SF-1 + 1 mg ml ⁻¹ G418	3.00	2.90	3.96	1.69	1.35/1.05
PF-1 – G418	2.25	3.70	6.82	1.22	1.84/0.97
PF-1 + 1 mg ml ⁻¹ G418	2.18	3.63	5.08	2.00	1.40/1.04
(b) BHK 570					
SF-1 – G418	2.30	6.76	12.48	0.81	1.83/1.07
SF-1 + 1 mg ml ⁻¹ G418	1.25	6.63	12.81	1.26	1.86/1.02
PF-1 – G418	2.10	6.53	12.04	0.81	1.85/1.10
PF-1 + 1 mg ml ⁻¹ G418	1.15	6.68	13.78	1.22	1.90/1.03

spinner and T-flask experiments, q_{glu} , q_{lac} and $Y_{\text{lac,glu}}$ were largely unaffected by G418, suggesting there was no shift of glucose flux into the TCA cycle or biosynthetic pathways, while q_{gln} and q_{amm} increased equally (q_{amm} by 49 and 56% in PF-1 and SF-1). The failure of BHK 570 to achieve the same cell density in media with and without G418 may indicate an inability to overcome the increased metabolic load as effectively as CHO 679.

Effect of G418 on the metabolism of untransfected parent cell lines

In any population under antibiotic selection, there will be a normal distribution with respect to the copy number of the resistance protein, with most cells possessing the required copy number enabling survival at the particular antibiotic concentration used, some possessing a higher copy number and some possessing a lower copy number. Those cells possessing a lower copy number may be subject to the toxicity effects of the antibiotic. In order to determine if any of the effects we observed on metabolism could be due to G418 toxicity on this part of the population, we tested the effect of G418 on the metabolism of the two parent, untransfected and therefore non-resistant cell lines (CHO K1 and BHK 570). Cells from a mid logarithmic culture growing in SF-1 without G418 were inoculated into T-flasks containing $1000 \mu\text{g ml}^{-1}$ G418 and the concentrations of glucose, lactate, glutamine and ammonia measured. Glutamine utilisation and ammonia

production remained unchanged in cells grown with and without G418 (data not shown) while glucose utilisation and lactate production was reduced by approximately 50% when compared to cells grown without G418. Although the alteration in glucose metabolism is a major effect, the fact that it was not observed in the recombinant cells and that $Y_{\text{lac,glu}}$ and q_{gln} and q_{amm} remained unchanged suggested that the effects on metabolism observed in the recombinant strains in the presence of G418 were not due to G418 toxicity.

Effect of insulin on the growth of BHK 570 and CHO 670

T-flask and spinner data for both cell lines showed that a higher cell density and faster doubling time were obtained in SF-1 than PF-1. One of the differences between the two media is that SF-1 contained 2.0 mg l^{-1} human recombinant insulin. It is known that insulin can shorten the cell cycle by facilitating passage through the restriction point and into the next cell cycle. The restriction point is a control mechanism, which ensures that cell division takes place only if certain criteria are met, for example nutrient concentration. Insulin may therefore benefit cells exposed to G418 by facilitating cell division under the conditions caused by a metabolic load, which would otherwise be inhibitory to cell division. Figure 5 shows that the presence of 10 mg l^{-1} insulin resulted in a significant improvement in the final cell number of both cell lines, with an increase of 68.8 and 26.2% for CHO

679 and 116.0 and 58.3% for BHK 570, in media with and without G418 respectively. The increase in insulin concentration also led to a reduction in the doubling time for cells grown with and without G418. The doubling time of CHO 679 was reduced by 10% from 19.9 to 18.0 h in media without G418 and by 14% from 22.5 to 19.4 h in media with G418. The doubling time of BHK 570 was reduced by 9% from 22.1 to 20.3 h in media without G418 and by 24.4% from 30.3 to 22.9 h in media with G418. Insulin therefore had a greater effect on the cell density and doubling time of cells grown in the presence of G418 and on the cell density of BHK 570. It may therefore facilitate cell division under what would normally be unfavourable conditions, whether this is glutamine limitation (in the case of CHO 679) or low pH, high lactate and ammonia concentration (for BHK 570).

Rates of utilisation q_{glu} , q_{lac} and q_{amm} in each cell line were largely unaffected by insulin (data not shown) so that the same pattern of increased q_{glu} , increased q_{amm} , decreased q_{lac} and decreased $Y_{\text{lac, glu}}$ due to G418 were observed.

The mean cell diameter of both cell lines measured in SF-1 with 2 mg l⁻¹ insulin was 16.0–16.5 μ . This decreased in the presence of 10 mg l⁻¹ insulin by approximately 15% to 13.5–14.0 μ . At the same time, the specific receptor production for both cell lines decreased by 10–20% (data not shown). The reduction in specific receptor production may be linked to the reduction in cell size. The overall volumetric production however increased due to the larger increase in final cell density. To ensure that it was the insulin that was responsible for these effects and not the presence of other medium components in SF-1, growth of both cell lines was tested in PF-1 with 10 mg l⁻¹ insulin. As in SF-1, the final cell number and doubling times of both cell lines improved significantly on addition of insulin and no effect of G418 could be observed (data not shown).

Conclusion

The growth and metabolism of both cell lines was affected by G418. The maximum cell density was significantly reduced and doubling times (for BHK 570) increased in the presence of G418, while the specific utilisation of glutamine increased and, in the case of CHO 679, the flux of glucose was directed away from lactate production and towards either the TCA cycle to provide energy or biosynthetic pathways. These

findings are indicative of an increased metabolic load, although it should be stressed that conclusive proof of a link between the observed effects on growth and metabolism requires further study, in particular a detailed study of the fluxes through the central metabolic pathways and energy metabolism should be carried out so that the re-direction of nutrients into energy metabolism and/or biosynthesis can be verified and the metabolic pathway fluxes quantified. This could be combined with a study of the expression levels of selected metabolic genes to see if they were up regulated by G418. An example would be to look at the enzymes which link glycolysis to the TCA cycle (pyruvate dehydrogenase complex, pyruvate carboxylase and phosphoenolpyruvate carboxykinase) in CHO 679 to see if these are up regulated as this may explain the fate of glucose that is diverted away from lactate in the presence of G418.

It is clear that the effects of G418 on cell metabolism and growth have important implications to the production of recombinant proteins. In the case of CHO 679, the effects of G418 on maximum cell density could be overcome by increased glutamine concentration while the effects on growth rate and cell density could be overcome by increased insulin addition. This demonstrated that unlike in bacteria, the response of mammalian cells to a metabolic load could be influenced by medium formulation. It also demonstrated the importance of using metabolic analysis, not only growth rates or cell density, in the measurement of metabolic load. The failure of BHK 570 to direct glucose away from lactate production and the growth inhibition even at high glutamine concentrations indicates that the response to G418 may be cell line dependent, which may have important implications in cell line selection.

That cells grown without G418 expressed the recombinant receptor proteins to the same level but showed none of metabolic or growth effects shown by cells grown with G418 suggested that it was expression of the neomycin resistance protein, not the receptor proteins, that was responsible for the observed effects on growth and metabolism. Furthermore, the correlation between increasing G418 concentration, increasing metabolic activity/growth inhibition, and increasing *neo*^r mRNA levels indicated that these effects were a result of increased *neo*^r expression. The effects of G418 on the growth and metabolism of the parent cell lines showed that the effects observed in the recombinant clones was not due to G418 toxicity against those cells with a reduced *neo*^r copy number.

The situation thus appears similar to the *dhfr* system reported for CHO cells (Gu et al., 1992, 1996) where expression of the resistance protein has been found to be responsible for the increased metabolic load. However, unlike the *dhfr* system, the G418 system is not amplifiable and so increases in the *neo^r* copy number may not necessarily lead to increases in expression of the protein of interest. This has implications in the use of G418 in the production of recombinant proteins as the lowest concentration of G418 should be chosen which maintains the highest expression level and where expression is stable without selection, G418 should be removed.

The kinetics of inactivation of aminoglycoside antibiotics by *neo^r* have been reported by McKay and Wright (1995). The *neo^r* gene encodes the protein 3'-aminoglycoside phosphotransferase, which mediates resistance to aminoglycoside antibiotics by a covalent phosphorylation. The first reaction is the binding of ATP to the enzyme to form an enzyme-ATP complex. This then binds G418 and phosphorylates it to produce an enzyme-ADP complex and the phosphorylated G418. The enzyme-ADP complex then dissociates to form free enzyme and ADP. However, kinetic studies have shown that the rate of dissociation of the enzyme-ADP complex is slower than the rate of enzyme-ATP formation. It was therefore postulated that at high aminoglycoside concentrations, the enzyme-ADP complex accumulates. This binds G418 which blocks ADP release and therefore also regeneration of the free enzyme. The only solution is for the cell to increase enzyme production and as this is under control of a constitutive promoter the only possibility is the selection of cells with an increased *neo^r* copy number. It is therefore proposed that it is this increase in copy number and overproduction of the *neo^r* protein that is responsible for the observed effects on growth and metabolism. This theory is supported by the findings Yu et al. (1996), who report that in the protozoa *Giardia lamblia*, production of *neo^r* mRNA increases with G418 concentration.

A possible route for increasing production efficiency for cell lines where expression is stable for a period of months may therefore be to remove selection, removing the effects of G418 and increasing productivity. Where expression is not stable, it may be possible to reduce G418 concentration so that the effects of G418 are reduced but that specific production is not affected. A further advantage for removing or lowering the concentration of G418 is faster adaptation of cells to serum or protein-free medium (data not

shown). Adaptation places a stress on the cells, which may be easier to overcome without the added stress of a high metabolic load.

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