Use of cell cycle analysis to characterise growth and interferon- γ production in perfusion culture of CHO cells

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Abstract

The importance of cell cycle analysis in cell culture development has been widely recognised. Whether such analysis is useful in indicating future performance of high cell density culture is uncertain. Using flow cytometric approach to address this question, we utilised the fraction of cells in the S phase to control specific growth rate and productivity in spin filter perfusion cultures and found a significant increase in the accumulated interferon- γ over that obtained from the nutrient-based controlled fed culture. While a general decrease with time exists in both percentage of S phase cells and specific growth rate, a clear oscillatory behaviour of both parameters is found in perfusion cultures.

Introduction

The cultivation techniques for mammalian cells that are developed for a more efficient production of important biochemicals such as viral vaccines, interferons, insulin and monoclonal antibodies must also meet stringent demands in purity, quantity and safety. The traditional batch mode of animal cell culture has several disadvantages, which include the low cell number achieved, the fact that cell death follows very shortly after the attainment of maximum cell number, the low product titre and the poor volume productivity normally achieved. To enhance reactor productivity, various methods using fed-batch and perfusion have been developed.

Perfusion culture can be operated either in a heterogeneous or a homogeneous manner. In heterogeneous systems, there are spatial variations in cell and substrate concentrations and, therefore, in growth or production rates. Global control of the reaction is possible only on a 'black box' basis given the complexity of the heterogeneous reaction system involved. In homogeneous perfusion systems, spatial variation of reaction conditions is reduced to an absolute minimum, but the cell density in the reactor is significantly

enhanced. Homogeneous perfusion cultures have the advantages of easy monitoring of cell culture condition, lack of nutrient and metabolite concentration gradients, better control of physical environmental factors and easy scale-up. A number of these perfusion systems have been described, characterised by total cell retention, in the case of membrane filtration systems, or partial cell retention, in the case of sedimentation, centrifugation or spin-filter systems.

The use of a spinning filter in continuous perfusion cell culture has been reported by a number of investigators (Himmelfarb et al., 1969; Tolbert et al., 1981; Reuveny et al., 1985; Van Wezel et al., 1985; Varecka and Scheirer, 1987; Avgerinos et al., 1990; Yabannavar et al., 1992; 1994; Jan et al., 1993). By retaining the cells inside the bioreactor, the spin filter permits operation at high perfusion rates with minimal cell washout. This allows the high rates of nutrient supply and waste product removal which are necessary to achieve high cell densities. The attainable cell concentration in such a bioreactor has been reported to be ten times as high as that in a typical standard type of bioreactor (Hu and Peshwa, 1991).

The kinetics of cellular growth and death, cell cycle and protein production in high cell density per-

fusion cultures are likely to be significantly different from those in low density cultures. Since one may then achieve rather low apparent specific growth rates at high cell concentrations, it is likely that the distribution of cells in the different phases of the cycle will be different. It is also likely that, should there be a relationship between the latter and product productivity, the expression of the desired product may also vary. In CHO batch culture, it is found that the change in productivity with time is correlated with both the progression of the cell cycle phases and the progression of the cell cycle phases and the progression of batch physiological/environmental phases (Leelavatcharamas et al., 1997). These latter results suggest that cell physiological activity, cell proliferation and productivity are associated and their respective rates are mainly influenced by the culture environment. Moreover, the analysis of cell cycle can allow one to predict the future status of the culture. A linear relationship is found in CHO cell culture between the proportion of S phase cells and the specific growth rate 10.5 hours later, demonstrating the ability of flow cytometry to give an early indication of a future process state (Leelavatcharamas et al., 1996). The objective of this work is to establish the association between growth rate and the proportion of cells in each cell cycle phase in continuous culture and in high density perfusion culture of CHO cells, and to use cell cycle analysis to control the nutrient medium flow rate to the latter.

Materials and methods

Cell line and growth

The cell line used in this was CHO-320, a DHFR deficient mutant of CHO-K1, which produces human interferon- γ . The interferon- γ gene is under the control of SV40 early promoter and enhancer sequences. The cell line was kindly provided by Glaxo Wellcome (Beckenham, Kent, UK). Cells were maintained in RPMI 1640 supplemented with 5% foetal calf serum (GIBCO, Paisley, UK) and 1 μ M methotrexate (Sigma, Poole, UK). Viable and non-viable cell numbers of random samples were counted using trypan blue exclusion in a haemocytometer.

Continuous culture

Continuous culture studies were conducted in a 500 ml Duran stirred bottle with a working volume of 300 ml.

The stirrer speed was 200 rpm. Fresh medium, supplemented with 5% FCS and 1 μ M methotrexate, was supplied from a 2 l reservoir which was kept in an ice-batch. The culture volume was held at a constant level by the overflow of spent medium and cells into a 10 l vessel. The medium flow rate was controlled using a peristaltic pump with periodic checks on the flow rate using a graduated reservoir in-line. The culture was surface aerated with 5% CO₂/air at 100 ml/min.

The culture was inoculated with cells from the mid-exponential phase of growth at a cell concentration of 2×10^5 cells/ml. The culture was allowed to grow in batch mode for 3 days before starting the medium feed at a dilution rate of either 0.25 or 0.5 d⁻¹. After initiation of the medium feed, the passage of at least three working volumes was allowed before the culture was considered to be at steady state and the dilution rate changed. This ensured that the effects of non-ideal mixing in the vessel, characterised by the 'Residence Time Distribution = RTD', do not influence the 'steady-state-values' recorded. Samples were taken daily for cell counting. After centrifuging, cells were fixed with 70% ethanol. Fixed cells and supernatants were kept in a freezer at -18°C for cell cycle and biochemical analysis.

Perfusion culture

Perfusion culture studies were performed in a standard 2l capacity cell culture vessel with 1 litre working capacity equipped with a spin-filter (The LSL Group, Luton, UK). A diagram of the spin-filter perfusion system is shown in Figure 1. The diameter of the spin-filter was 40 mm and the immersed depth 50 mm. The nominal pore size of the spin-filter is 25 μ m. After autoclaving and cleaning with 10% 7X-PF phosphate-free laboratory detergent (ICN Biomedicals Inc.) in an ultrasonic bath (L & R Transistor/ultrasonic T-9) for half an hour, the spin-filter was rinsed several times with distilled water and mounted with a four-blade Rushton turbine impeller below it on the centrally located shaft. Perfusion rates were controlled by a peristaltic pump (Watson Marlow 101U). The culture volume was held at a constant level by flow over a weir inside the filter using a small top pressure from surface aeration at a constant flow rate of 100 ml/min. Oxygen was supplied via silicone tubing. The % dissolved oxygen was controlled by a gas flow controller (Brooks Instruments) with on-off valves for blending O₂-N₂ flows.

Figure 1. Schematic diagram of the spin filter perfusion system.

Interferon- γ assay

Interferon- γ was assayed by an ELISA using the human IFN- γ DuoSet kit (Genzyme Corporation, Cambridge, U.S.A.). The procedure was as follows: The plate was coated, by adding 100 μ l of capture antibody coating solution to each well, and incubated overnight at 4 °C. The plate was washed 5 times with wash buffer and 250 μ l of blocking buffer was added before it was incubated at 37 °C for 2 hours. The blocking buffer was decanted and the plate was blotted dry. Then, duplicate 100 μ l aliquots of each standard or diluted sample were added to the appropriate wells. The plate was then incubated at 37 °C for 2 hours. After washing 5 times with wash buffer, 100 μ l of diluted secondary antibody was added to each well and the plate was incubated at 37 °C for 30 min. The plate was washed 5 times with wash buffer, before adding 100 μ l of substrate reagent to each well, incubated at room temperature for 30 min and then the reaction was stopped by adding 100 μ l of 2 N H₂SO₄ to each well. The absorbance of the plate was read at 450 nm.

Absorbance values were converted to IFN- γ (pg/ml) by interpolating against the standard curve.

Other biochemical assays

Glucose concentration was measured by the enzymatic GOD-Perid method (Boehringer Mannheim, E. Sussex, UK). Lactic acid was analysed by a spectrophotometric method based on the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase and the coenzyme NAD⁺ (Sigma, Poole, UK). Glutamine in the culture supernatants was detected using the Sigma glutamine/glutamate determination kit. The concentration of ammonium ions was detected by the Sigma spectrophotometric method based on the conversion of NH₄⁺ ions to NH₃ which reacts with sodium phenate and hypochloride to produce a blue colour.

Cell cycle analysis

Dead cells were removed for subsequent analysis by DNase treatment. 100 mg of DNase (Sigma, UK) was

Figure 2. Cell cycle distribution of CHO cells deconvoluted by the Multicycle software. Single cells from a dot-plot distribution of propidium iodide peak vs. integral were selectively gated for subsequent cell cycle analysis.

added to 200 ml sucrose magnesium and tris buffer solution. 5×10^5 cells/ml were resuspended in 1 ml of DNase solution and incubated for 30 min, then centrifuged. The cells were resuspended in cold 70% ethanol and stored at 4 °C for at least 30 min prior to staining. Fixed cells were washed in PBS, resuspended in 500 μ l RNase/PBS solution and incubated for 20 min at 37 °C. The cells were double washed with PBS and resuspended in 1 ml PBS. 50 μ l/ml propidium iodide was added and the sample incubated for 15 min at room temperature. After washing with PBS, cells were resuspended in 1 ml PBS and analysed in a Coulter EPICS Elite flow cytometer. Cell cycle distributions were deconvoluted using the Multicycle software programme (Phoenix Flow Systems) as described in Al-Rubeai et al. (1995) and shown in Figure 2.

Results and discussion

Growth rate vs cell cycle

To establish the relationship between growth rate and cell cycle duration a chemostat was operated continuously over a period of 170 days. Four dilution rates, 0.008, 0.010, 0.014 and 0.016 h^{-1} , were used. After each increase in the dilution rate, at least up to 0.014 h^{-1} , the viable cell number increased and reached a new steady state (Figure 3). Increasing the dilution rate then to 0.016 h^{-1} did not lead to a significant increase in the cell concentration. The % viability of CHO 320 cells at each dilution rate was very high, there being no difference of % viabilities among the dilution rates used. Plotting the average of viable cell number at each steady state against the dilution rate, (Figure 4), showed that the cell number increased with the increase of dilution rate. The numbers of dead cells

Figure 3. Steady state cell numbers at various dilution rates in continuous culture. Bars indicate standard errors.

Figure 4. Percentage of S phase cells and specific growth rate at steady state cell numbers at various dilution rates in continuous culture. Bars indicate standard errors.

(also shown in Figure 3) at the different dilution rates were almost the same. These results were different from those reported by Hayter et al. (1993) and Marten et al. (1993) in which the dead cell numbers increased with the decrease of dilution rate. Growth rate is generally a function of cell cycle duration and cell death. Thus, in the absence of death, any difference in growth

rate must be due to a change in the cell cycle time. Cell cycle analysis and growth rate measurement (Figure 4) indicated that the increase in dilution rate resulted in shortening of the cell cycle and that the changes in the duration of the G1 phase accounted for the differences in cycling times. Prolongation of the residence time may have influenced the interdivisional time and consequently reduced the growth rate by prolonging the G1 phase. These results are in agreement with the widely held view that the length of G1 is highly variable. In contrast, S, G2 and mitosis are relatively uniform in duration and unaffected by environmental changes.

These results concerning changes in cell cycle duration and in the percentage of cells in each phase of the cycle at different dilution rates coincided with the results we have reported on the effects of serum concentration on batch growth rate (Leelavatcharamas et al., 1996). One obvious trend in all these results is that, on increasing the dilution rate, the proportion of S phase cells was increased while the proportion of cells in the G1 phase was reduced. The percentage of cells in the G2/M phase was more or less constant at all different dilution rates/growth rates. This behaviour was also found in continuous cultures of hybridoma cells (Al-Rubeai et al., 1992; Marten et al., 1993).

Growth of CHO cells in spin-filter perfusion culture

The spin-filter perfusion culture was started at 6.0×10^5 cells/ml and was grown as a bath for 24 h before starting perfusion at a 'dilution rate' of $0.25 d^{-1}$. Oxygen was supplied through surface aeration and the dissolved oxygen tension (DO) was controlled at 50% for 8 days. Thereafter this was supplemented by bubble-free aeration through immersed silicone tubing, which gave similar overall oxygen transfer rates to those achieved with sparging with air (Emery et al., 1995), and the DO continued to be controlled at 50%. The dilution rate was then stepped up successively to $0.5 d^{-1}$ at 7 days, $1.0 d^{-1}$ at 15.5 days and $2.0 d^{-1}$ at 25.5 days.

The viable cell number and % viability of the CHO cells in spin-filter perfusion culture are shown in Figure 5. Cell numbers increased with the increase of dilution rates and eventually reached a steady state at a dilution of $1.0 d^{-1}$, at which the viable cell number was about 1.2×10^7 cells/ml. The maximum cell numbers in batch and chemostat cultures had been about 1.2×10^6 cells/ml 1.6×10^6 cells/ml respectively but these had been passed within about 5–6 days of the

Figure 5. Growth of CHO 320 cells in spin filter perfusion culture.

start of the spin-filter perfusion culture. The maximum cell number achieved in spin-filter perfusion culture was about 7.5–10 times higher than those seen in batch and continuous cultures. There were effectively no viable or dead cells in the outlet spent medium. Thus, cells were completely retained in the reactor. As cells were retained in the reactor, it was expected that the cell number at each dilution rate would increase until limited by the nutrient supply. Then, the culture would enter a steady state. This could be seen at the dilution rate of 0.25 d^{-1} . However, when cells entered a steady state at the dilution rate of 1.0 d^{-1} this was not because of nutrient limitation but because of oxygen limitation. pH was not controlled and varied between 6.91 and 7.15 before it went down to 6.6 after increasing the dilution rate of 2.0 d^{-1} towards the end of the experiment.

The specific growth rate decreased sharply after the perfusion was started (Figure 6). This was possibly because the perfusion culture was started at the time that the specific growth rate from the batch mode started to decline. An interesting cyclic change and a general declining trend in growth rate were observed independent of the dilution rate. However, toward the end of the run the oscillatory behaviour of the growth rate

was lost even when the dilution rate was increased to 2.0 d^{-1} . This may have been due to oxygen limitation causing cell cycle arrest in G₂ phase (a phenomenon observed in similar situations). The arrest of cells in the G₂ phase was possibly because of the increase of cyclic adenosine monophosphate (cAMP) concentration in the oxygen-limited conditions. Zeilig et al. (1976) showed that elevation of cAMP levels in S or G₂ caused arrest of HeLa cells in G₂ and suggested the existence of a cAMP-mediated checkpoint in G₂. Marti et al. (1994) and Firth et al. (1995) suggested that the cAMP levels could be increased by hypoxia. The oxygen limitation at the end of the perfusion culture, thus, could induce the cAMP production, which is generally low in the G₂ phase, and lead to the arrest of cells in the G₂ phase. The oxygen uptake rate of this high percentage G₂ phase culture ($1.6 \times 10^{-16} \text{ mol O}_2/\text{cell}/\text{sec}$) was much higher than those of the cultures which were taken from the exponential and death phases (2.3×10^{-17} and $3.5 \times 10^{-17} \text{ mol O}_2/\text{cell}/\text{sec}$ respectively) of normal batch culture. This result supported the positive correlation seen between oxygen uptake rate and the percentage of S + G₂ cells in batch cultures (Leelavatcharamus, 1997). The mean cell size in oxygen-limited culture was also apparently larger

Figure 6. Specific growth rate, cell numbers and % S phase cells in spin filter perfusion culture of CHO cells.

than that of cells in normal exponential phase and death phase cultures. The higher oxygen uptake rate in G₂/M phase cells was therefore, possibly, because of their larger size.

The relationship between the specific growth rate and the proportion of cells in S phase was quite clear (Figure 6). The percentage of S phase cells decreased from more than 40% to about 10% when the specific growth rate declined from 0.35 d⁻¹ to 0.05 d⁻¹ over the 33 days of culture duration. The percentage of S phase cells seen during the exponential phase of a batch culture varies between 25–45%. The change of the percentage of S phase cells in the spin-filter perfusion culture was, however, found to be similar to those found in the late exponential phase of batch cultures. After 10 days of cultivation, the percentage of S phase cells had declined to less than 25%. Nevertheless, after increasing the dilution rates, the percentage of S phase cells increased each time before declining again. This partial synchronisation phenomenon was also found in batch cultures when the inoculum was taken from the very late exponential phase (Leelavatcharamas et al., 1996). The partial synchronisation phenomenon in perfusion culture could be explained in the same way as is discussed earlier in the batch culture ex-

periments. As cells were retained in the reactor, the cell number at a constant dilution rate would increase until the demand for nutrients exceeded the supply. As is known from the previous study, when the nutrients were exhausted, e.g. at the end of a batch culture, CHO cells accumulated in the G₁ phase. After the limiting nutrients were added by increasing the dilution rate, the accumulated G₁ phase cells would traverse to the S and G₂/M phases.

Comparing the cell cycle distribution in spin-filter perfusion and chemostat culture, it can be seen first that increasing the dilution rate in the chemostat culture led to an increase of the percentage of S phase cells. The percentage of S phase cells at the steady state in the chemostat culture was dependent on the dilution rate used. However, increasing the dilution rate in the spin-filter perfusion culture could only lead to a transient increase in the percentage of S phase cells but the general trend was of decrease with increasing cell number. It is therefore plausible to suggest that in the absence of significant cell death the decrease in growth rate with increasing cell number is due to the prolongation of G₁ phase which in turn leads to increased cell cycle duration. Al-Rubeai and Emery (1990) showed that a reduction in DNA and protein

Figure 7. Percentage of cells in G₁, S and G₂/M phases in spin filter perfusion culture of CHO cells.

synthesis was associated with the decrease in growth rate during batch culture of hybridoma cells. It seems that cells spend more time in the G₁ phase when subjected to the chemical or physical stresses which may be expected to increase with increasing cell density in perfusion cultures.

The use of percentage of S phase cells as a parameter for the control of the feeding rate in perfusion culture

Since it has been shown that the percentage of S phase cells can be used as an indicator of the future specific growth rate, it should be possible to use this parameter as an indicator for the control of the perfusion rate required in order to maintain the specific growth rate at the desired level.

An attempt has been done to use this indicator to control the feed rate in a spin-filter perfusion culture. The target range was 35–40% and the feed rate was increased when the percentage of S phase declined to below this.

The percentage of cells in each phase of the cell cycle is shown in Figure 7. At the beginning of the ex-

periment, cells were grown in a batch mode for about 3 days before the perfusion culture was started. The cell cycle results, in agreement with the previous report (Leelavatcharamas et al., 1996), showed that during this period, cells were cycling in a partially synchronised manner. As the percentage of G₁ phase declined, the percentage of S phase increased. The percentage of G₂ phase declined, as a result of its movement to G₁ phase, and increased when the percentage of S phase decreased. The percentage of S phase in the second cycle, which possessed a lower degree of synchronisation, reached its peak and began to decline thereafter just before the perfusion culture was started. The increase in G₁ cells was therefore coincidental with the start of the perfusion of the medium. The tail of the partial synchronisation could be seen at the end of the dilution rate of 0.5 d⁻¹ but the percentage of S phase could be held at the desired value for at least another 5 days. At day 9, after the dilution rate was increased to 2.0 d⁻¹, the percentage of S phase increased slightly before declining in the following day to 28%. Increasing the dilution rate to 4.0 d⁻¹ could not enhance

Figure 8. Cell number, specific growth rate and % dissolve oxygen in spin filter perfusion culture of CHO cells.

the percentage of S phase to the desired value (above 30%). The decline in the proportion of S phase cells was possibly a consequence of DO limitation. It was clear that the % DO started to decline once medium perfusion was switched on (Figure 8). At day 6 it rose slightly when the gas mixture was first fed through the silicone tube, before it declined continuously throughout the run. At the end of the run, the % DO went down to about 10%. The specific rates of nutrient (glucose and glutamine) utilisation and product (lactic acid and IFN- γ) production had the same profile as the specific growth rate (results not shown).

The viable cell number reached 1.0×10^7 cells/ml within 11 days of cultivation (Figure 8) compared to the 20 days needed in the first run although this latter started with a cell number 3 times higher (6.0×10^5 cells/ml). The % viability during perfusion culture was always very high, more than 90%. The specific growth rate varied between 0.18 and 0.5 d^{-1} which was higher than in the uncontrolled run (about 0.18 d^{-1}) but it oscillated and declined continuously with time in a similar fashion. In a synchronous culture, the cell number doubles within a short period of time

as the cells enter mitosis and divide together. Consequently, a gradual increase in the specific growth rate is observed. As new daughter cells begin their cell cycle and their progress through G₁, S and G₂ phases, a decline in growth rate is now observed. The degree of oscillation in growth rate then depends on population heterogeneity – the more heterogeneous the system the less oscillation is found in the growth rate. In a completely homogeneous system the growth rate should approach zero for a period equivalent to the duration of the G₁, S and G₂ phases. On the other hand, no change should be observed in a stable heterogeneous system, such as that found during a steady state in chemostat culture.

Figure 9 shows a comparison of the accumulative IFN- γ produced in perfusion culture as seen in both the first and second runs. Clearly, the accumulative IFN- γ produced in the second run, where the specific growth rate was higher, was significantly more than in the first run. More importantly, this amount (36 g) was accumulated within one third of the time needed to accumulate 23 g in the first run. These results, again, suggested that the percentage of S phase cells could be

Figure 9. Cumulative interferon- γ produced in perfusion culture.

used successfully in process optimisation of perfusion culture.

Conclusions

A spin-filter perfusion system was used to study the growth, cell cycle and IFN- γ production in recombinant CHO cells. Increasing the dilution rate led to an initial increase of nutrient concentrations and consequently the percentage of S phase cells and specific growth rate. At each dilution rate change, both of these parameters increased at the beginning and then decreased through to the next change in dilution rate, presumably because of nutrient limitation. These findings supported the results from batch culture that IFN- γ production was growth associated. The relationship between the percentage of S phase cells and the specific growth rate was also confirmed. The increase of percentage of G₁ phase cells and the decrease of specific growth rate at the end of each dilution rate suggested that the spin-filter perfusion culture system was suitable for a non-growth associated product, e.g. monoclonal antibody in hybridoma cell lines, more than a growth associated product (for further discussion see Al-Rubeai and Emery, 1990; Al-Rubeai et al., 1992; Banik et al., 1996).

Controlling the percentage of S phase cells at a level above 30% in spin-filter perfusion culture led

to higher specific rates of growth and production, and shorter cultivation time in order to achieve the maximum cell concentration. There was an enormous difference in the accumulated IFN- γ at day twelve between the cell cycle and non cell cycle based fed cultures (36 g and 3.4 g, respectively). A general decrease with time in both percentage of S phase cells and specific growth rate with a clear oscillatory behaviour of growth rate was found in both cultures. The oxygen transfer rate was found to be the factor limiting maximum cell concentration.

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