Optimization of culture conditions for high cell density proliferation of HL-60 human promyelocytic leukemia cells

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Summary

The purpose of the present investigation was to optimize the culture conditions in suspension of the HL-60 cell line for high-density production. The optimized HL medium was a mixture of RPMI-1640, DMEM, HamF12 and IMDM media supplemented with transferrin, insulin, Primatone RL, Pluronic F68, ethanolamine and selenite. Under these conditions, whether serum was added or not, cells grew to up to 8×10^6 cells ml^{-1}, which was at least three times higher than the maximum cell density usually described. Glucose and four amino acids: cystine, glutamine, methionine and serine, were highly consumed and disappeared quickly from the medium. Nutrient supply and metabolic end-product accumulation were the most probable growth-limiting factors. Different propagation systems were used to increase the cell density further. Cells were grown in dialysis tubing where relatively high levels of nutrients and low levels of waste products were maintained, leading to cell densities of 60×10^6 to 70×10^6 cells ml^{-1}. A perfusion-culturing method with cell retention was found to be most effective for high-density production of HL-60 cells at a 2 l as well as a 60 l fermentor scale. Average concentrations of 40×10^6 to 50×10^6 cells ml^{-1} were achieved. Expression and distribution of both tumor necrosis factor receptors (55 and 75×10^3 M_r) on the surface of HL-60 cells were analysed as a control of the physiological integrity of the cells during the perfusion course; 1.3 μg of tumor necrosis factor receptors/10^{10} cells was regularly expressed on the surface of HL-60 cells.

Key words: HL-60, proliferation, high cell density, tumor necrosis factor receptors.

Introduction

The HL-60 cell line, originally derived from a human promyelocytic leukemia, is a unique human leukemic cell line capable of seemingly unlimited proliferation in suspension culture (Collins *et al.* 1977). HL-60 cells can be induced to differentiate *in vitro* into a number of different cell types (granulocyte, monocyte, macrophage) and studies with this leukemic cell line have proved invaluable in a variety of different areas including: proliferation, differentiation and cellular oncogene expression. HL-60 cells also provide a ready source of specific human cDNA for cloning enzymes and monokines (for review, see Collins, 1987). The HL-60 cell line has been found to express a relatively high level of tumor necrosis factor binding proteins at the cell surface (Roodman *et al.* 1987). Two distinct tumor necrosis factor receptor proteins of about 55 and 75×10^3 molecular weight have been identified on human cell lines using monoclonal antibodies (Hohmann *et al.* 1989; Brockhaus *et al.* 1990). Both proteins specifically bind tumor necrosis factors α and β with a high affinity. The two receptor types have been recently isolated and purified from HL-60 cells (Loetscher *et al.* 1990).

In order to produce large quantities of tumor necrosis factor receptors we initiated this study for optimization of the cultural environment for HL-60 cells at high cell densities. Mammalian cells proliferate *in vitro* in a dynamically complex and rather undefined environment. Glucose and glutamine are the most essential nutrients for energy requirement as well as for anabolic functions (Zielke *et al.* 1978; Reitzer *et al.* 1979). End-products of this catabolism, like ammonia, lactate and CO_2, may be toxic to cell growth when accumulated to critical concentrations (Imamura *et al.* 1982; Butler *et al.* 1983; Glacken *et al.* 1986; Nahapetian *et al.* 1986; Reuveny *et al.* 1986*a*, Hassell *et al.* 1987). The mechanisms of lactate and ammonia inhibition are not well understood. It has been proposed that at least part of the inhibitory effect may be the result of pH perturbation of electrochemical gradients affecting all membrane transport (Dean *et al.* 1984; Glacken *et al.* 1988). For example, a correlation has been found between the effects of ammonia on growth and on intracellular pH (McQueen and Bailey, 1989). Ammonia has also been shown to inhibit the release of iron from transferrin inside acidic vesicles (Karin and Mintz, 1981), and lactate has been found to interfere with multiple activities involving calcium by acting as a very potent calcium chelator (Glacken *et al.* 1988). Lactate is the main source of nonvolatile acid in mammalian cell cultures and excessive accumulation of this product often exceeds the buffering capacity of the medium, thereby lowering the culture pH (Imamura *et al.* 1982; Glacken *et al.* 1986). Cells have also been shown to be highly sensitive to oxygen (Werrlein and Glinos, 1974; Miller *et al.* 1987) and to osmolality (Waymouth, 1970; Schlaeger and Schumpp, 1989). Fur-

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thermore, growth conditions are usually complicated by the use of serum, which is a complex mixture of poorly defined constituents. HL-60 are known to grow in serum-free RPMI-1640 medium when supplemented with transferrin and insulin (Breitman *et al.* 1980). More recently, medium completely devoid of proteins and hormones other than insulin was shown to promote HL-60 cell growth when supplemented with a source of iron (Bang *et al.* 1989). However, the maximum cell concentrations usually achieved are rather low.

It is known from the literature that cells entrapped within physical boundaries proliferate abundantly to dense concentrations provided there is continuous nutrient replenishment and waste removal. A variety of such perfusion systems with cell retention have been described including: spin filter with a stainless steel mesh (Himmelfarb *et al.* 1969; Reuveny *et al.* 1986*b*; Varecka and Scheirer, 1987), dialysis tubing (Adamson *et al.* 1983), microencapsulation (Posillico, 1986; Reilly *et al.* 1990), hollow fiber (Knazek *et al.* 1972; Tharakan and Chau, 1986), encapsulation in alginate beads (Familietti and Fredericks, 1988), stirred membrane tubing reactor (Lehmann *et al.* 1987), tangential cross-flow filtration (Murphy, 1989; Velez *et al.* 1989), etc. Such technologies are very attractive, since high cell concentrations can easily be obtained in small-scale reactors. They have proved invaluable for the production of recombinant proteins as well as for monoclonal antibodies.

In this paper we report on experiments that were designed to: (1) develop a low-cost protein-reduced culture medium sustaining optimal proliferation of HL-60 cells; (2) analyse the factors (nutrients, metabolites, osmolality, etc.) limiting cell proliferation; (3) test the capacity of the HL-60 cells to grow to higher cell densities in dialysis tubing; (4) perform continuous cultures without cell retention in a more constant environment; and (5) establish a perfusion system with cell retention by using a cross-flow filtration unit connected to the bioreactor, in order to reach higher cell concentrations. Under our optimized growth conditions, dense HL-60 cultures were achieved in reproducible 60 l perfusion runs yielding 2.5×10^{12} viable cells, which were subsequently used for isolation and purification of the tumor necrosis factor receptors (Loetscher *et al.* 1990). Expression and distribution of both types of tumor necrosis factor receptors during the course of the perfused culture were analysed to check the integrity of the cells.

Materials and methods

Cells

The human promyelocytic leukemia HL-60 cell line (American Type Culture Collection, CCL 240) was routinely maintained in T-flasks incubated at 37 °C under 5% CO_2–balance air and 96% water saturation. Growth experiments were carried out in roller bottles incubated under the same conditions.

Media and chemicals

DMEM, HamF12, IMDM and RPMI-1640 media were purchased from Gibco BRL (Basel, Switzerland) and mixed in the respective proportions: 1:1:2:2 (by vol.). This medium (designated HL medium) containing 5 μM L-glutamine and 22 mM glucose was supplemented with insulin (5 μg ml^{-1}), selenite (20 nM), ethanolamine (20 μM), human transferrin (15 μg ml^{-1}, Boehringer Mannheim, Rotkreuz, Switzerland), Primatone RL ultrafiltrated (10K) (3.5 mg ml^{-1}, Humko Sheffield Products Norwich, NY, USA), Pluronic F68 (0.1 mg ml^{-1}, Serva, Heidelberg, FRG) and β-mercaptoethanolamine (2.5 mM). The medium was sterilized by Durapore® membrane filtration (0.2 μm) (Millipore, Kloten, Switzerland). Fetal calf serum (Amimed, Muttenz, Switzerland) was added to the medium in various concentrations up to 3%. Modified media without $NaHCO_3$, glucose, glutamine and NaCl were supplied by Amimed. In some experiments (see text) vitamin C (Hoffmann-LaRoche Ltd, Basel, Switzerland) was added at 20 μM and ferric citrate at 250 μM. The feeding solution consisted of glucose (5.5 mM), glutamine (1 mM), Primatone (0.25 mg ml^{-1}) and vitamins (Gibco). All chemicals were purchased from Sigma Chemical and Co. (St Louis, MO, USA) unless otherwise specified.

Growth determination

Growth counts were determined by the use of a hemocytometer and cell viability was assessed by exclusion of Trypan Blue (Patterson, 1979). The colorimetric assay for cell survival and proliferation described by Mosmann (1983) was used when a great number of samples had to be analysed, after verification of the linearity between cell growth and increase in absorbance. This microtiter plate assay is based on the cleavage of the MTT salt (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide) in active mitochondria of living cells. A linear relationship between HL-60 cell number and formazan production has been reported by Wiedermann *et al.* (1990).

Sample analysis

The cell-free culture supernatants were kept frozen until measurement of glucose (glucose oxidase test/Glucose Analyser 2/Beckman Instruments, Fullerton, CA, USA), glutamine (glutamine test/Boehringer Mannheim), lactate (L-lactate oxidase test/L-lactate Analyser/Yellow Springs Instrument, Yellow Springs, OH, USA), ammonia (specific gas-detecting electrode/Metrohm, Herisau, Switzerland) and osmolality (micro-osmometer 3MO/Advanced Instruments, Needham Heights, MA, USA). Amino acids from protein-free supernatants were resolved after *o*-phtaldialdehyde (OPA) derivatization *via* high-performance liquid chromatography on a reverse-phase column (Beckmann Instruments) and analysed on a amino acid analyser (D450, Kontron, Zürich, Switzerland) according to Büntemeyer *et al.* (1990). Cystine, proline and hydroxyproline were analysed after chemical transformation (M. Manneberg, unpublished). The given concentrations of cystine correspond to those of cystine and cysteine together. For the determination of the 55 and 75×10^3 M_r tumor necrosis factor receptors, 4×10^8 cells were solubilized with detergent and the amount of either receptor quantified in a solid-phase enzyme-linked ligand-binding assay using anti-receptor monoclonal antibodies (H. Gallati, unpublished).

Dialysis tubing growth system

Pieces of dialysis tubing (Spectra/Por 6, 2×10^3 molecular weight cutoff, 10 mm diameter/Medical Industries, Los Angeles, CA, USA) were prepared as described before (Schumpp and Schlaeger, 1989). They were filled with growing cells in 1 ml medium and incubated in roller bottles containing HL medium, which was exchanged every other day.

Cultures in bioreactors

Cell cultures were performed in 3 l (2 l working volume) airlift type of bioreactors (Bellco, Vineland, NJ, USA) or in 75 l (60 l working volume) airlift bioreactors (Chemap, Zürich, Switzerland), and oxygen (30±10%), pH (7.1±0.1) and temperature (37(±0.1) °C) were continuously monitored. A Minikros hollow-fiber cartridge (Microgon, Brussels, Belgium), made up of a mixed cellulose ester membrane, polysulfone housing and polyurethane potting material, was used as cross-flow filtration module (0.2 μm pore diameter, 0.09 m^2 surface area) for the removal of cell-free waste medium when working with a 2 l bioreactor. For larger working volumes (60 l) a cassette–membrane system was used, namely the Prostac-Cell-Processing-System (Millipore) with a membrane surface of 0.32 m^2. Cells were circulated in silicon tubes (15.4 mm diameter) through the module (5 l min^{-1}) using a peristaltic pump (Watson Marlow, 603U, New Brunswick Scientific, Falmouth Cornwall, England); spent medium was drawn off

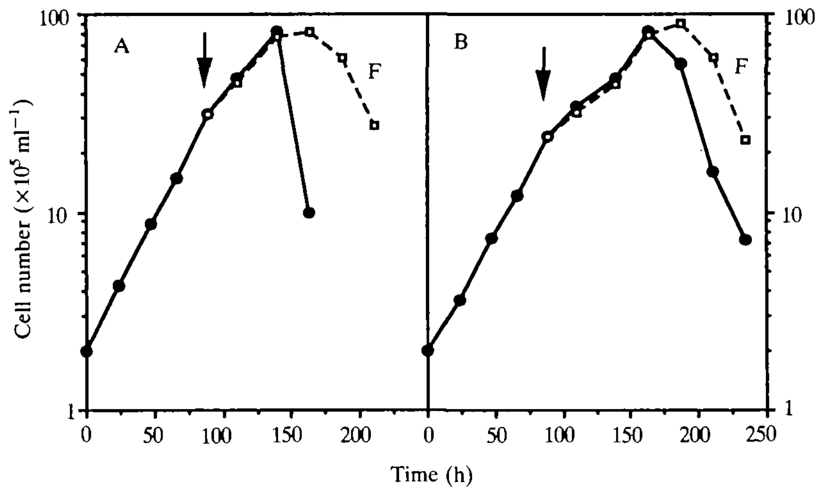

Fig. 1. Comparison of growth of HL-60 cells in HL medium supplemented with 3% fetal calf serum (A) or without serum (B). Cells were grown in roller bottles in 500 ml HL medium and feeding (F) occurred daily from the day indicated by the arrow. Cell proliferation without feeding (●——●) and with feeding (□---□).

and cells recycled in the reactor, which was perfused with fresh medium at the same rate as cell-free supernatant was removed (1 to 2 volumes day^{-1}).

Results

Characterization of HL-60 cell growth in HL medium

HL-60 cells grown in suspension culture usually reach final cell concentrations of 10^6 to 3×10^6 cells ml^{-1} in several described basal media supplemented with 5 to 10% fetal calf serum (Collins, 1987). In the first attempt to optimize the environmental growth conditions a basal culture medium was developed that sustains optimal growth of HL-60 cells. The so-called HL medium, which consisted of a mixture of DMEM, HamF12, IMDM and RPMI-1640 media was supplemented with transferrin, insulin, Primatone RL (a tryptic meat extract) and Pluronic F68 (a polymeric protectant) as described in Materials and methods. The growth properties of HL-60 cells in this optimized medium supplemented with 3% fetal calf serum or serum-free are shown in Fig. 1. In both cases cells cultured in a batch mode were able to reach a cell density of about 8×10^6 cells ml^{-1}, which was a threefold increase as compared to our initial conditions (not shown). The doubling time (t) of the cell population from 10^6 to 2×10^6 cells ml^{-1} was 22.5 h, which corresponds to a growth rate (μ) of 0.0305 h^{-1} in the presence of serum as well as in the absence of serum. However, the serum-free culture revealed a significant decrease in growth rate in the late exponential growth phase, most probably due to a deficiency of (an) essential nutrient(s). Furthermore, in the absence of serum the cells appeared to be more sensitive to environmental changes like pH (see also Martinez *et al.* 1988), osmolality, shear stress or oxygen starvation. Therefore, further growth studies were carried out in the presence of 0.3 to 3% fetal calf serum. Prolongation of the maximum cell density phase could be achieved by feeding glucose, glutamine, Primatone and vitamins to the cells coming in the late exponential growing phase. The cell concentration, however, did not increase further, indicating that exhaustion of major nutrients (glucose, glutamine and other amino acids) was not the only factor limiting growth and survival of HL-60 cells.

The growth-promoting effects of the additives transferrin, insulin and Primatone were studied in more detail in serum-free medium. For unknown reasons the maximal cell concentration reached in this set of experiments was only 4×10^6 to 5×10^6 cells ml^{-1}. Nevertheless, Fig. 2A clearly shows the importance of transferrin for the proliferation of HL-60 cells although the doubling time was drastically increased (about 55 h). By adding insulin together with transferrin the doubling time significantly decreased without a change in the maximum cell density reached. In presence of insulin ferric citrate was able to substitute partially for transferrin in our HL medium, suggesting the existence of an alternative iron-uptake system in HL-60 cells depending on the nutritional environment, as previously described for other cell lines

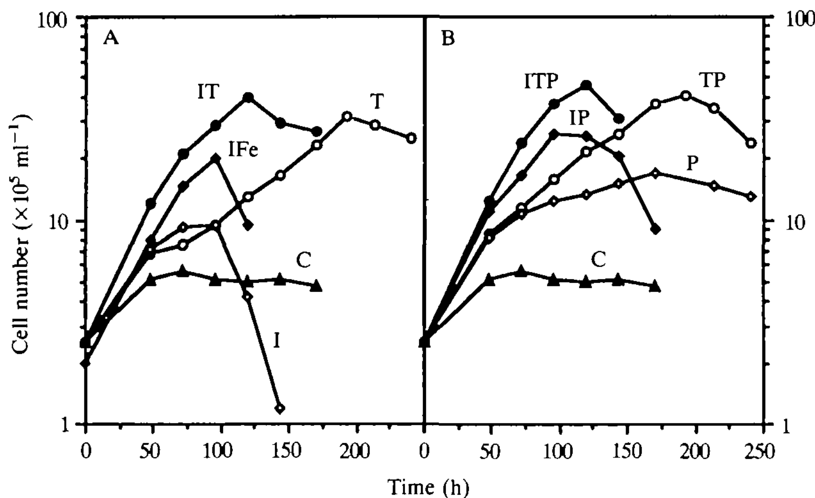

Fig. 2. Response of HL-60 cells to the addition of specific supplements in serum-free medium. Cells were cultured in roller bottles in 100 ml HL medium. Influence of transferrin (T), insulin (I) and ferric citrate with vitamin C (Fe) is shown in (A), and of Primatone (P) in (B). These supplements were added at the concentrations indicated in Materials and methods. Cells were first harvested by centrifugation and thoroughly washed in 37 °C warm HL medium without any supplement (C) before inoculation in the fresh medium.

(Basset *et al.* 1986; Kovar and Franek, 1987; Schneider, 1989; Sturrock *et al.* 1990). Primatone supported a significant increase in the cell number and in the growth rate only when added together with insulin (Fig. 2B). Addition of both Primatone and transferrin was not found to be better than that of transferrin alone. Obviously, Primatone was more important for its iron content than for its high concentration in amino acids and short peptides.

Metabolic activities

As a result of active cell growth there were sharp declines in concentrations of glucose and glutamine with concomitant increases in concentrations of lactate and ammonia in the culture medium up to 28 and 4 mM, respectively (Fig. 3). The complete consumption of glucose and glutamine occurred as cells approached their highest density showing that availability of these two essential nutrients played an important role in cell growth. Specific consumption or production rates were always maximal in the early exponential growing phase and decreased approximately tenfold during culture (Table 1). It is suggested that specific consumption rates increase when high glucose or glutamine concentrations are available.

In addition, as amino acid concentrations in the medium are known to be of tremendous importance for the proliferation of animal cells (Eagle, 1955), kinetic studies of amino acid utilization by HL-60 cells were performed. Roughly three major categories of amino acids could be characterized. The first group included alanine, aspartic acid, glutamic acid, glycine, histidine, ornithine and proline, which were produced at concentrations three to five times the original level. The second group comprised the majority of the amino acids, which were consumed at 10–30%: arginine, asparagine, hydroxyproline, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, tyrosine and valine. The four notable exceptions, which formed the third group, were cystine, methionine, serine and glutamine, consumed at 60, 60, 85 and 100%, respectively, after 5 days of cell growth. The graph in Fig. 4 compares the change in each amino acid tested, i.e. consumption or production as percentage of the initial concentrations in the HL medium. An identical analysis was performed in RPMI-1640 medium (not shown) and the same three groups were characterized. It appears that the utilization of amino acids is not drastically altered when using several media with different amino acid concentration ratios. Differences in the utilization of several amino acids (especially of serine) were found when compared with values in the literature (Eagle, 1955; Butler *et al.* 1983; Wagner *et al.* 1988; Duval *et al.* 1989; Büntemeyer *et al.* 1990; Schmid and Johannsen, 1990). The species origin of the cell line studied could explain the observed differences.

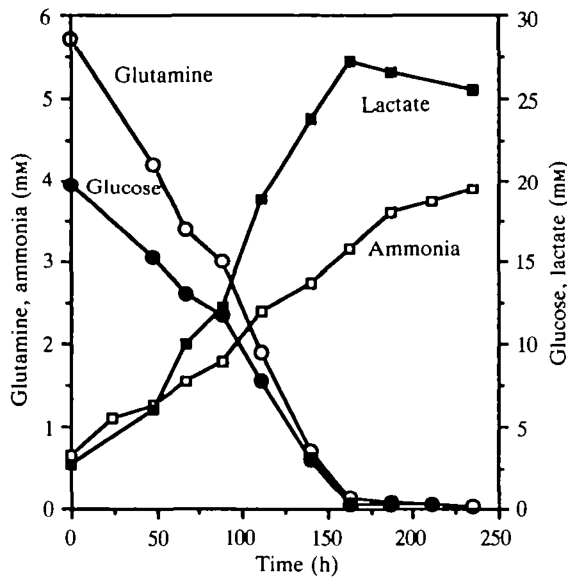

Fig. 3. Changes in concentrations of glucose (●——●), glutamine (○——○), lactate (■——■) and ammonia (□——□). The samples correspond to the cultures shown in Fig. 1 (B) without feeding.

Table 1. *Specific rates of HL-60 cells grown in HL medium*

	Early log phase	Late log phase
μ (h^{-1})	0.030	0.020
Glucose*	3500	400
Glutamine*	1200	100
Lactate*	4500	400
Ammonia*	400	50

* nmol per 10^6 cells day^{-1}.

Cell growth in dialysis tubing

In order to improve cell densities further and to test the growth capacity of HL-60 cells a dialysis membrane system with a molecular weight cutoff of 2×10^3 was used (Schumpp and Schlaeger, 1989). The advantage of this simple method is a cell proliferation without complication by essential nutrient exhaustion or interference by accumulated low molecular weight metabolic end-products. Under these conditions the final cell concentration after 8 days increased about tenfold compared to that of conventional culture in roller bottles (Fig. 5). This result was remarkable in two ways. First, the dialysis tubing functioned as a very compact small high cell density module allowing HL-60 cells to reach final densities of 60×10^6 to 70×10^6 cells ml^{-1} at approximately similar growth rates (μ=0.033 h^{-1}, t=21 h) as measured in conventional cultures. Second, the stationary phase of the HL-60 cells could be maintained at a high cell density over a period of 6 days, indicating survival of non-dividing cultures with a rather low death rate. Growth limitation in the dialysis tubing might be due to: changes in the microenvironment (clogging of the dialysis membrane leading to loss in transport efficiency), availability of oxygen, formation of loose aggregates at higher cell concentration and/or accumulation of released proteins like proteases.

Influence of osmolality, lactate and ammonia

After development of the optimized HL medium, studies were undertaken to identify the potential factor(s) limiting cell proliferation. The optimal range of osmolality (controlled by sodium chloride addition) was measured using the MTT assay after 48 h incubation and appeared to be rather broad. Optimal growth of HL-60 cells was obtained at osmolalities ranging from 260 mosmol kg^{-1} to 320 mosmol kg^{-1}; thereafter cell growth decreased with increasing values of osmolality and was drastically inhibited beyond 350 mosmol kg^{-1} (Fig. 6). The optimal range of osmolality of HL-60 cells differs significantly from other cell lines which usually exhibit values between 320 and 350 mosmol kg^{-1} (Schlaeger and Schumpp, 1989).

Next, cell proliferation in medium containing various concentrations of ammonium chloride or sodium lactate

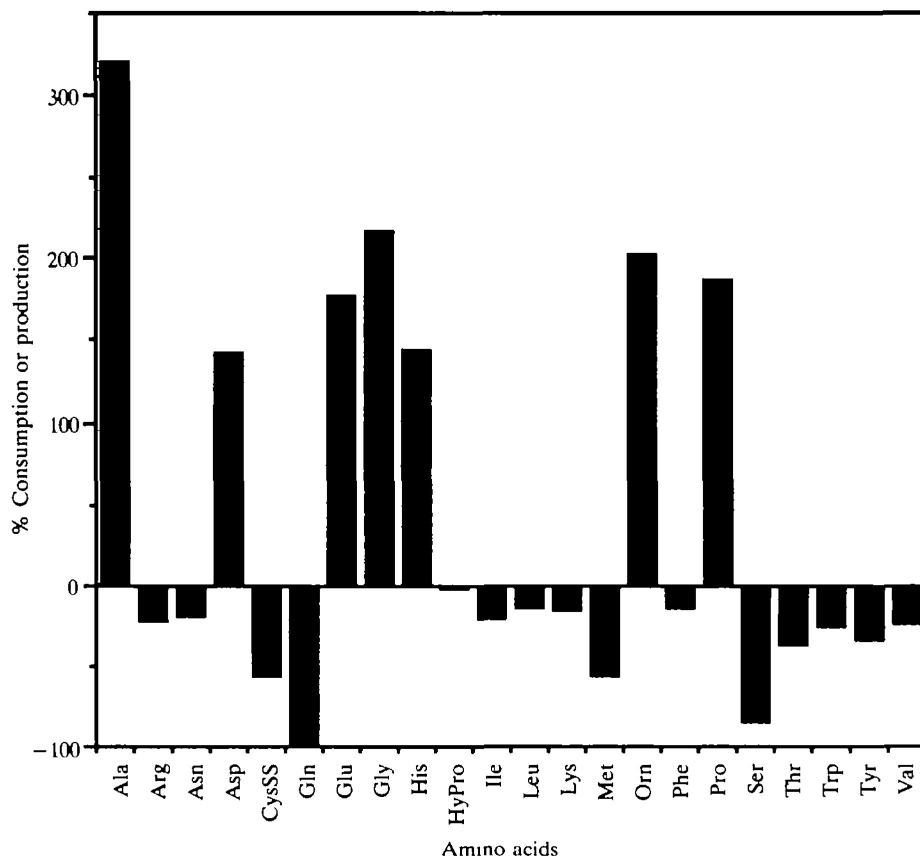

Fig. 4. Changes in concentrations of amino acids in a HL-60 batch culture. Final consumption (above) and production (over) as percentages of the initial concentrations in the HL medium supplemented with 3% fetal calf serum after 7 days of culture.

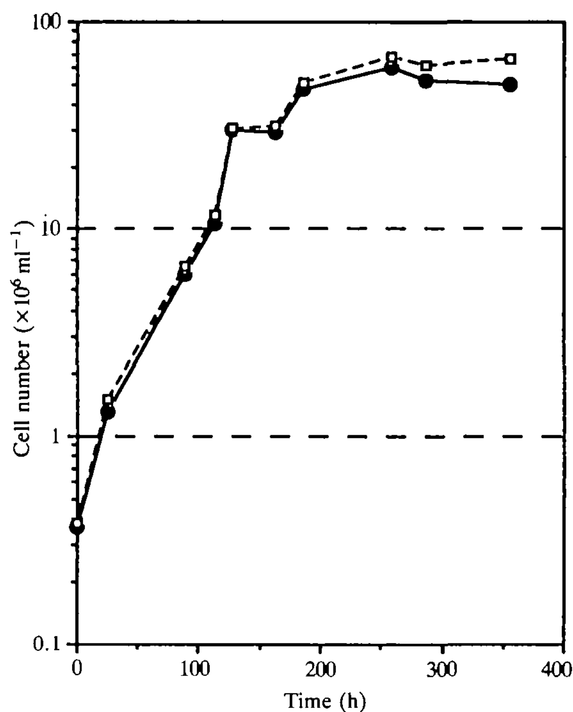

Fig. 5. Culture of HL-60 cells in dialysis tubings. 20 pieces of dialysis tubing containing 1 ml cells in HL medium supplemented with 3% fetal calf serum were incubated in 300 ml of this same medium, which was changed every second day. Each point shows the average of two replicates. Viable cell number (●——●); total cell number (□---□).

was examined using the dialysis tubing system. Both products, especially sodium lactate, increased the osmolality of the medium. To avoid interference by various osmolalities (Fig. 6) in the effects of ammonia or lactate, media with fixed well-controlled osmolalities were used. Increasing inhibition of cell proliferation was observed with increasing concentrations of ammonia or lactate and increasing exposure time (Fig. 7). Growth inhibition in the presence of both ammonia and lactate was not strictly additive, suggesting that the two products have overlapping effects on cell metabolism (not shown). Earlier experiments using the MTT assay gave similar results (Schlaeger and Schumpp, 1989). Considering the data shown in Fig. 7 final concentrations of 22 mM lactate would inhibit cell growth by about 0 and 35%, and 4 mM

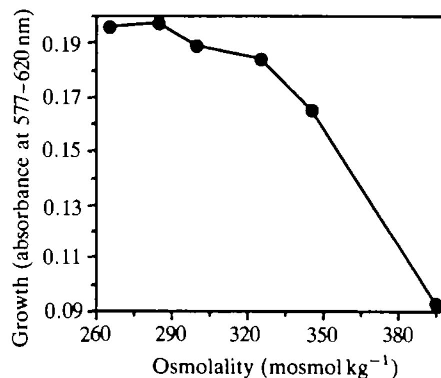

Fig. 6. Influence of osmolality (increased by addition of sodium chloride) on HL-60 proliferation was measured after 48 h incubation with the MTT assay.

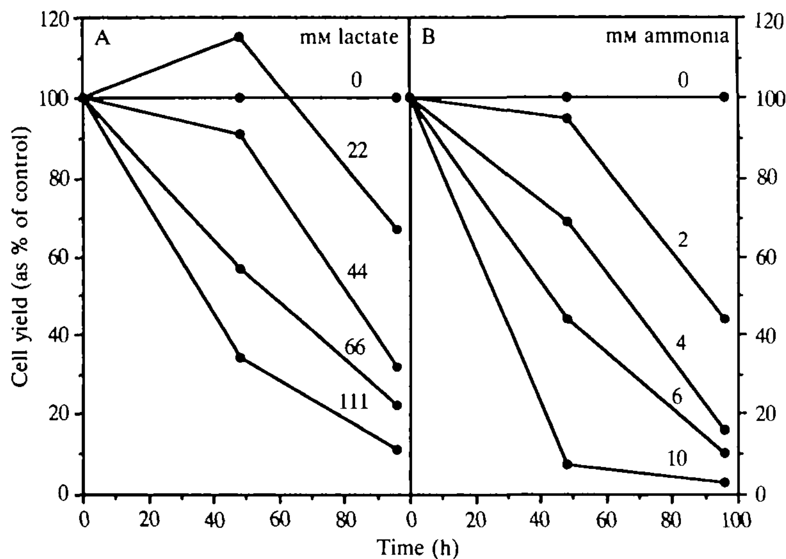

Fig. 7. Response of HL-60 cells to the addition of exogenous sodium lactate and ammonium chloride. For each concentration of sodium lactate or ammonium chloride tested four pieces of dialysis tubing filled with cells were incubated in 150 ml HL medium supplemented with 1% serum and the desired concentration of product. Each point shows the average of two replicates.

ammonia by about 35 and 85% after 48 and 96 h, respectively. During growth, however (Fig. 1 and Fig. 3), cells stopped proliferating without being exposed to these metabolite concentrations for a long period, suggesting a more complex growth limitation process. From these results it appears that other unknown factors also inhibit cell growth and survival.

Continuous culture in a 2 l bioreactor

Growth properties of HL-60 cells in a continuous culture mode were studied in a 2 l bioreactor. Cells were seeded at 1×10^{6} cells ml^{-1} in HL medium and after exponential growth up to 6×10^{6} cells ml^{-1} in a batch mode the medium circulation was established at a flow rate of about 1.5 l day^{-1}. After a relatively short adaptation time, cells reached densities close to 10×10^{6} cells ml^{-1} with a viability greater than 90% as illustrated in Fig. 8A (μ=0.030 h^{-1}, t=22 h). This cell density could be maintained over more than 10 days even when working with serum-free medium. The specific oxygen consumption rate measured at the beginning of the culture in the early exponential growing phase was 1.1×10^{-10} mmol cell^{-1} h^{-1}. As the cells reached 6×10^{6} cells ml^{-1} glutamine was almost totally consumed and HL medium with 6 mM glutamine was therefore used (Fig. 8B,C). Interestingly, the expected increase in the glutamine concentration went with an increase in glucose concentration, illustrating a close connection between glucose and glutamine metabolism. Thereafter, the glucose concentration decreased slowly whereas the glutamine level stabilized at about 0.8 mM. Following correction of the glucose concentration, constant levels of both nutrients were achieved. The osmolality remained stable as expected at a value of about 290±10 mosmol kg^{-1} (not shown).

Perfusion culture in a 2 l and a 75 l bioreactor

The cell density was further increased by connecting a tangential cross-flow filtration module as an external loop to the bioreactor. Using a peristaltic pump the culture medium, including the cells, was permanently circulated through the filtration unit, which allowed the removal of cell-free conditioned medium. Fresh medium was added directly to the bioreactor at the same rate as waste medium was drawn off from the filtration cassette. Fig. 9 shows a perfusion run in a 2 l bioreactor where the cells

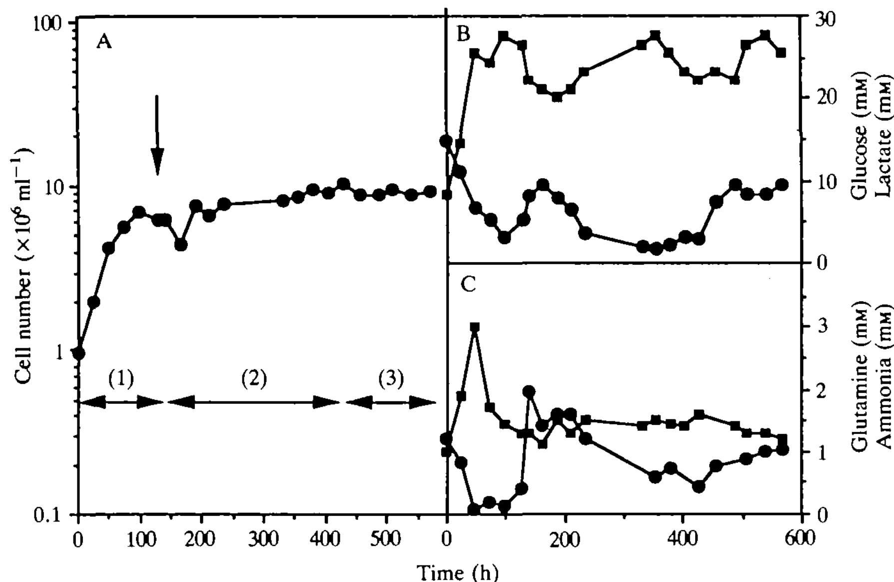

Fig. 8. Continuous culture of HL-60 in a 2 l Bellco bioreactor (A). After 120 h of incubation the medium circulation was established at a flow rate of 1.5 l day^{-1} as indicated by the arrow. The composition of the media used varied as follows: (1) HL medium with 22 mM glucose, 5 mM glutamine and 1% fetal calf serum; (2) HL medium with 22 mM glucose, 6 mM glutamine and 1% serum; (3) HL medium with 33 mM glucose, 6 mM glutamine and no serum. Results of metabolites analysis are shown in B: glucose (●——●) and lactate (■——■) and in (C): glutamine (●——●) and ammonia (■——■).

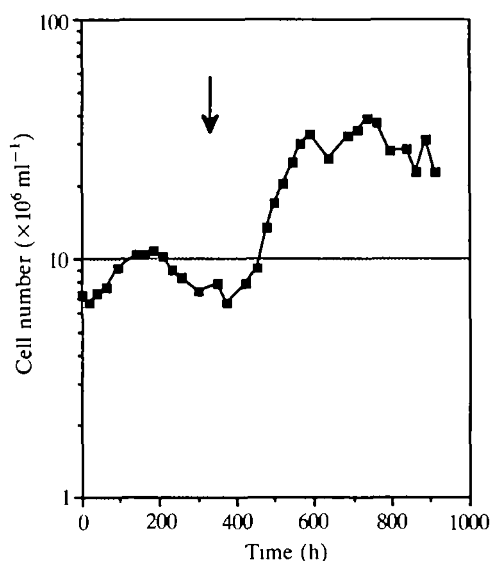

Fig. 9. Perfusion culture with cell retention in a Bellco bioreactor. After 14 days continuous propagation mode of culture with a flow rate of 1.5 l day^{-1} the perfusion culture was started as indicated by the arrow. The medium was then exchanged at a flow rate of 2 l day^{-1}. Cell-free conditioned medium was harvested from the hollow fiber as described in Materials and methods.

were cultured first in a continuous mode for 14 days before the perfusion with cell recycling was started at a flow rate of 2 l day^{-1}. A cell density of 40×10^6 cells ml^{-1} could be achieved and this was stable over a period of 5 days. This experiment showed that cells cultured continuously for a long time retain their potential to grow to higher cell densities. In a scale-up procedure, a 75 l airlift bioreactor with 60 l working volume was used, and again the cell yield increased about five to seven times compared to a batch culture (Fig. 10). The cell viability remained higher than 90 %.

In order to analyse further the integrity of the cells we also assayed the expression of a surface protein. Because HL-60 cells express relatively high level of tumor necrosis factor receptors (Brockhaus *et al.* 1990) the two types of tumor necrosis factor receptors were more thoroughly characterized during cell growth. The results showed that the expression and the distribution of the $75\times10^3 M_r$ protein remained relatively constant during the fermentation process (Fig. 10). The expression of the $55\times10^3 M_r$ protein however decreased by about 25 %, the decrease being more dramatic at later stages of culture. This could be due to a particular sensitivity of the $55\times10^3 M_r$ protein to the continuous circulation of the cells. Average amounts of 1.3 μg of both tumor necrosis factor receptors/10^{10} cells were regularly measured.

Discussion

Several environmental and cultural factors affecting cell growth and viability of the HL-60 cell line have been investigated with the aim of increasing the final cell density. A mixture of the well-known media, RPMI-1640, DMEM, IMDM and HamF12, was found to sustain optimum proliferation of HL-60 cells. The beneficial contribution of the individual media to the final mixture could be to provide a better balance in the nutrient

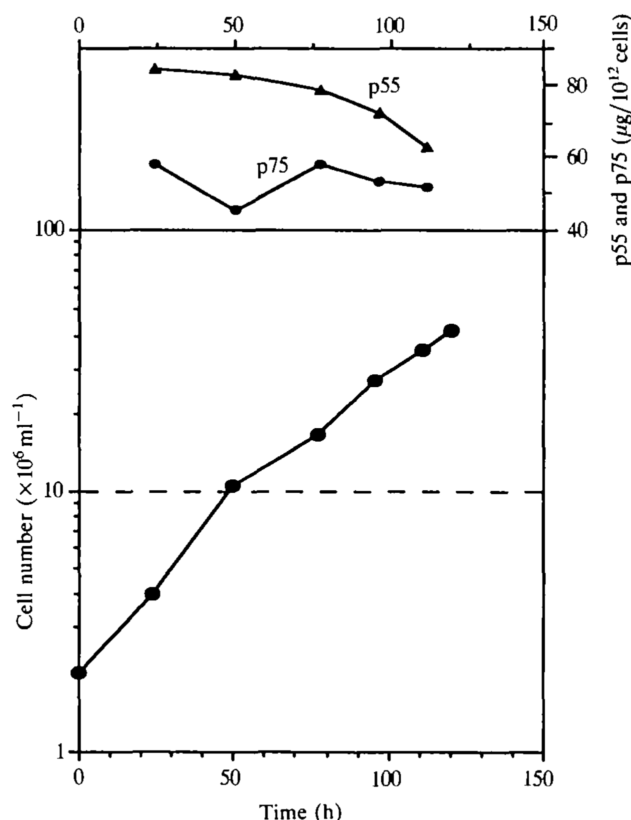

Fig. 10. Scaling up of the perfusion culture system to a 60 l working volume bioreactor. The medium perfusion rate was gradually increased from 30 l day^{-1} to 120 l day^{-1} at day 4. Cell-free waste medium was harvested with the Prostac-Cell-Processing-System as described in Materials and methods. Samples of 4×10^8 cells were taken and extracted for the determination of 55 and $75\times10^3 M_r$ tumor necrosis factor receptors (p55 and p75).

composition and the presence of specific substances (glutathione, vitamins, Hepes, nucleotides precursors, metal cations) that are not constituents of each individual medium. In this medium final cell densities were at least threefold higher and the doubling time was reduced to 21–23 h compared to the results usually described (Collins, 1987; Bang *et al.* 1989). Transferrin and insulin were shown to be the only proteins needed in this HL medium for optimal growth of HL-60 cells. Transferrin can be partially replaced by Primatone or ferric citrate, indicating the existence of an alternative iron-uptake mechanism. So far, growth studies with ferric citrate alone were not very successful but experiments are in progress to adapt the cells to grow in a protein-free medium. HL-60 cells did not require any source of lipids other than the very low quantity provided by the HamF12 medium.

During our optimization of the HL-60 growth conditions we aimed to achieve an understanding of factors limiting cell proliferation. Lactate (22 mM) and ammonia (4 mM) accumulation occurred in concentrations which could inhibit HL-60 cell proliferation in the later stages of culture when cells are exposed to them for several hours. However, our results indicate that neither ammonia nor lactate alone can be the unique factor limiting HL-60 cell growth in conventional batch culture. This assumption is supported by the results of preliminary studies of lactate- and ammonia-resistant subclones obtained by limiting dilution experiments performed in the presence of these

metabolites. Further experiments are in progress to test their proliferating capacity in the presence or absence of lactate and ammonia compared with the non-resistant cell population. Furthermore, accumulation of lactate and ammonia to 22 mM and 4 mM, respectively, was observed in two cultures with different final densities of 4×10^6 cells ml^{-1} and 8×10^6 cells ml^{-1}. This suggests that under optimized growth conditions the specific consumption or production rates decrease, certainly as a result of more efficient metabolism for energy production. The specific consumption rates measured for the HL-60 cells are similar to those found by other investigators for mouse hybridoma cell lines (Miller *et al.* 1988; Büntemeyer *et al.* 1990). In general, growth inhibition by ammonia and lactate depends widely on the cell line, on the time of exposure to the toxic product and on the physiological stage of the cells used in the experiments (Schlaeger and Schumpp, 1989). Besides the accumulation of metabolic end-products, cessation of growth could be the result of nutrient deficiency, including oxygen. Specific nutrients that are rapidly consumed have been identified: glucose, glutamine, cystine, methionine and serine. However, feeding these substrates at the end of the exponential growing phase did not improve cell yields further in our HL medium. Furthermore, an increase in cell density did not occur in the bioreactor, where a controlled continuous oxygen supply was achieved. From these observations it is suggested that limitation of cell proliferation is a complex process not restricted to a single factor but rather to the accumulation of several limitation causes.

Our primary goal for growing HL-60 cells under optimal conditions in continuous perfusion culture was to maximize the cell density over an extended culture life and to express and maintain high levels of tumor necrosis factor receptors on the cell surface for subsequent purification. Since the extracellular environment of the cells should remain optimal, the daily perfusion rate (1–2 bioreactor volume(s) per day) was chosen to maintain pH and sufficient levels of the energy sources glucose and glutamine while minimizing toxic metabolites accumulation. It is noteworthy that the distribution (expression) of both types of tumor necrosis factor receptors on the HL-60 cell surface was approximately constant during cell growth to high densities, suggesting that cells were not highly stressed by the perfusion system used. The production of about 2.5×10^{12} viable HL-60 cells by perfusion culture was sufficient to isolate and purify the two tumor necrosis factor binding proteins in the 100 μg range (Loetscher *et al.* 1990). Using the perfusion technique together with the optimized growth conditions we were able to reduce the bioreactor scale by a factor 10. Furthermore, tumor necrosis factor receptor analysis during the culture showed that the process leading to high cell densities did not dramatically influence the expression of both receptor types.

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References

Adamson, S. R., Fitzpatrick, S. L., Behie, L. A., Gaucher, G. M. and Lesser, B. H. (1983). *In vitro* production of high titre monoclonal antibody by hybridoma cells in dialysis culture. *Biotechnol. Lett.* **5**, 573–578.

Bang, B. E., Sager, G. and Aarbakke, J. (1989). Growth, differentiation and the β-adrenergic signal system of HL-60 cells. *Biochem. Pharmac.* **38**, 3723–3729.

Basset, P., Quesneau, Y. and Zwiller, J. (1986). Iron-induced L1210 cell growth: evidence of a transferrin-independent iron transport. *Cancer Res.* **46**, 1644–1647.

Breitman, T. R., Collins, S. J. and Keene, B. R. (1980). Replacement of serum by insulin and transferrin supports growth and differentiation of the human promyelocytic cell line, HL-60. *Expl Cell Res.* **126**, 494–498.

Brockhaus, M., Schoenfeld, H.-J., Schlaeger, E.-J., Hunziker, W., Lesslauer, W. and Loetscher, H. (1990). Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proc. natn. Acad. Sci. U.S.A.* **87**, 3127–3131.

Büntemeyer, H., Lütkemeyer, D. and Lehmann, J. (1990). Optimization of serum-free fermentation processes for antibody production *Cytotechnology* (in press).

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

Collins, S. J. (1987). The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood* **70**, 1233–1244.

Collins, S. J., Gallo, R. C. and Gallagher, R. E. (1977). Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature, Lond* **270**, 347–349.

Dean, R. T., Jessup, W. and Roberts, C. R. (1984). Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. *Biochem. J.* **217**, 27–40

Duval, D., Geahel, I., Dufau, A. F. and Hache, J. (1989). Effect of amino acids on the growth and productivity of hybridoma cell cultures. In *Advances in Animal Cell Biology and Technology for Bioprocesses* (ed. R. E. Spier, J. B. Griffiths, J. Stephenne and P. J. Crooy), pp. 257–259. Butterworths, Sevenoaks, Kent.

Eagle, H. (1955) The specific amino acid requirements of a mammalian cell (strain L) in tissue culture. *J. biol. Chem.* **214**, 839–853.

Familletti, P. C. and Fredericks, J. E. (1988). Techniques for mammalian cell immobilization. *Bio/Technology* **6**, 41–44.

Glacken, M. W., Adema, E. and Sinskey, A. J. (1988). Mathematical descriptions of hybridoma culture kinetics: initial metabolic rates. *Biotechnol. Bioengng* **32**, 491–506.

Glacken, M. W., Fleischaker, R. J. and Sinskey, A. J. (1986). Reduction of waste product excretion *via* nutrient control: possible strategies for maximizing product and cell yields on serum in cultures of mammalian cells. *Biotechnol. Bioengng* **28**, 1376–1389.

Hassell, T. E., Allen, I. C., Rowley, A. J. and Butler, M. (1987). The use of glutamine-free media for the growth of three cell lines in microcarrier culture. In *Modern Approaches to Animal Cell Technology* (ed. R. E. Spier and J. B. Griffiths), pp. 245–261. Butterworths, Sevenoaks, Kent.

Himmelfarb, P., Thayer, P S. and Martin, H. E. (1969). Spin filter culture: the propagation of mammalian cells in suspension. *Science* **164**, 555–557.

Hohmann, H., Remy, R., Brockhaus, M. and van Loon, A. P G. M. (1989). Two different cell types have different major receptors for human tumor necrosis factor (TNFα). *J. biol. Chem.* **264**, 14 927–14 934

Imamura, T., Crespi, C L., Thilly, W. G and Brunengraber, H. (1982). Fructose as a carbohydrate source yields stable pH and redox parameters in microcarrier cell culture *Analyt. Biochem.* **124**, 353–358.

Karin, M. and Mintz, B. (1981) Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse tetracarcinoma stem cells. *J. biol. Chem.* **256**, 3245–3252.

Knazek, R. A., Gullino, P. M., Kohler, P. O. and Dedrick, R. L. (1972). Cell culture on artificial capillaries: an approach to tissue growth *in vitro*. *Science* **178**, 65–66.

Kovar, J. and Franek, F. (1987). Iron compounds at high concentrations enable hybridoma growth in a protein-free medium. *Biotechnol. Lett.* **9**, 259–264.

Lehmann, J., Piehl, G. W. and Schulz, R. (1987). Bubble free cell culture aeration with porous moving membranes. *Develop. biol. Standard.* **66**, 227–240.

Loetscher, H., Schlaeger, E.-J., Lahm, H.-W., Pan, Y.-C. E., Lesslauer, W. and Brockhaus, M. (1990). Purification and partial amino acid sequence analysis of two distinct tumor necrosis factor receptors from HL-60 cells. *J. biol. Chem.* (in press).

Martinez, R., Gillies, R. J and Giuliano, K. A. (1988). Effect of serum on the intracellular pH of BALB/c-3T3 cells: serum deprivation causes changes in sensitivity of cells to serum. *J. cell. Physiol.* **136**, 154–160.

McQueen, A and Bailey, J. E. (1989). Ammonium ion effects on hybridoma cell physiology. Presented at the Cell Culture Engineering II Meeting, December 3–8, 1989, Santa Barbara, California U.S.A., abstract no. 85.

Miller, W. M., Wilke, C. R. and Blanch, H. W. (1987). Effects of dissolved oxygen concentration on hybridoma growth and metabolism in continuous culture. *J. cell. Physiol.* **132**, 524–530.

Miller, W. M., Wilke, C R. and Blanch, H. W. (1988). Transient responses of hybridoma cells to nutrient additions in continuous culture: glucose pulse and step changes. *Biotechnol. Bioengng* **33**, 477–486.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. immun. Meth.* **65**, 55–63.

Murphy, T. J (1989). Continuous perfusion of stirred or airlift bioreactors using hollow fibers for cell separation and oxygenation. Presented at the Cell Culture Engineering II Meeting, December 3–8, 1989, Santa Barbara, California U.S.A., abstract no. 62.

Nahapetian, A. T., Thomas, J. N. and Thilly, W. G. (1986). Optimization of environment for high density Vero cell culture: effect of dissolved oxygen and nutrient supply on cell growth and changes in metabolites. *J. Cell Sci.* **81**, 65–103.

Patterson, M. K. (1979). Measurement of growth and viability of cells in culture. *Meth. Enzym.* **58**, 141–152.

Posillico, E. G. (1986). Microencapsulation technology for large-scale antibody production. *Bio/Technology* **4**, 114–117.

Reilly, E. B., Antognetti, G., Wesolowski, J. S. and Sakorafas, Jr and P. (1990). The use of microcapsules for high density growth of human tumor infiltrating lymphocytes and other immune reactive T cells. *J. immun. Meth.* **126**, 273–279.

Reitzer, L. J., Wice, B. M and Kennell, D. (1979). Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J. biol. Chem.* **254**, 2669–2676.

Reuveny, S., Velez, D., Macmillan, J. D. and Miller, L. (1986*a*). Factors affecting cell growth and monoclonal antibody production in stirred reactors. *J. immun. Meth.* **86**, 53–59.

Reuveny, S , Velez, D., Miller, L. and Macmillan, (1986*b*). Comparison of cell propagation methods for their effect on monoclonal antibody yield in fermentors *J. immun. Meth.* **86**, 61–69.

Roodmann, G. D., Bird, A., Hutzler, D. and Montgomery, W. (1987). Tumor necrosis factor-α and hematopoietic progenitors: effects of tumor necrosis factor on the growth of erythroid progenitors CFU-E and BFU-E and the hematopoietic cell lines K562, HL-60 and HEL cells. *Expl Hemat.* **15**, 928–935.

Schlaeger, E.-J. and Schumpp, B. (1989). Studies on mammalian cell growth in suspension culture. In *Advances in Animal Cell Biology and Technology for Bioprocesses* (ed. R E. Spier, J. B. Griffiths, J. Stephenne and P. J. Crooy), pp. 386–396. Butterworths, Sevenoaks, Kent.

Schmid, G. and Johannsen, R. (1990). Metabolic quotients for recombinant CHO and BHK cell lines producing human antithrombin III. *Biotechnol. Lett.* **12**, 317–322.

Schneider, Y.-J. (1989). Optimisation of hybridoma cell growth and monoclonal antibody secretion in a chemically defined, serum- and protein-free culture medium. *J. immun. Meth* **116**, 65–77.

Schumpp, B. and Schlaeger, E.-J. (1989). Physiological studies of high cell density culture of different cell lines. In *Advances in Animal Cell Biology and Technology for Bioprocesses* (ed. R. E. Spier, J. B. Griffiths, J. Stephenne and P. J. Crooy), pp. 224–229. Butterworths, Sevenoaks, Kent.

Sturrock, A., Alexander, J., Lamb, J., Craven, C. M. and Kaplan, J. (1990). Characterization of a transferrin-independent uptake system for iron in HeLa cells. *J. biol. Chem.* **265**, 3139–3145.

Tharakan, J. P. and Chau, P. C. (1986). A radial flow hollow fiber bioreactor for the large-scale culture of mammalian cells. *Biotechnol. Bioengng* **18**, 329–342

Varecka, R. and Scheirer, W. (1987) Use of a rotating wire cage for retention of animal cells in a perfusion fermentor. *Devl biol. Standard.* **66**, 269–272.

Velez, D., Miller, L. and Macmillan, J. D. (1989). Use of tangential flow filtration in perfusion propagation of hybridoma cells for production of monoclonal antibodies. *Biotechnol. Bioengng* **33**, 938–940.

Wagner, R., Ryll, T., Krafft, H and Lehmann, J. (1988). Variation of amino acid concentrations in the medium of HU β-IFN and HU IL-2 producing cell lines. *Cytotechnology* **1**, 145–150.

Waymouth, C. (1970). Osmolality of mammalian blood and of media for culture of mammalian cells. *In Vitro* **6**, 109–127.

Werrlein, R J. and Glinos, A. D (1974). Oxygen microenvironment and respiratory oscillations in cultured mammalian cells. *Nature, Lond.* **251**, 317–319.

Wiedermann, C. J., Niedermühlbichler, M., Bilgeri, R., Zilian, U. and Konwalinka, G. (1990). Analysis of proliferation and terminal differentiation of HL-60 human promyntiation of HL-60 human promyelocytic leukemia cells by a rapid colorimetric assay. *J. immunol. Meth.* **128**, 147–148.

Zielke, H. R., Ozand, P T, Tildon, J T., Sevdalian, D. A. and Cornblath, M (1978). Reciprocal regulation of glucose and glutamine utilization by cultured human diploid fibroblasts. *J. cell. Physiol.* **95**, 41–48.

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