Analysis of Nutritional Factors and Physical Conditions Affecting Growth and Monoclonal Antibody Production of the Hybridoma KB-26.5 Cell Line

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The effect of medium composition and physical conditions on the growth pattern and monoclonal antibody production of the hybridoma cell line KB-26.5 has been studied in batch and fed-batch cultures. Different aspects have been analyzed both individually and in combination, as it is expected that not only one component plays a predominant role in this process but a combination of a number of them. Modification of the concentrations of glucose and glutamine, supplementation of the medium with vitamins and amino acids, influence of lactate and ammonium accumulation, and pulse addition of fetal calf serum have been studied in detail, contributing to an improvement in the cell growth and IgG_3 production. Finally, the combined effect of all these factors in a fed-batch culture leads clearly to a major improvement in the cultures, with a 10-fold increase in the final monoclonal antibody concentration with respect to nonoptimized batch experiments.

Introduction

Current and potential applications of monoclonal antibodies in fields such as diagnostic assays, biosensors, catalysis, downstream processing, therapeutics, and other areas of biotechnological interest (Birch et al., 1985; Werner and Noé, 1993) have resulted in an increasing demand for monoclonal antibody *in vitro* production techniques.

One method to optimize the monoclonal antibody production is to increase the monoclonal antibody specific production rate (q_{Ab}). The addition of epidermal growth factor (EGF), fibroblast growth factor (FGF), and interleukin 2 (IL-2) (Pendse and Bailey 1990), mouse peritoneal factors (Mikami et al., 1991), or cyclic nucleotides such as cAMP and cGMP (Dallili and Ollis, 1988) markedly improved the productivity per cell. In some other cases, it has been shown that different physiological conditions which perturb cell growth also enhance antibody production in hybridomas. Addition of thymidine (Suzuki and Ollis, 1990; Hayter et al., 1992), serum-depleted or serum-free medium (Hayter et al., 1992), suboptimal oxygen concentrations (Reuveny et al., 1986a; Phillips et al., 1987), low pH (Hayter et al., 1992), and high osmolarities (Ozturk and Palsson, 1991; Reddy et al., 1992) are examples of perturbation of cell growth with a favored antibody production resulting in enhanced specific productivities. Recently, enhanced antibody concentrations have been achieved by adaptation to high-osmolarity medium and addition of sodium butyrate (Oh et al., 1993).

Another strategy to improve the monoclonal antibody production is to increase the cell concentration of the culture. The density of hybridoma cell cultures is often low and the duration of the culture short, showing an abrupt transition from exponential growth phase to death phase, resulting in a steep population decline. Consequently, the total amount of monoclonal antibodies produced is low. The factors causing the arrest of cell growth vary with the different cell lines, media, and growth conditions. Some of them have been studied, quite often in an individual basis (Lavery et al., 1985; Reuveny et al., 1986a,b), showing that the nutritional environment is an important factor because the culture medium has to replace the complex body fluids and will affect all physiological and metabolic events in the cell. The metabolism of transformed cells is significantly different from the original cell lines showing new properties, such as high glycolytic and glutaminolytic rates, leading to a high piruvate production (Baggetto, 1992). Most of the pyruvate is transformed to lactate instead of being oxidized via the TCA cycle in the mitochondria under aerobic conditions. Therefore, glucose and glutamine are known to be main requirements for growth (McKeehan, 1982). However, cells require in smaller amounts other nutrients as well, such as amino acids, vitamins, and also serum compounds that will cause cell death if they are depleted from the medium. The importance of amino acids and vitamins for *in vitro* growth of animal cells has long been recognized (Luan et al., 1987a; Duval et al., 1991; Jo et al., 1993). Amino acids are required for the formation of nucleotides, lipids, proteins, and thus monoclonal antibodies. Vitamins do not furnish energy but are essential for energy transformation and regulation of metabolism. Another factor that can be limiting cell growth is fetal calf serum (FCS), especially in the case when cultures are performed with low concentrations in FCS, as many authors do for many motivations (Glassy et al., 1988).

Glucose, glutamine, and other nutrients are converted into biomass, antibody, energy as ATP, reducing power (NADPH) for biosynthesis, CO_2, and waste products, like lactate and ammonium, which can inhibit cell growth. The effect of lactate and ammonium is well discussed in the literature (Martinelle and Häggström, 1993; Ozturk et al., 1992; Newland et al. 1994) and markedly changes depending on each cell line. The environmental conditions are also an important factor in the culture (Reuveny et al., 1986a; Phillips et al., 1987), basically, DO concentration and pH. The amount of oxygen requirement varies with each cell line and should be determined in every case (Ozturk and Palsson, 1990).

As a consequence of this complex physiological behavior, an improvement of cell growth and/or antibody

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production by hybridomas requires a global analysis of the different aspects that take place in their metabolism (nutrient supply, accumulation of toxic products, culture conditions). It can be anticipated that not a single factor can be expected to improve the culture output, but a combination of a number of them, in a given sequence. In this article, such an analysis is made for the hybridoma cell line KB-26.5, from both the individual and global perspective.

Material and Methods

Cell Line. The KB-26.5 murine hybridoma has been used in this work. This cell line produces an IgG_3 antibody directed against antigen A_1 of red cells that is used for the determination of the ABO human blood group. The cell line was kindly provided by Laboratorios Knickerbocker SAE (Barcelona) in frozen state growing in 5% serum supplemented medium. Cells were adapted to grow at 1% FCS maintaining their ability to produce antibodies at the same level (Damgaard et al., 1993). Once adapted, they were frozen and stored in liquid N_2. The cell culture was replaced every 2 months with frozen stock culture. The absence of mycoplasma infection in the cell stock was regularly checked.

Media. The basal medium used in all the cultures was Dulbecco's Modified Eagle's medium (DMEM) (Sigma D5030) supplemented with sodium bicarbonate (3.7 g L^{-1}) (Probus), phenol red (15 mg mL^{-1}), β-mercaptoethanol (0.391 mg L^{-1}) (Sigma), insulin (0.836 mg L^{-1}) (Novo), and 1% fetal calf serum (FCS, Biological Industries). This basal medium was also supplemented with glucose (Merck) and glutamine (Merck) at different concentrations specified in the Results and Discussion. In some experiments, the medium was further supplemented with four times the normal concentraction of amino acids (Sigma) and vitamins (Merck):

amino acids	mg mL^{-1}	vitamins	mg mL^{-1}
serine (Ser)	126	Ca pantothenate	12
histidine (His·HCl·H_2O)	126	choline chloride	12
glycine (Gly)	90	folic acid	12
threonine (Thr)	285	inositol	21.6
arginine (Arg)	209.04	nicotinamide	12
tyrosine (Tyr)	250.02	riboflavine	1.2
cystine (CysCys·2HCl)	187.71	thiamine	12
valine (Val)	282	biotine	2
methionine (Met)	90	B_{12}	1
phenylalanine (Phe)	198	benzoic acid	1
isoleucine (Ile)	315		
leucine (Leu)	315		
lysine (Lys·HCl)	438		
tryptophan (Trp)	48		

In this fortified medium, the DMEM concentration was reduced by 20% to maintain medium osmolarity. No antibiotics were used except for the experiments in the fermenter where 125 units mL^{-1} penicillin and 167 μg mL^{-1} streptomycin were added.

Cell Propagation Systems. ***T-Flasks.*** Cell line maintenance was carried out in 25 cm^2 t-flasks (Costar). Cultures were maintained in 10 mL volumes, and sampled and diluted every 2 or 3 days with fresh medium to give an initial seeding density of 1.0×10^5 viable cells mL^{-1}. Some of the batch experiments were performed in 45 mL t-flasks.

Spinner flasks (Techne) with a working volume of 125 or 250 mL and stirred at 40 rpm. In both cases, cells were cultivated in a humidified atmosphere of 5% CO_2 in air (Forma Scientific CO_2 incubator).

Fermenter. Cells were cultivated in a Biostat MCD bioreactor (Braun Biotech) with a 2 L working volume. Temperature and pH were kept constant at 37° C and 7.1, respectively. Various dissolved oxygen (DO) concentrations were used, as reported in the Results and Discussion. The pH and dissolved oxygen concentration could be controlled by CO_2 supply and by reversible supply of air and nitrogen via a gas mixing station. Bubble free aeration was performed by means of a tubular microporous silicone membrane. Agitation was provided by large bladed paddle stirrers operating at 60 rpm. An inoculum density of 1×10^5 cells mL^{-1} with cells grown to middle exponential phase was used in all the reported experiments.

Analytical Methods. ***Cell Number.*** Cells were counted using an haemocytometer (Neubauer improved, Brand). Cell viability was determined by the trypan blue exclusion method (1:1 mixture of 0.2% trypan blue in normal saline and cell sample). This method has an error of 5%. After cell counting, the remainder of each sample was centrifuged (500 g, 5 min) to remove the cells and the supernatant was frozen for further analysis.

Metabolite Determinations. *Glucose and lactate* concentrations were determined with an YSI 2000 automated glucose and L-lactate analyzer. The error in these analysis was 5%.

Ammonium concentration was determined by a flow injection analysis system as previously described (Campmajó et al., 1994). The error of this analysis was 3%.

Glutamine and other amino acids concentrations were measured by HPLC (Hewlet Packard 1090) using a reversed phase column (aminoquant 200 × 2.1 mm) after derivatization of the samples with *O*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) for the analysis of both primary and secondary amino acids. As the cyst(e)ine sulfhydryl group competes with the thiol compound, which is required for the derivatization reaction and becomes part of the product, cyst(e)ine was stabilized and capped to block participation in the OPA chemistry using 3,3′-dithiodipropionic acid at 80 °C for 15 min. A diode array detector at 338 nm was used to detect primary amino acids and at 262 nm for secondary amino acids. A two-solvent gradient was used for the separation of all the amino acids. Solvent A had the following composition: 20 mM sodium acetate/0.3% tetrahydrofuran/0.018% triethylamine. Solvent B was prepared with 100 mM sodium acetate, acetonitrile, and methanol at a 20/40/40 ratio. The errors in these analyses were less than 5%.

Product Analysis. *Antibody Concentration.* The monoclonal antibody IgG_3 was quantified by the enzyme-linked immunosorbent assay (ELISA) as described before (Damgaard et al., 1993). The error of this method is 10%.

Results and Discussion

Effect of Dissolved Oxygen Concentration. Oxygen is required for energy generation as the terminal electron receptor in oxidative phosphorylation. The influence of oxygen on cell growth, antibody production, and cell metabolism of hybridoma KB-26.5 has been investigated in various batch experiments carried out in the fermenter at DO levels between 0 and 40% of O_2 saturation. Initial concentrations of 25 mM glucose and 6 mM glutamine were used. Results are shown in Table 1.

Cells in the absence of oxygen did not survive, indicating its essential role for the cell growth. DO concentrations with 10−40% O_2 saturation did not greatly influence cell growth, since the three cultures showed the same growth profiles reaching very similar cell densities. Typically, the batch growth profiles showed an initial period of exponential growth followed by a rapid decline

Table 1. Effect of the Dissolved O_2 Concentration on IgG_3 Production and Cell Metabolism

	dissolved O_2 concn		
	10%	20%	40%
IgG_3 (μg mL^{-1})	24.0	36.0	34
glucose consumed (mM)	21.5	21.3	17
lactate produced (mM)	50.7	42.0	34
glutamine consumed (mM)	6.0	6.0	6
max cell concn ($\times 10^5$ viable cells mL^{-1})	18.0	20.34	20.1

in the cell viability. The peak in viable cell concentration took place at the time at which glutamine was exhausted. This fact suggested that glutamine could be the limiting nutrient in these experiments. However, it was not evident whether other nutrient limitations caused cell growth to stop. Glucose consumption by the cells was also stopped at the point of glutamine depletion (data not shown).

The main difference that could be observed in different DO concentrations was that the amount of lactate produced increased as the concentration of dissolved oxygen decreased (Table 1). By comparing the results obtained in DO of 10 and 20%, it can be observed that for an equivalent glucose consumption, the level of lactate is higher for 10% than 20%, indicating that under oxygen limitation the pathway to lactate via the malate shunt seemed to be stimulated. Regarding the total amount of IgG_3 produced, DO concentration had no significant effect on it, except at 10% O_2 saturation, where the IgG_3 produced was slightly lower.

Batch Culture: Effect of Different Initial Concentrations of Glucose and Glutamine. A series of batch experiments was carried out to determine the effect of different initial glucose and glutamine concentrations on cell growth, monoclonal antibody production, and metabolism. Cells were inoculated into 45 mL of medium in t-flasks to give an initial concentration of 1×10^5 viable cells mL^{-1}. Three different initial glucose concentrations (6.5, 12.5, and 25 mM) and three glutamine concentrations (2, 4, and 8 mM) were combined in nine experiments.

As shown in Table 2, it can be observed that the maximum number of viable cells obtained at the end of the exponential growth phase increased with the initial glucose concentration in the medium. However, the influence of glutamine seemed to be irrelevant regarding this aspect. The maximum growth rate did not change with different initial glucose and glutamine concentrations, and the same observation could be made with respect to the glucose and glutamine uptake rates (data not shown). These results are in agreement with those obtained by Miller et al. (1988) in hybridoma cell cultures. The total amount of antibody produced was higher for the highest glucose concentrations.

With respect to cell growth cessation, two different possible causes could be pointed out from the results obtained. Firstly, the most plausible cause for cell death in a good number of the experiments was the depletion of either glucose or glutamine. Secondly, in experiment I, where neither glucose nor glutamine was totally consumed, the cell death pattern was quite similar to the rest of the experiments. Consequently, the accumulation of a toxic product (i.e., lactate, ammonium) or the depletion of another key nutrient could be taking place.

In essence, these basic tests show that, although glucose and glutamine can become limiting when they are exhausted, not only is the analysis of cell growth limited by these two main nutrients, but it is more complex.

Growth Inhibition by Toxic Compounds (Fed-Batch Culture). One possibility to reduce waste products, as lactate and ammonium, and investigate their effect on the growth pattern of the KB-26.5 cell line is to maintain the concentration of glucose and glutamine at low levels during the culture by means of a controlled addition of them (Glacken et al., 1986). Four fed-batch experiments were performed in parallel in spinner flasks (125 mL) in order to reduce ammonia and lactate accumulation. Glucose and glutamine were added daily at different concentrations: 3 and 5 mM for glucose and 1 and 1.5 mM for glutamine. Results are shown in Table 3.

Addition of 1 mM glutamine was not enough to prevent its depletion from the medium (experiments C and D). In cultures A and B, neither glucose nor glutamine was exhausted throughout the experiments. In culture B, after 72 h, when the viability started to decrease, the highest concentrations of lactate and ammonium obtained were only 20.44 and 2.35 mM, respectively. The four experiments showed the same cell growth and antibody production curves with the peak cell density at the same point (72−96 h). The lactate or ammonium concentrations were very low compared to the previous series of batch experiments and lower than the levels found to be toxic in the literature for different cell lines. Therefore, the hypothesis of lactate and/or ammonium accumulation cannot explain the cell growth pattern in these experiments. Cell death in the presence of residual glucose and glutamine could be due to the depletion of other nutrients or the possible accumulation of an inhibitory or toxic compound, other than lactate or ammonium, in the culture medium.

Nutrient Limitation. The addition of amino acids, vitamins, and serum was studied to confirm that the factor preventing cells from further growth was a nutrient limitation in the culture medium. Five experiments were run in parallel in spinner flasks (125 mL), and results are shown in Figures 1−3. Cultures A and C were the control; in cultures B, D, and E, the medium was four times more concentrated in amino acids and vitamins with respect to DMEM concentrations, as

Table 2. Glucose, Glutamine, Lactate, and Ammonium Concentrations Corresponding to the Peak Cell Density of Experiments Using Different Initial Glucose and Glutamine Concentrations in the Medium, as Well as the Total Amount of IgG_3 Produced

culture	initial GLUC (mM)	initial GLN (mM)	GLUC (mM)	GLN (mM)	lactate produced (mM)	NH_4 produced (mM)	IgG_3 (μg mL^{-1})	max cell density ($\times 10^5$ viable cells mL^{-1})
A	6.5	2	0.3	0.18	11.33	2.19	6.05	4.7
D	6.5	4	0.2	1.07	11.44	2.97	4.13	4.9
G	6.5	8	0.2	2.90	11.44	3.59	4.02	4.9
B	12.5	2	0.4	0.00	22.66	2.00	8.42	7.2
E	12.5	4	1.5	0.90	21.55	2.71	9.88	8.1
H	12.5	8	0.9	3.08	19.88	4.45	8.04	7.5
C	25.0	2	13.1	0.00	22.00	2.10	9.14	7.3
F	25.0	4	8.4	0.30	30.22	2.96	18.52	9.2
I	25.0	8	11.5	2.04	32.00	5.10	13.88	8.1

Table 3. Glucose, Glutamine, Lactate, Ammonium, and IgG_3 Concentrations Corresponding to the Peak Cell Density of Experiments on Growth Inhibition by Lactate and Ammonia Accumulation

culture	initial GLUC (mM)	initial GLN (mM)	GLUC (mM)	GLN (mM)	lactate (mM)	NH_4 (mM)	IgG_3 (μg mL^{-1})	max cell density ($\times 10^5$ viable cells mL^{-1})
A	1.5	3	0.33	1.091	12.44	1.90	23.96	13.1
B	1.5	5	2.44	0.929	20.44	2.35	24.22	14.0
C	3.0	3	0.66	0.011	11.00	1.68	24.32	12.5
D	3.0	5	3.05	0.011	21.33	1.58	26.32	12.0
A	1.5	3	0.33	1.091	12.44	1.90	23.96	13.1

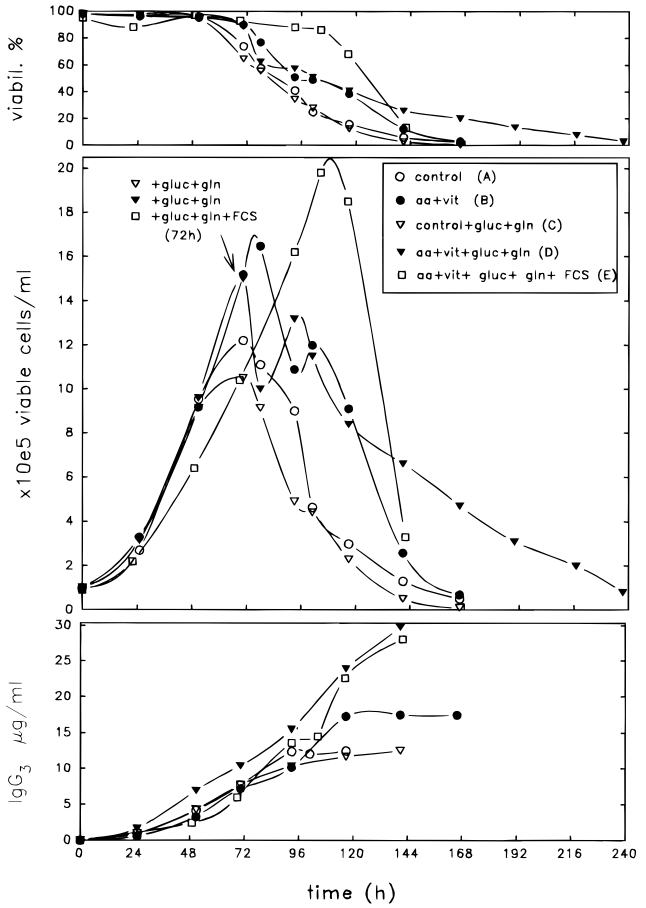

Figure 1. Effect of the addition of glucose, glutamine, amino acid, vitamins, and serum on cell growth and IgG3 production (spinner flasks, 125 mL).

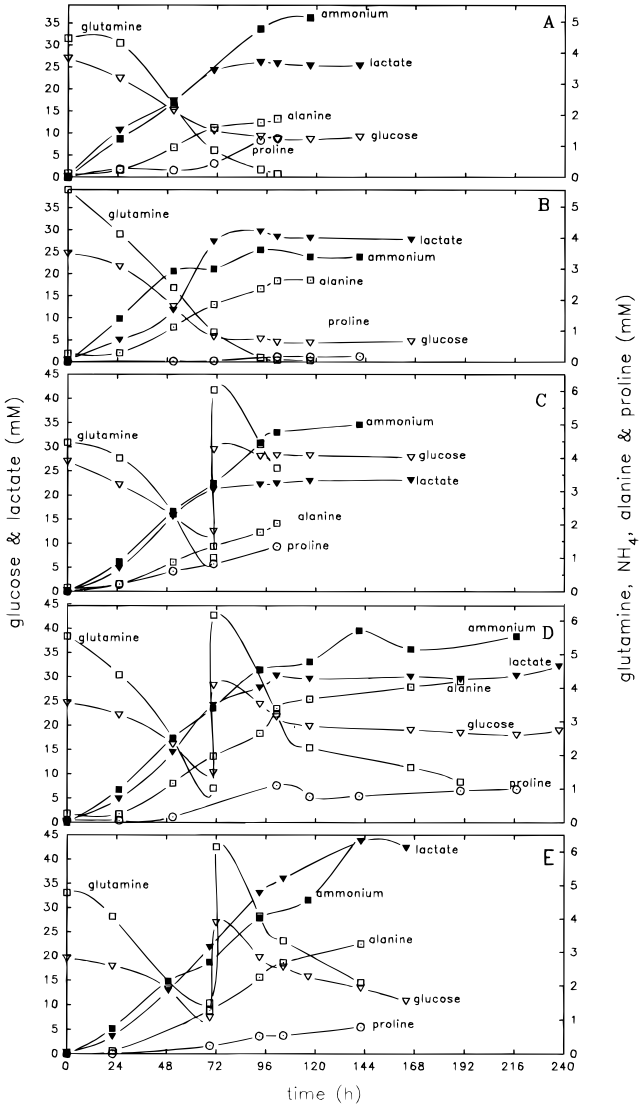

Figure 2. Time evolution of the concentration of glucose, glutamine, lactate, ammonium, alanine, and proline corresponding to the experiments in Figure 1.

specified in the Material and Methods. In cultures C, D, and E, glucose (20 mM) and glutamine (5 mM) were added at 72 h to prevent them from being totally depleted and 1% FCS was added at 72 h in culture E.

Effect of Glutamine and Glucose. The effect of the pulse addition of glucose and glutamine at 72 h on cell growth is only notorious when the medium contains enough amino acids and vitamins: it increases the life span of the culture, as can be seen by comparing the growth curves of the cultures B and D (Figure 1). This glutamine effect leading to a delay in cell death is in agreement with the results obtained by Geaugey et al. (1989). However, in control cultures (A and C), an addition of glucose and glutamine does not have any effect: both show the same growth profile, probably because they are limited by other essential amino acids and/or vitamins.

Effect of Amino Acids and Vitamins. In Figure 1, the comparison of the growth curves for the cultures B and D with those for cultures A and C leads to the conclusion that a medium four times more concentrated in amino acids and vitamins significantly enhances the life span of the culture as well as markedly increases the maximal cell density reached during the culture and the final monoclonal antibody concentration. The nutrient requirements of the cells have been satisfied by the supply of sufficient amino acids and vitamins, allowing maintenance of the cells for a longer time.

The evolution of the amino acid concentration at different stages in the experiments is presented in Figures 2 (only alanine and proline) and 3. It can be observed that none of the amino acids was totally depleted, with the exception of cysteine, which was soon totally consumed. Therefore, these experiments show that amino acids and/or vitamins can also limit cell growth before their complete depletion. In fact, it has been proved that a critical threshold level exists below

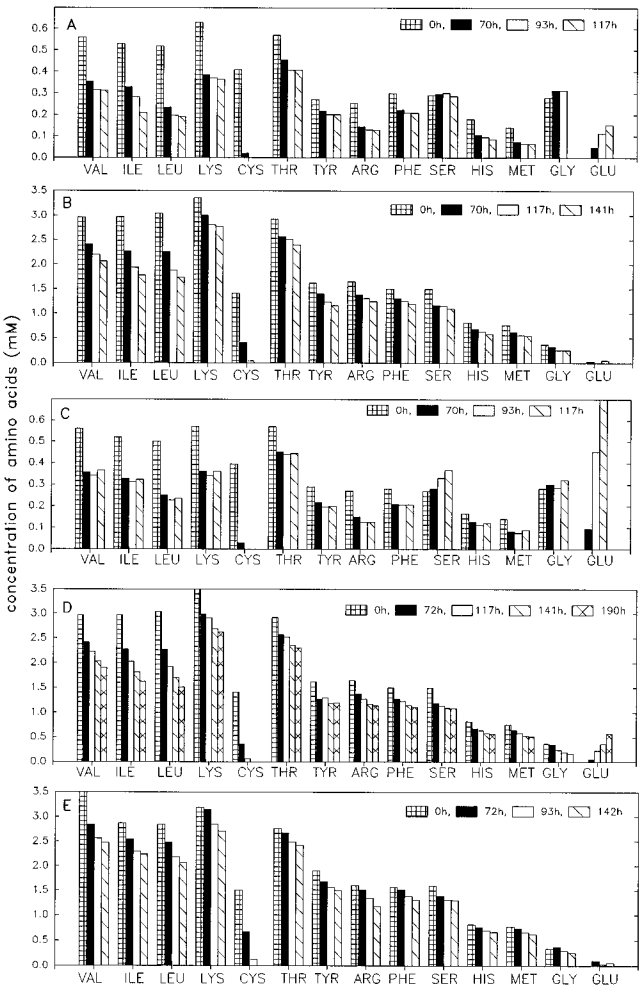

Figure 3. Amino acid profiles corresponding to the experiments in Figure 1.

which there is no appreciable protein synthesis and no demonstrable cellular division (Eagle and Levintow, 1969).

The profiles of the different amino acids could be divided in three groups:

(a) The most rapidly consumed are Val, Ile, Leu, Lys and Cys, Cys being the one which is totally consumed and at the highest rate. Cys is not an essential amino acid, as it is synthesized from Met in normal cells, but is very important for cell line KB-26.5. This requirement for Cys has also been found by other authors (Lambert and Pirt, 1975; Jo et al., 1990), and Duval et al. (1991) reported that its depletion irreversibly inhibited cell growth. Häggström (1993) described that the availability of Cys is very important for the uptake of some other amino acids. The depletion of Cys from the medium at the early stage of the culture, which can be observed in Figure 3, could be a possible cause of cell death. The rapid consumption of branched chain amino acids (Val, Ile, Leu, and Lys) has also been described by other authors using various cell lines and media (Lazo, 1981; Geaugey et al., 1989).

(b) The amino acids that are only slightly consumed or remain constant are Thr, Tyr, Arg, Phe, Ser, His, Met, and Gly. In contrast to the control cultures (A and C), cultures B and D showed higher consumption of Ser and Gly. Gly can be synthesized from Ser, and it is very important as a nucleotide precursor.

(c) The amino acids that are produced and released by the cell are Ala, Pro (Figure 2), and Glu. Ala is produced in large amounts and is formed from pyruvate and glutamic acid. Thus its production is related to the glucose and glutamine metabolism; approximately 42% of the nitrogen of the glutamine is converted to Ala in the five experiments of this series. In this way the cell decreases ammonium formation by transamination of glutamate to alanine.

Glu is formed from glutamine either by transamination or by direct deamidation. Its appearance in the medium does not take place until 72−96 h, once the cells start dying and stop consuming glutamine, and it is especially high in cultures C and D, where a pulse of glucose and glutamine has been added at 72 h. This means that the glutamic acid released by the cells comes from an intracellular excess which earlier on had been converted to nucleotides, proteines, or TCA intermediates. Pro is synthesized directly from excess intracellular Glu and appears also at the later stages of the culture. The production of Asp via transamination of glutamic with oxalacetate, which has been reported by other authors in hybridoma cultures (Moreadith and Lehninger, 1984), was negligible in this case. This can be an indication that the Asp produced by the cell is used rapidly in the protein, nucleotide synthesis and in the malate−aspartate shuttle for the transfer of reducing equivalents from the cytosol to the mitochondria as the mitochondrial membrane is not permeable to NADH (Kovacevic and McGivan, 1983).

Another fact that can be observed in Figure 3 is that, after cell peak concentration, in the cultures with the fortified medium (C and D), cells are still consuming amino acids, whereas in the control cultures, all metabolic routes are stopped, including consumption of amino acids, glucose, and glutamine.

Effect of Serum. By adding serum (1%) in culture *E*, cell growth was significantly enhanced, reaching 20 $\times$ 10^5 viable cells/mL in comparison to control cultures where maximum cell densities are (10−12) $\times$ 10^5 viable cells mL^{-1} (Figure 1). In culture E a high lactate concentration (40 mM) was produced (Figure 2). This lactate production overcame the buffering capacity of the medium ($NaHCO_3$), and as a consequence, the pH of the culture suddenly dropped, cell growth was then inhibited, and the culture presented a steep loss in viability, which was more pronounced than in the parallel experiments (Figure 1). Hybridoma cells are very sensitive to pH variation, and cells can maintain an intracellular pH at control values down to an external pH of approximately 6.5. Below that value, cytoplasmatic acidification takes place, leading to cellular quiescence or death (Musgrove et al., 1987).

In Figure 2, at approximately 72 h, the levels of lactate and ammonium were very similar in all cultures, being 20−25 mM for lactate and 3−3.5 M for ammonium. Whereas the number of viable cells starts to decrease in control cultures (A and C), in the cultures with fortified medium (B and D), the number of viable cells still increases to some extent and is maintained for a longer time, probably because the amino acids and/or vitamins may supply energy and intermediates for biosynthesis. When serum is added to the medium (E), viable cells still grow to higher concentrations, showing that there is a key component in the serum for the growth of KB-26.5 cell line. These results also corroborate the fact that the concentrations of lactate and ammonium mentioned above are not toxic for this cell line and are not the reason for the cells to start the death phase.

In this experiment cell growth and monoclonal antibody production have increased considerably. However, even in supplemented cultures, cell decline still occurred. To conclude this series of experiments, the two effects

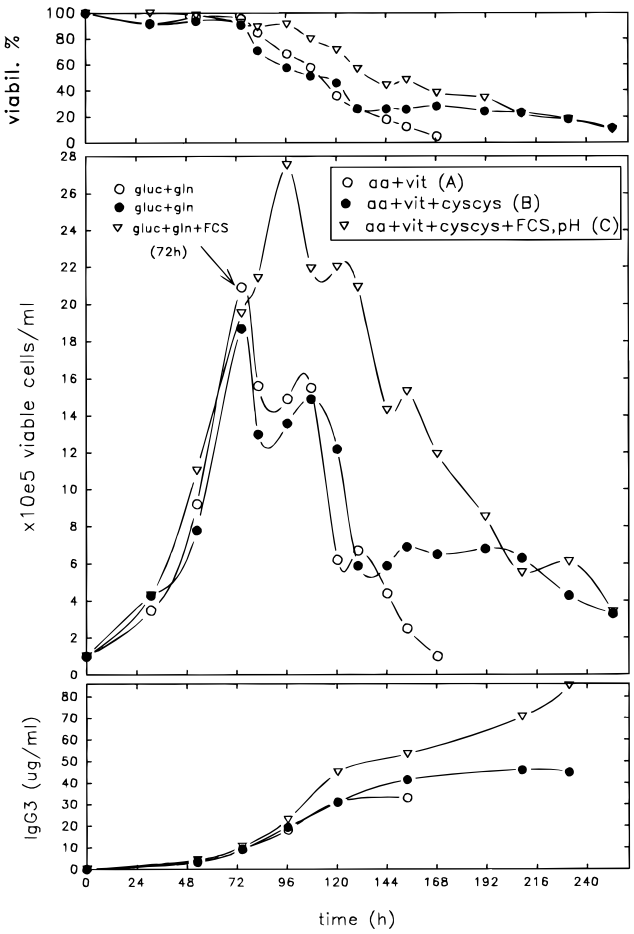

Figure 4. Effect of the higher initial cystine concentration and pulse addition of serum together with an additional control of pH by NaOH (0.2 mM) on cell growth and IgG3 production (spinner flasks, 125 mL).

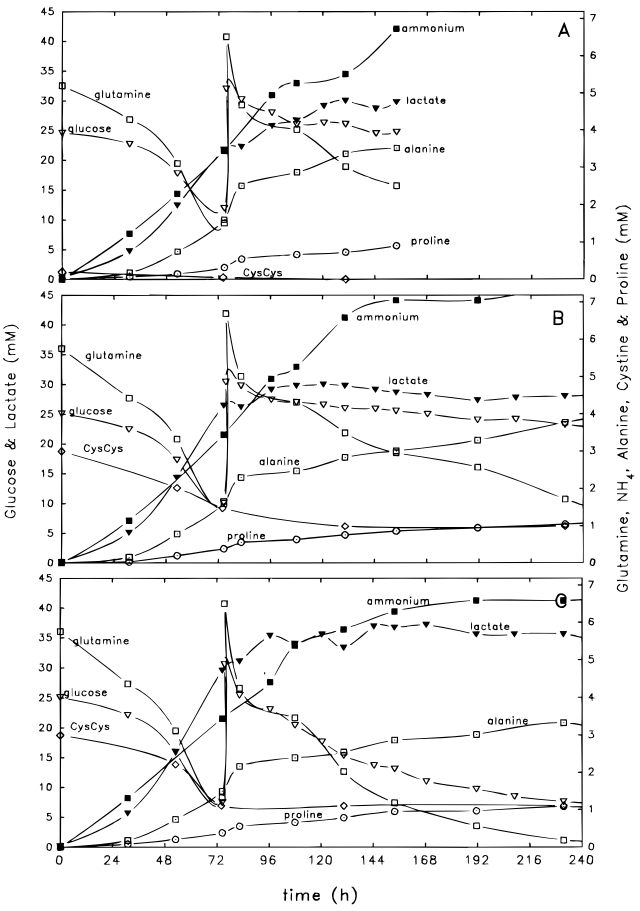

Figure 5. Time evolution of the glucose, glutamine, lactate, ammonium, alanine, cystine, and proline concentrations corresponding to the experiment in Figure 4.

that required a further investigation were the addition of cysteine and the regulation of the pH in the case of high accumulation of lactate. Therefore, a new series of experiments was carried out.

The medium was formulated in a way that an excess of all defined medium constituents (amino acids, vitamins) and serum were present and that the pH was maintained constant throughout the culture. Three spinner flask experiments were run in parallel: a culture with a high initial concentration of CysCys (cystine, dimeric form of cysteine) of 3 mM (culture *B*) and a culture with 3 mM CysCys plus a pulse addition of serum (1%) at 72 h were compared to a control culture (A). After 72 h, the three cultures were supplemented with glucose (20 mM) and glutamine (5 mM), and in culture C, pH was additionally controlled by NaOH (0.2 M). In this set of experiments, the control culture also contained four times more amino acids and vitamins, except for CysCys, which was kept at its normal level (0.2 mM). Results are shown in Figures 4 and 5.

In Figure 4, the effect of cysteine and serum can be observed. The pulse addition of serum clearly enhances the cell growth as long as pH is controlled. Cys was depleted from the medium at 72 h in culture A, whereas in the other experiments, there was a high residual Cys concentration (Figure 5). Cys has no effect in preventing the transition of the culture to the death phase, but it keeps cells viable for a longer time; it has an effect in delaying cell death. Looking at Figure 5, one can see that, around 72 h, the lactate and ammonium concentrations are very similar in all the cultures, being 25−30 mM for lactate and 3.5 mM for ammonium. Whereas in cultures A and B, the number of viable cells starts to decline, in culture C, viable cells continue to grow to higher concentrations with high viability and the culture shows a longer life span. In culture B, the final cell yield was not limited by exhaustion of any of the defined nutrients (glucose, gutamine, amino acids) or by an excess accumulation of lactate or ammonium; the cessation of growth before any defined nutrient in the medium was exhausted suggested that any nutritional limitation of the cell yield must be due to exhaustion of some unknown component of serum.

Regarding amino acid analysis, the same trends as in the last experiment were observed, that is to say, a high production of alanine and proline as well as a high consumption of the branched chain amino acids and cysteine. The profile concentrations for Ala, Pro, and CysCys are shown in Figure 5.

In culture C, a change in the metabolism seems evident (Figure 5). Cells stop producing lactate when NaOH is added and start consuming glucose for other purposes. Cells change from a fermentative state into a more aerobic one. Luan et al. (1987b) have also observed that high glucose concentration leads to extensive lactate formation only during growth phase but not during stationary phase, which suggests a correlation between growth rate and formation of lactate. However, they observed that an increase of lactate concentration occurs after pH regulation, leading to a second growth phase. Our results are different as we have observed that lactate formation stops after pH regulation.

Regarding monoclonal antibody production, the amount of IgG_3 achieved in these experiments was considerably

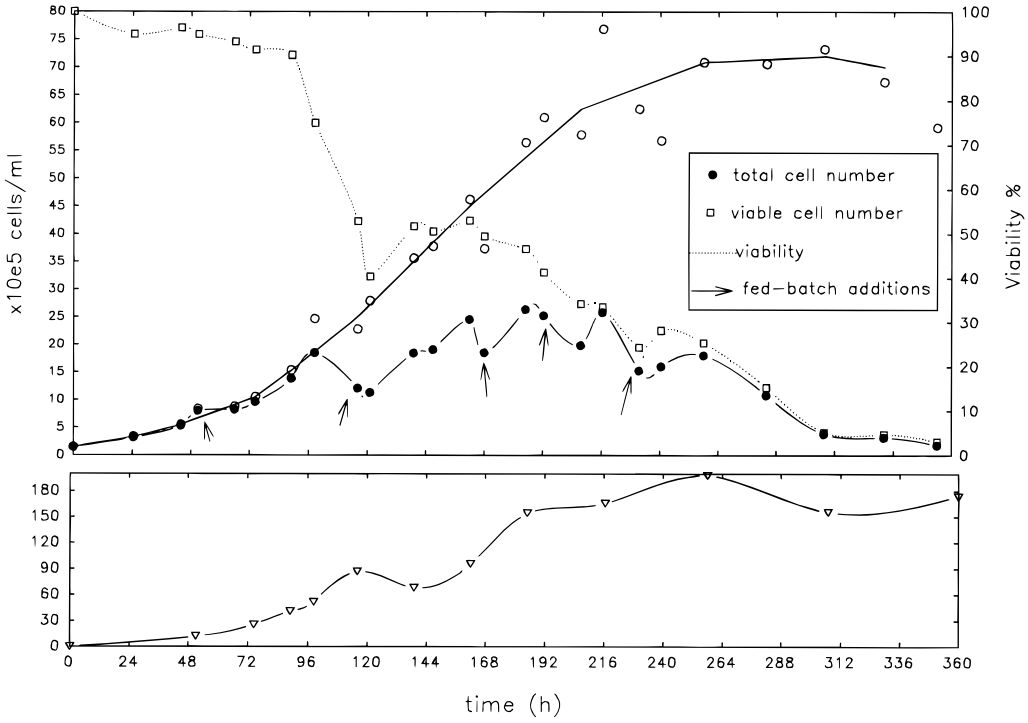

Figure 6. Fed-batch culture with addition of a solution containing amino acids, vitamins, glucose, glutamine, and FCS (0.5%).

increased (from 30 to 80 μg/mL) with respect to the previous experiments.

Combined Fed-Batch Addition. The results obtained in the previous experiments revealed the individual effect that different factors had on the cell culture, and some improvements were obtained with respect to the initial nonoptimized batch conditions. However, it is quite possible that when the medium is supplemented with one of the key compounds for the culture, that has been detected to become exhausted in previous experiments, then a new one can be the limiting compound. One strategy to enhance the culture could be to make fed-batch additions of a combination of these compounds previously identified. In order to investigate this possibility, a fed-batch experiment was designed with several additions of a solution containing amino acids, vitamins, glucose, glutamine, and FCS (0.5%). This was carried out in a fermenter (2 L) with 40% O_2 saturation. Also, after the initial phase of the cell growth, pH was additionally controlled by 0.2 M NaOH. The results are presented in Figure 6.

Compared to most of the initial experiments, where the number of living cells always suffered a sharp decrease around 96 h, in this case, the viable cell concentration was maintained for more than 250 h and the antibody produced reached almost 200 μg/mL, which represents more than a 10-fold increase with respect to the level usually obtained in most of the previous control experiments. It seems therefore evident that the different key nutrients identified in the previous experiments should be combined to obtain better results and that the effect of the simultaneous addition of the compounds is more positive than that of each individual compound.

Another aspect that could be observed in this experiment is that, although the number of living cells remained relatively constant, the number of dying cells significantly increased after the 96 h. Therefore, in spite of the enhancement obtained, this experiment also shows that there are still limiting factors in the medium, such as high osmolarities caused by the nutrient additions or accumulation of toxic compounds different than lactate and ammonium, and in consequence, the fed-batch culture of the KB-26.5 cell line could be further improved.

Conclusions

From the study of the effect of the different components of the medium, both individually or combined, it can be concluded that the addition of amino acids, vitamins, and serum stimulates cell growth, increasing the maximum cell density reached in the culture as well as prolonging the life of the culture. This effect is much more relevant when a combination of compounds is used to supplement the medium than when any of these compounds is used individually. As a consequence, the monoclonal antibody production is considerably improved. When medium formulation enables a very pronounced growth, the final products of the metabolism, lactate in particular, overcome the buffer capacity of the medium and an additional pH control is required. Furthermore, it has been shown that serum contains one or more unknown factors which enhance KB-26.5 growth and that cyst(e)ine is particularly required at higher concentrations in the medium. It has also been demonstrated that lactate and ammonium do not appear to inhibit growth for the KB-26.5 cell line, as long as pH is controlled.

Summarizing, the development of optimized cultures for mammalian cell growth and protein production requires the combination of a number of factors. Although in some cases these factors are well-known and have been described, they need to be investigated comprehensively in order to achieve improvements in the culture output. A balance has to be reached between the supplementation of the necessary metabolites and the accumulation of toxic compounds. The control of physical conditions such as O_2 and pH levels is also very important. The results obtained in this work showed that these effects are not independent, and the best results are obtained for combined fed-batch addition of key nutrients with appropiate regulation of the physical parameters. However, the hypothesis of some toxic compound accumulation other than lactate and ammonium throughout the culture is one possibility that

requires further research. Furthermore, the need for the implementation of on-line measurements for the key nutrients or products in this type of culture is very important for its further development.

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