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The role of vitamins and amino acids on hybridoma growth and monoclonal antibody production

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Abstract

A balanced supplementation method was applied to develop a serum and protein- free medium supporting hybridoma cell batch culture. The aim was to improve systematically the initial formulation of the medium to prevent limitations due to unbalanced concentrations of vitamins and amino acids. In a first step, supplementation of the basal formulation with 13 amino acids, led to an increase of the specific IgA production rate from 0.60 to 1.07 pg cell^{-1} h^{-1}. The specific growth rate remained unchanged, but the supplementation enabled maintenance of high cell viability during the stationary phase of batch cultures for some 70 h. Since IgA production was not growth- related, this resulted in an approximately 4-fold increase in the final IgA concentration, from 26.6 to 100.2 mg l^{-1}. In a second step, the liposoluble vitamins E and K_3 were added to the medium formulation. Although this induced a slightly higher maximal cell concentration, it was followed by a sharp decline phase with the specific IgA production rate falling to 0.47 pg cell^{-1} h^{-1}. However, by applying a second cycle of balanced supplementation with amino acids this decline phase could be reduced and a high cell viability maintained for over 300 h of culture. In this vitamin- and amino acid- supplemented medium, the specific IgA production rate reached a value of 1.10 pg cell^{-1}h^{-1} with a final IgA concentration of 129.8 mg l^{-1}. The latter represents an increase of approximately 5-fold compared to the non- supplemented basal medium.

Introduction

Serum- free media have been developed as early as in the 1960s and 1970s (Evans et al. 1964; Higushi 1973) and are now widely used to overcome the problems associated with serum- containing media (Leist et al. 1990; Chen et al. 1993). The formulations typically include a basal medium and supplements containing additional vitamins, growth factors and other trace elements. However, cell growth and metabolism may be significantly different in serum-containing and serum- free media (Chua et al. 1994) with precise nutrient requirements specific for each cell line. Therefore there is considerable room for improving the basal formulation of most serum- free media, in order to avoid limitations and to achieve higher cell growth and productivity. Despite the very large number of supplements that are used in serum-free media to replace some functions of serum (Barnes and Sato 1980; Shacter 1989; Hewlett 1991; Barman and Rajput 1993), few successful systematic methods for supplementation and optimization of the concentrations of these supplements have been proposed. Among them, a model based on the stoichiometric nutritional demands for animal cell growth has been developed (Xie and Wang 1994) to avoid toxic waste accumulation in the culture. Stoll et al. (1996) reported that a balanced supplementation strategy was necessary to prevent toxicity of some amino acids at high concentration. In this study, a systematic method was proposed for the development and optimization of a serum- and protein- free medium, which led to

higher cell and product concentrations in hybridoma cell cultures. Other authors have stressed the importance of the initial composition of amino acids in the fresh medium, due to competition for common uptake systems by the cells (Marquis et al. 1996a). These authors also report an increase in the specific productivity of a murine hybridoma cell line, during the decline phase of a batch culture, through the enrichment of the medium with amino acids (Marquis et al. 1996b). The importance of balanced supplementation has been stressed by a number of authors, especially for amino acids (Zetterberg and Engström 1981; Engström and Zetterberg 1984; Geaugey et al. 1989; Jo et al. 1990), and toxicity due to an excess of certain vitamins (Baker et al. 1988).

This article describes the effect of a systematic supplementation of a serum- and protein- free medium, with vitamins and amino acids, on hybridoma growth and monoclonal antibody production. The specific growth rate of the cells, the maximal cell concentration, the final product concentration and specific production rate were used as criteria to compare the different media formulations.

Materials and methods

Cell line

Zac3, a murine hybridoma cell line made by fusion of mouse myeloma P3X63/AgU.1 with a lymphocyte from Peyer's patches of a BALB/c mouse, was used

Table 1. Partial formulation of FMX-Turbodoma- derived media FMX-1, FMX-3, FMX-5 and FMX-6 (concentrations in mM).

Components	FMX-1	FMX-3	FMX-5	FMX-6
D-glucose	25.0	25.0	25.0	25.0
Amino acids				
L-alanine	0.11	0.11	0.11	0.11
L-arginine	1.00	1.10	1.10	1.10
L-asparagine	0.10	0.12	0.12	0.12
L-aspartic acid	0.11	0.18	0.18	0.18
L-citruline	0.03	0.03	0.03	0.03
L-cysteine	0.19	0.45	0.45	0.45
L-glutamine	5.00	5.00	5.00	7.62
L-glutamate	0.10	0.10	0.10	0.10
L-glycine	0.13	0.13	0.13	0.13
L-histidine	0.33	0.33	0.33	0.33
L-isoleucine	1.22	1.60	1.60	1.60
L-leucine	0.99	1.50	1.50	1.50
L-lysine	0.60	0.60	0.60	0.60
L-methionine	0.10	0.22	0.27	0.27
L-ornithine	0.06	0.06	0.06	0.06
L-phenylalanine	0.33	0.40	0.40	0.40
L-proline	0.30	0.30	0.30	0.30
L-serine	0.48	0.80	0.80	1.05
L-threonine	0.13	0.40	0.54	0.54
L-tryptophan	0.02	0.06	0.07	0.07
L-tyrosine	0.11	0.25	0.25	0.25
L-valine	0.77	1.00	1.00	1.00
Vitamins				
D-biotin	0.0004	0.0004	0.0008	0.0008
Ca-pantothenate	0.011	0.011	0.022	0.022
choline chloride	0.11	0.11	0.22	0.22
cyanocobalamine	0.0005	0.0005	0.0010	0.0010
folic acid	0.0029	0.0029	0.0058	0.0058
myo-inositol	0.01	0.01	0.02	0.02
niacinamide	0.016	0.016	0.032	0.032
pyridoxine HCl	0.0097	0.0097	0.0194	0.0194
riboflavine	0.0005	0.0005	0.0010	0.0010
thiamine HCl	0.0074	0.0074	0.0074	0.0074
menadione sodium bisulfite	0	0	0.0018	0.0018
D-alpha-tocopherol acetate	0	0	0.0011	0.0011

in this study. This cell line produces an IgA monoclonal antibody of allotype 2, directed against the lipopolysaccharide of the envelope of *Vibrio cholerae*.

Media and additives

Cells were grown in the serum- and protein- free medium FMX-Turbodoma (Dr. F. Messi, Cell Culture Technologies, Zürich, Switzerland). This commercial medium is referred to as FMX-1 in this study. Modifications of FMX-1, referred to as FMX-3, FMX-5 and FMX-6, were used where stated. The supplements, which were added to the basal medium FMX-1, are reported in Table 1.

Four liposoluble vitamins have been tested: retinol acetate (A) (Sigma R-7011), ergocalciferol (D_2) (Sigma E-9007), D-alpha-tocopherol acetate (E) (Sigma-T-1157) and menadione sodium bisulfite (K_3) (Sigma M-2518). The concentration of the following water soluble vitamins was also modified: D-biotin (Sigma B-4639), Ca-pantothenate (Sigma P-5155), choline chloride (Sigma C-7527), cyanocobalamine (Sigma V-6629), folic acid (Sigma F-8758), myo-inositol (Sigma I-7508), niacinamide (Sigma N-0636), pyridoxine HCl (Sigma P-6280), riboflavine (Sigma R-9504) and thiamine HCl (Sigma T-1270).

Cell culture

Cell cultures was performed in 25-cm^2 T-flasks, incubated at 37 °C in a humidified incubator under a 5% CO_2/95% air atmosphere. Cells were adapted for two weeks to any new medium before undertaking growth experiments. For each growth curve, 20 identical T-flasks were prepared, each containing 6 ml of fresh medium and a cell inoculum concentration equal to 5.0×10^4 cells ml^{-1}. Three times a day a T-flask was removed from the incubator and cells counted microscopically. Samples from the flasks were then centrifuged and the supernatant stored at −20 °C in small aliquots (500 μl) for further analysis.

For the screening of the many vitamin concentrations and combinations, this procedure was reduced to enumeration of cells only once, after 3 days of culture.

Analyses

Cells were counted microscopically using a Neubauer improved haemocytometer. Cell concentration and viability were determined using the Trypan blue exclusion method (0.4%, Sigma T-8154).

Standard enzyme assays were used to quantify glucose (Boehringer Mannheim 716 251), lactate (Sigma 826-10) and ammonia (Boehringer Mannheim 1112 732) concentrations.

Amino acids were quantified by HPLC analysis following pre-derivatisation with orthophtaldialdehyde (OPA). For this purpose, a HPLC system (450-MT2, Kontron Instruments, Zürich, Switzerland) fitted with a UV detector and a Superspher RP18 EC column (15 × 0.4 cm, 4 μm, Tracer Analitica, Barcelona, Spain) were used. OPA reagent was prepared in borate solution (H_3BO_3 0.05 M; KCl 0.05 M; NaOH 0.05 M) at a concentration of 1 g l^{-1} and supplemented with 1 ml l^{-1} of ethanethiol. The mobile phases were: buffer A: 96% Na_2HPO_4 9 mM, pH 7.2 containing 4% tetrahydrofuran; buffer B: 40% Na_2HPO_4 9 mM, pH 7.2 containing 36% methanol and 24% acetonitrile. All samples were first deproteinized by the addition of methanol.

Water- soluble vitamins were quantified by HPLC analysis using a ET Nucleosil 5C18 column (4 × 125 mM, 5 μm, Macherey-Nagel, Düren, Germany). The mobile phases were; buffer A: NaH_2PO_4 5mM; buffer B: 50% NaH_2PO_4 5mM containing 50% methanol.

IgA concentration was quantified using a sandwich ELISA, involving an antimouse IgA α-chain specific (Sigma M-7144) as coating, a biotinylated anti-mouse IgA α- chain specific (Amersham RPN 1175), a streptavidin-horseradish peroxidase conjugate (Amersham RPN 1231) and o-phenyl-diamine (Sigma M-2895) as substrate. A purified mouse IgA, κ- chain (Sigma M-2895) was used as standard.

The molecular weight pattern of the produced antibody was determined by SDS-PAGE. A gradient gel (4–12% acrylamide) was prepared in a Mini-PRO-TEAN II cell (Bio-Rad). Biotinylated molecular weight standards (Bio-Rad, No 161-0319) were used. Western blotting was performed and band revelation made with the same reagents that were used in the ELISA method, except for the last step, which was replaced by a treatment with Luminol (Amersham, RPN 2106). Autoradiogram paper (X-OMAT AR, Kodak) was used for final documentation.

Results and discussion

Culture in FMX-1 and FMX-3

Results of *Zac3* hybridoma cell cultures in the basal

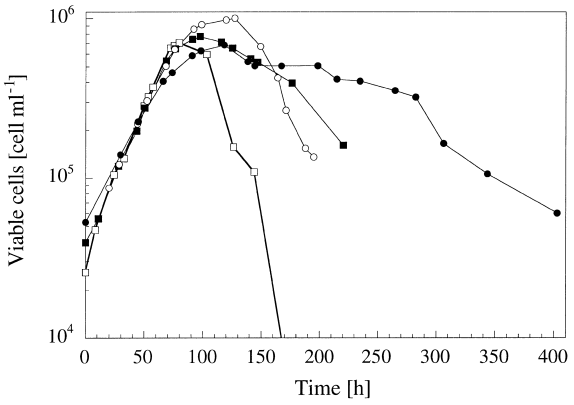

Figure 1. Variation of the viable cell concentration during batch cultures of *Zac3* hybridoma cells in media FMX-1 (□), FMX-3 (■), FMX-5 (○) and FMX-6 (●).

medium FMX-1, and in the supplemented media FMX-3, FMX-5 and FMX-6, are presented in Figure 1.

During the exponential growth phase in FMX-1, the cells grow with a specific growth rate (μ) of 0.039 h^{-1} and reach a maximal viable cell concentration ($X_{v,max}$) of 7.0×10^5 cell ml^{-1}(Table 2). The exponential growth phase is then immediately followed by a sharp decline phase, and viability falls from 83% to 16% in less than 24 h. This effect was shown to be due to an unbalanced formulation of the medium in terms of amino acid concentration, which led to limitation of the culture by amino acids (Stoll et al. 1996). A rapid decline in viability has also been reported in the literature for deprivation of any single amino acid in hybridoma culture (Simpson et al. 1998). As a result, a balanced supplementation method, to systematically improve the culture medium, has been proposed (Stoll et al. 1996). In this method amino acids that should be added to the initial medium are identified from HPLC analysis, using the following criterion:

$$C_{f,i}^{FMX-1} \leq C_{0,i}^{FMX-1} \quad (1)$$

where $C_{f,i}^{FMX-1}$ represents the concentration of the i^{th} amino acid in the basal medium FMX-1 at the end of the growth phase, and $C_{0,i}^{FMX-1}$ the initial concentration. The proportionality coefficient α was arbitrarily given the value of 0.6. Thus, amino acids whose concentration at the end of the growth phase is decreased by 40% or more satisfy Inequality (1) and are potentially limiting and have to be supplemented. A balanced supplementation has been proposed (Stoll et al. 1996) in order to avoid possible toxic effects due to excessive concentrations of individual amino acids:

$$C_{0,i}^{new} = C_{0,i}^{FMX-1} + (\beta C_{0,i}^{FMX-1} - C_{f,i}^{FMX-1}) \quad (2)$$

where $C_{0,i}^{new}$ represents the concentration of the i^{th} amino acid in the new medium formulation. The proportionality coefficient β was given the value of 0.7 (β was chosen to be 15% higher than α, since supplementation of the medium is expected to increase cell concentration and thus amino acid consumption).

This procedure was repeated until no amino acid satisfied Inequality (1), as shown in Figure 2, and led to a new formulation of the medium: FMX-3 (Table 1). *Zac3* hybridoma cells were grown in this supplemented medium and results are shown in Figure 1. The exponential growth phase in FMX-3 is very similar to the one in the basal medium FMX-1. The cells grew with the same specific growth rate (0.039 h^{-1}), and attained a maximal viable cell concentration only 10% higher (7.7×10^5 cell ml^{-1}) than in FMX-1. However, the sharp decline phase that is observed with FMX-1, after the end of the exponential growth phase, does not take place in the culture with FMX-3. Instead, a high level of cell viability could be maintained for over 70 h. This increase in cell viability induces a significant improvement of the production parameters. As shown in Figure 3 and Table 2, the final IgA concentration ($c_{f,IgA}$) in FMX-3 reached

Table 2. Growth and production parameters of *Zac3* cells in the different FMX-Turbodoma- derived media FMX-1, FMX-3, FMX-5 and FMX-6.

Parameters	FMX-1	FMX-3	FMX-5	FMX-6
μ [h^{-1}]	0.039	0.039	0.036	0.031
$X_{v,max}$ [cell ml^{-1}]	7.0×10^5	7.7×10^5	9.8×10^5	6.8×10^5
$c_{f,IgA}$ [mg l^{-1}]	26.6	100.2	43.6	129.8
q_{IgA} [pg $cell^{-1}h^{-1}$]	0.60	1.07	0.47	1.10

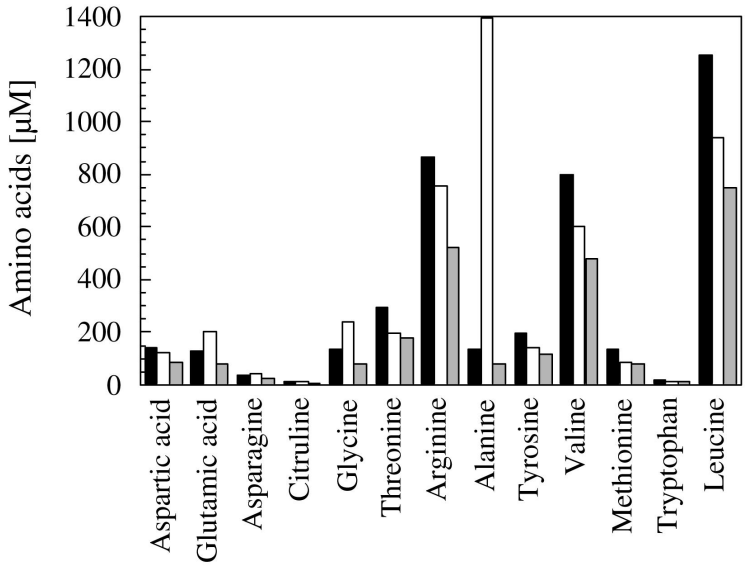

Figure 2. Analysis of amino acids in medium FMX-3: initial concentration $C_{0,i}^{FMX-3}$ (black columns), final concentration $C_{f,i}^{FMX-3}$ (white columns) and critical concentration α $C_{0,i}^{FMX-3}$(gray columns).

100.2 mg l^{-1}, instead of 26.6 mg l^{-1} in FMX-1, which represents an approximate 4-fold increase in titre. In the case of FMX-3, IgA production does not stop after $X_{v,max}$ has been reached, indicating that antibody production is not strictly growth- related for *Zac3* cells.

In batch culture, the specific IgA production rate (q_{IgA}) is determined from the production rate (R_{IgA}) divided by the cell concentration:

$$q_{IgA}=\frac{R_{IgA}}{X_v}=\frac{1}{X_v}\frac{dc_{IgA}}{dt} \quad (3)$$

q_{IgA} may then be calculated by the integral method

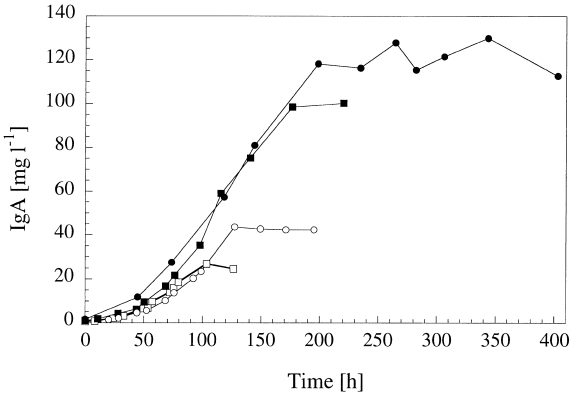

Figure 3. Variation of IgA concentration during batch cultures of *Zac3* hybridoma cells in media FMX-1 (□), FMX-3 (■), FMX-5 (○) and FMX-6 (●).

(Ducommun et al. 2001), and Equation 3 reformulated as follows:

$$c_{IgA}=q_{IgA}\int_0^t X_v dt \quad (4)$$

Figure 4 illustrates the q_{IgA} determination for each culture. It is obtained from the slope of a plot of IgA concentration against the integral term $\int X_v dt$ as a function of time. In FMX-3, q_{IgA} has a constant value of 1.07 pg $cell^{-1}$ h^{-1} throughout the culture. This represents a 79% increase compared to FMX-1 (Table 2). As in Figure 3, results show that IgA production is not growth- related but is proportional to the integral term. This fact has also observed in the literature (Reuveny et al. 1986; Renard et al. 1988).

Hence, the balanced supplementation of amino acids in FMX-3 increases q_{IgA} and improves the cell maintenance, preventing the sharp decline phase that is observed with FMX-1. This leads to a final IgA concentration increase of approximately 4-fold. However, it does not improve cell growth, which is still limited by other factors.

Supplementation with water- and liposoluble vitamins

The possibility of growth limitation due to vitamin depletion has been suggested in the literature (Kurano et al. 1990) and step changes of vitamin concentration

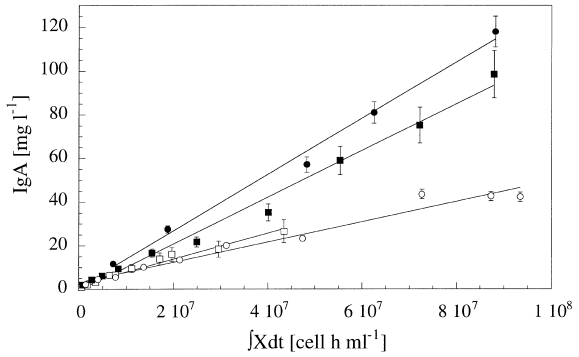

Figure 4. Determination of the specific IgA production rate for batch cultures of *Zac3* hybridoma cells in media FMX-1 (□), FMX-3 (■), FMX-5 (○) and FMX-6 (●).

Linear correlations:

FMX-1: $y = 1.929 + 0.597 \times 10^{-6} \times$, coefficient of correlation: 0.987

FMX-3: $y = -0.686 + 1.071 \times 10^{-6} \times$, coefficient of correlation: 0.995

FMX-5: $y = 3.116 + 0.465 \times 10^{-6} \times$, coefficient of correlation: 0.984

FMX-6: $y = 1.028 + 1.290 \times 10^{-6} \times$, coefficient of correlation: 0.996

have been reported to have a positive effect on cell growth and viability (Hiller et al. 1994). In order to test this the concentration of five water- soluble vitamins was quantitatively determined by HPLC analysis throughout cultures in FMX-1 and FMX-3. At the end of the exponential growth phase, the concentration of pyridoxine, folic acid, cyanocobalamine and riboflavine is still higher than the criterion defined in Equation 1. However, the concentration of niacinamide decreased by 54% in FMX-1 and by 52% in FMX-3, thus satisfying Inequality (1), and should be supplemented according to Equation 2. No degradation of the five water- soluble vitamins was detected in non-inoculated medium after 19 days incubation at 37 °C under the same conditions as the cell cultures. Thus the measured decrease in vitamin concentration during the cultures is not due to chemical degradation, but due to cell consumption. However, since five of the water- soluble vitamins, out of the ten that are present in FMX-1 and FMX-3, could not be quantified by the HPLC method, supplementation with niacinamide alone may not be sufficient to avoid any vitamin limitation. Therefore, it was decided to double the concentration of all 10 water-soluble vitamins present in the basal medium: D-biotin, Ca-pantothenate, choline chloride, cyanocobalamine, folic acid, myo-inositol, niacinamide, pyridoxine HCl, riboflavine and thiamine HCl. A batch culture carried out under these conditions exhibited growth rates, maximum cell density and production parameters identical to those obtained in FMX-3 (data not shown). Hence, this new formulation of water- soluble vitamins induced no toxic effect, while ensuring no limitation by niacinamide (66% of initial concentration remaining). However, supplementation with water- soluble vitamins alone did not improve either cell growth or IgA production.

In the next stage liposoluble vitamins were considered since, although the role in cell metabolism and maintenance is important, they are not present in the basal medium formulation. Four liposoluble vitamins were chosen, based on literature results showing positive effects on cell culture (Kurano et al. 1990): retinol acetate (vitamin A), ergocalciferol (vitamin D_2), D-alpha-tocopherol acetate (vitamin E) and menadione sodium bisulfite (vitamin K_3). First, screening experiments were performed in order to determine the effect on cell growth of all 4 vitamins, either alone or in combination. The screening was performed in duplicate and for two different concentrations of vitamin: 5.0 and 0.5 mg l^{-1}. The liposoluble vitamins were added, as soluble solutions in 100 μl ml^{-1} of ethanol and cell cultures performed in T-flasks. The cell concentration was determined after 10 days of culture and compared to one from a culture to which no vitamins had been added but contained 100 μl ml^{-1} of ethanol. A positive or negative effect was considered when a variation in cell density of more than 20% was determined, compared to the culture to which only ethanol was added. The results (Table 3) show a negative effect for all combinations containing 5 mg l^{-1} of vitamin K_3. Indeed complete inhibition of cell growth was observed indicating that vitamin K_3 is toxic at this concentration. This is in contrast to the results of Xie and Wang (1994), who report that vitamins have no toxic effect even at high concentration. However, vitamin K_3 was shown to have a positive effect on cell concentration at reduced levels (0.5 mg l^{-1}) as shown in Table 3. At this level, combinations of vitamins E and E + K_3 resulted in increased maximal cell numbers compared to the reference culture. Other combinations of vitamins, containing either E or K_3, resulted in maximal cell concentrations just below the threshold limit of 20% and were therefore considered to have no effect. Hence, only the two vitamins E and K_3 were added to the medium formulation at a concentration of 0.5 mg l^{-1}. The resulting medium (Table 1), containing supplementation with both water- and liposoluble vitamins was termed FMX-5.

Table 3. Effect of the four liposolubles vitamins retinol acetate (A), ergocalciferol (D_2), D-alpha-tocopherol acetate (E) and menadione sodium bisulfite (K_3) on the concentration of *Zac3* cells growing in FMX-3.

Combinations	5 [mg l^{-1}][a]	0.5 [mg l^{-1}][a]
A	0	0
D_2	0	0
E	0	+
K^3	−	+
A+D_2	0	0
A+E	0	0
A+K_3	−	0
D_2+E	0	0
D_2+K_3	−	0
E+K_3	−	+
A+D_2+E	0	0
A+D_2+K_3	−	0
A+E+K_3	−	0
D_2+E+K_3	−	0
A+D_2+E+K_3	−	0

[a] Each combination was tested with concentrations of 5 and 0.5 mg l^{-1}. A positive (+) or negative (−) effect is considered where there is a variation of more than 20% compared to the blank assay. Variations smaller than 20% are considered as not significant (0).

Hybridoma cells (*Zac3*) were subsequently grown in the vitamin- supplemented medium FMX-5 and results are shown in Figure 1. In this medium, the specific growth rate of the cells during the exponential growth phase (Table 2) is very similar to those determined in FMX-1 and FMX-3 (0.036 h^{-1}), although the maximal cell concentration (9.8×10^5 cell ml^{-1}) was between 27–40% higher. However, the exponential growth phase is followed by an immediate sharp decrease in cell viability, similar to that observed in FMX-1. Hence, the high level of cell viability that could be maintained in FMX-3, through a balanced supplementation with amino acids, is not observed when the same medium is further supplemented with vitamins (FMX-5). Moreover, the specific antibody production rate (q_{IgA}) in FMX-5 and FMX-1 are similar (0.47 pg $cell^{-1}$ h^{-1}) while the final IgA concentration obtained in FMX-5 (43.6 mg l^{-1}) is 56% lower than that obtained in FMX-3, despite a higher maximal cell concentration (Table 2).

The reduced cell viability observed in FMX-5 is neither due to glucose depletion, since more than 50% of the initial concentration remains at the end of the batch culture, nor water- soluble vitamins deficiency, since the final concentrations do not satisfy Inequality (1). However, amino acid analysis indicates that only 15% of the initial concentration of serine and glutamine remain at the end of the exponential growth phase. Thus, as in the case of the basal medium FMX-1, FMX-5 appears to be limited by the depletion of certain amino acids, whose concentration at the end of the growth phase satisfies Inequality (1).

To summarize, supplementation of media with water- and liposoluble vitamins (FMX-5) enabled the cells to overcome the limitation observed during growth in FMX-1 and FMX-3 and to achieve a higher maximal viable cell concentration. However, this increased growth led to a consumption of serine and glutamine to limiting levels, resulting in a sharp decline phase, similar to that observed with FMX-1. Thus it is once again necessary to make a balanced supplementation of amino acids to improve cell maintenance and viability.

Supplementation with amino acids

The amino acids serine and glutamine, the concentrations of which satisfy Inequation (1) at the end of the growth curve, were added to FMX-5 in order to reproduce the stable phase observed in cultures with FMX-3. In this medium (FMX-6; Table 1) *Zac3* cells grew to a maximal cell concentration of only 6.8×10^5 cell m l^{-1}, at a specific growth rate of 0.031 h^{-1}. These values, surprisingly, are the lowest of all those presented in Table 2. However, a high level of cell viability is maintained during over 300 h in FMX-6, whereas viability is only 16% after only 220 h with the other media formulations. The influence of glutamine on the prolongation of cell viability has similarly been reported elsewhere (Geaugey et al. 1989). However, the specific IgA production rate obtained in FMX-6 (1.290 pg $cell^{-1}h^{-1}$; Figure 4) is similar to that observed with the amino acid- supplemented medium FMX-3. Such variations of specific antibody production rate due to a higher concentration of glutamine are in agreement with the results of Banik and Heath (1996), although are in contradiction with other published results which suggest that the supply of glutamine and other amino acids does not increase the specific antibody production rate (Duval et al. 1991). Thus the final IgA concentration in FMX-6 reached a value of 129.8 mg l^{-1}, which is the highest of all media tested in this study and represents an increase of approximately 5-fold compared to the value obtained in the basal medium FMX-1 (26.6 mg l^{-1}).

Finally, the molecular weight pattern of the IgA produced with media FMX-1, FMX-3, FMX-5 and FMX-6 was determined by SDS-PAGE. Results were

similar and the apparent molecular weight of the IgA was situated between the bands of the standard at 97.4 and 116 kDa. Hence, the product appeared to be identical in all media formulations. However, since these experiments were all performed in small-scale, static cell culture flasks, the feasibility of using FMX-6 medium would have to be confirmed in stirred bioreactor systems for further process development.

In summary, a balanced supplementation of amino acids led to the formulation of FMX-3 and FMX-6 and enabled maintenance of high cell viability once growth had ceased, thus preventing the sharp decline phase observed with FMX-1 and FMX-5. The final IgA concentration in FMX-3 and FMX-6 reached much higher values since IgA production is not growth- related but proportional to the integral term in Equation 4. However, the balanced supplementation of amino acids directly affects the metabolism of *Zac3* hybridoma cells. The lactate/glucose yield is lowered from 1.4 (FMX-1 and FMX-5) to 1.0 (FMX-3 and FMX-6) and the specific IgA production rate reaches much higher values, as shown in Table 2. The specific glucose and glutamine consumption rates are also lowered by the balanced supplementation. Thus, the specific glucose consumption rate has a value of 676 fmol $cell^{-1}$ h^{-1} during the growth phase in FMX-1 compared with 153 fmol $cell^{-1}h^{-1}$ in FMX-6. The specific glutamine consumption rate is lowered from 183 fmol $cell^{-1}$ h^{-1} in FMX-1 to 119 fmol $cell^{-1}$ h^{-1} in FMX-6. Hence, the balanced supplementation method allows cells to metabolize directly the amino acids that are present in adequate quantity and thereby reducing the energy expenditure associated with *de novo* synthesis.

Finally, supplementation with water- and liposoluble vitamins alone (FMX-5) did not enhance monoclonal antibody production directly. However, when the balanced supplementation of amino acids was combined with vitamin addition (FMX-6) the result was an important prolongation of the stationary phase as well as in an increase of the specific IgA production rate, compared to FMX-3 that was only supplemented with amino acids. Hence, the combined supplementation of both vitamins and amino acids resulted in the highest IgA concentration.

Conclusions

The results clearly show the importance of balanced supplementation methods in the formulation of cell culture media. In particular the maximum cell density, growth rate, antibody production rate and concentration may be manipulated by applying suitable medium formulations. The importance of amino acids has been clearly identified, with medium supplementation resulting in improved maintenance and viability, as well as leading to higher specific IgA production rates.

Moreover, cultures performed with various supplemented media show that not only the absolute concentration of each vitamin and amino acid is important, to prevent limitation or inhibitory effects of individual components, but also – and in particular – the ratio between these concentrations. For example, the addition of vitamins in the medium FMX-3 induces first a significant decrease in IgA productivity, until the concentration of amino acids was adjusted, by application of the balanced supplementation method, leading to the highest production level of monoclonal antibody for the hybridoma cell line employed. While this work has been undertaken with a single hybridoma cell line, and the results may be specific for this line, the general concept of the need for quantitative analysis of metabolite consumption/production and the relationship between these and growth and productivity, are almost certainly generally applicable.

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