Hybridoma cell behaviour in continuous culture under hyperosmotic stress

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Received 14 January 1998; accepted 15 June 1998

Key words: bioreactor, continuous culture, hybridoma cells, hyperosmolality, monoclonal antibody production, non-producing subpopulation

Abstract

In this paper, we propose an alternative strategy to the ones proposed before (Oh et al., 1993; Øyaas et al., 1994a) to get real increases of global final antibody titer and production at hyperosmotic stress, by reducing the detrimental effect of such a stress on cell growth, and conserving the stimulating effect on antibody production. It consists of cultivating the cells in continuous culture and increasing the osmolality stepwise. In this way, the cells could progressively adapt to the higher osmolality at each step and antibody titers could be nearly doubled at 370 and 400 mOsm kg⁻¹, compared to the standard osmolality of 335 mOsm kg⁻¹. Surprisingly, the stimulation of antibody production was not confirmed for higher osmolalities, 425 and 450 mOsm kg⁻¹, despite the minor negative effect on cell growth. Intracellular IgG analysis by flow cytometry revealed at these osmolalities a significant population of non-producing cells. However, even when taking into account this non-producing population, a stimulating effect on antibody production could not be shown at these highest osmolalities. It seems to us that osmolality has a significant effect on the appearance of these non-producing cells, since they were not observed in continuous cultures at standard osmolality, of comparable duration and at an even higher dilution rate. The appearance of the non-producing cells coincides furthermore with modifications of the synthesised antibody, as shown by electrophoretic techniques. It is however not really clear if these two observations reflect actually the same phenomenon. Hyperosmolality affects the cell behaviour in continuous culture in multiple ways, independently of the growth rate, counting all at least partially for the observed stimulation of antibody production: acceleration of the amino acid, and in particular the glutamine metabolism, increase of the cell volume, increase of the intracellular pH and accumulation of cells in the G1 cell cycle phase.

Introduction

It is well known by now that hyperosmotic stress can stimulate antibody production by hybridoma cells (Øyaas et al., 1989; Ozturk and Palsson, 1991; Oh et al., 1993). In general under such conditions, an inhibitory effect on cell growth was observed, accompanied by a stimulating effect on monoclonal antibody production. Ozturk and Palsson (1991) for instance observed in batch culture a reduction of the cell specific growth rate from 0.040 to 0.024 h⁻¹, and an

increase of the specific monoclonal antibody production rate by a factor 2.2, at an osmolality of 435 mOsm kg⁻¹, compared to its standard physiological value of 290 mOsm kg⁻¹.

However, in general no increase of the final antibody titer has been obtained at hyperosmotic stress, since the increase of the antibody production rate is not enough to compensate for the slower growth rate and lower cell density. Several strategies can be considered to obtain a real increase of antibody production at the end of a culture. They have of course the common objectif that they try to reduce the growth inhibitory effect at hyperosmotic stress on cell growth, while maintaining the stimulating effect on antibody pro-

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Table 1. Preparation of the media used at the different osmolalities studied, as percentages of the three basal media used: the standard medium reconstituted in 3.7 g L⁻¹ of sodium bicarbonate (335 mOsm kg⁻¹), the medium 1 reconstituted in 3.7 g L⁻¹ of sodium bicarbonate and 9.6 g L⁻¹ of PBS (565 mOsm kg⁻¹) and the medium 2 reconstituted in 3.7 g L⁻¹ of sodium bicarbonate and 19.1 g L⁻¹ of PBS (840 mOsm kg⁻¹)

	Osmolality of the experimental media (mOsm kg ⁻¹)				
	335	370	400	425	450
Standard medium	100%	85%	87%	82%	77%
Medium 1		15%			
Medium 2			13%	18%	23%

duction. Oh et al. (1993) for instance adapted their hybridoma cells to higher osmolalities by successive subculturing. In this way, the growth inhibitory effect became indeed negligible, for osmolalities up to 350–400 mOsm kg⁻¹, and the final antibody titer was doubled compared to the standard osmolality. Another strategy adapted by Øyaas et al. (1989) is the addition of so-called osmoprotective components to the medium, such as glycine betaine. For instance, a 15 mM addition of this component to hyperosmotic batch cultures (465 to 610 mOsm kg⁻¹) reduced again the growth inhibitory effect, and final antibody concentration was also doubled (Øyaas et al., 1994a). It seems that the cells accumulate these osmoprotective compounds in their cytoplasm permitting to counterbalance, at least partially, the increased extracellular osmolality. Their intracellular concentration and their accumulation rate increase in a linear way with respect to the medium osmolality (Øyaas et al., 1994b).

An alternative strategy is the one presented in this paper. It consists of cultivating the hybridoma cells in continuous culture, and increasing the medium osmolality by successive steps. In this way, the cells might be able to adapt at each step to the increase of the osmolality. If the stimulating effect on antibody production will be maintained under these conditions, higher antibody titers should be obtained. The only attempt to cultivate hybridoma cells in continuous culture at hyperosmolality found in the literature until now at our knowledge (Reddy and Miller, 1994) was only a very short term experiment compared to the one we present here and did therefore not permit to achieve such a result, probably since osmolality increased too rapidly to permit cells to adapt. The osmolalities used in this continuous culture are derived from preliminary experiments in batch culture, which will also be briefly presented. The results of the continuous culture

presented concern cell growth, monoclonal antibody production and cell metabolism at the different osmolalities tested. Complementary results concerning intracellular IgG content, cell cycle position and cell volume will equally be presented, since they revealed to be very useful to understand the hybridoma cell behaviour in continuous culture at hyperosmotic stress. In addition, the results of an electrophoretic study of the produced MABs will be shown at the different osmolalities tested.

Materials and methods

Cell line and medium

The cell line used was the mouse-mouse hybridoma OKT3 (CRL 8001, ECACC, Salisbury, UK), producing IgG_{2a} monoclonal antibodies directed against the CD3 antigen of human T lymphocytes. The basal medium consisted of DMEM, buffered with bicarbonate at 3.7 g L⁻¹, supplemented with 7% (v/v) FCS, 25 mM glucose, 4.5 mM glutamine, 2% (v/v) MEM essential amino acids, 1% (v/v) MEM non essential amino acids and 1% (v/v) antibiotics (of a stock solution containing 10 000 U mL⁻¹ penicillin and 10 000 µg mL⁻¹ streptomycin). The medium and its supplements were supplied by ATGC (Intermed, Strasbourg, France) with exception of the glucose (Sigma, St. Quentin Fallavier, France). Cells were propagated in T-175 flasks (Falcon, ATGC, Strasbourg, France) at 37 °C in an incubator (Jouan, St. Herblain, France) at 6% CO₂, with a medium renewal every two days, before inoculation of the spinner or bioreactor cultures.

Spinner and bioreactor cultivation

The spinner cultures were performed in 500 mL vessels (Techne, New Brunswick Scientific, Wezembeek-Oppem, Belgium) with a working volume of 250 mL. Cells were inoculated at a density of 3×10^5 cells mL⁻¹ and incubated at 37 °C in the above mentioned incubator at 6% CO₂ at a rotation speed of 30 rpm. Two medium osmolalities, namely 400 and 425 mOsm kg⁻¹, were studied in spinner batch culture, and compared with a standard culture with a medium osmolality of 335 mOsm kg⁻¹. The medium osmolality was increased using PBS, permitting to maintain the ratio Na⁺/K⁺ constant at its physiological level. Table 1 synthesises the preparation of the different media used.

The bioreactor culture in continuous mode was performed in a 4 L stirred tank reactor (SGI, Toulouse, France) with a working volume of 1.8 L. Cells were inoculated at a density of 3×10^5 cells mL⁻¹. Temperature was maintained at 37 °C and the rotation speed at 50 rpm. The dissolved oxygen concentration was kept at 50% of air saturation by injection of oxygen in the liquid through a sparger and nitrogen in the headspace. The medium pH was controlled at 7.00 ± 0.02 by addition of CO₂ in the headspace and NaOH 0.2 N in the liquid. The feed medium with increasing osmolality was added at a constant dilution rate of 0.020 h⁻¹. It contained only 5% (v/v) FCS and 6.5 mM glutamine, compared to the basal medium. The medium osmolality was increased four times, compared to its standard value of 335 mOsm kg⁻¹, namely to 370, 400, 425 and 450 mOsm kg⁻¹ (preparation, see Table 1). At each increase of the osmolality of the feed medium, the osmolality of the medium in the culture vessel was increased in the same way, by addition of the adequate volume, negligible compared to the working volume of the reactor, of a concentrated solution of PBS having an osmolality of 2200 mOsm kg⁻¹.

Analytical methods

Cell concentration was determined with a hemacytometer and viability by trypan blue exclusion. Glutamine, glucose and lactate concentrations were measured by enzymatic methods (Boehringer Mannheim, Meylan, France; Biomérieux, Marcy L'Etoile, France and Sigma respectively) and NH₄⁺ ions by means of a selective electrode (Orion, Espoo, Finland). MAb concentration was determined using an ELISA technique. Osmolality was measured using an automatic

micro-osmometer (Roebbling, Bioblock Scientific, Illkirch, France).

Cell volume measurement

Cell volume distribution measurement was performed almost immediately after removal of the sample from the reactor. 1 to 3 mL of the cell suspension was diluted in 150 mL of Isoton II (Coulter, Margency, France) and analyzed on a Coulter Counter Multisizer II with a 100 μm orifice. Calibration was done with 8.6 μm diameter standard latex microspheres (Coulter). The distributions were stored on an IBM PC for later analysis with the Coulter Multisizer Accucomp software (Windows).

Determination of specific rates

The actual specific growth rate (μ_{act}) was calculated, either in batch or in continuous culture, as follows:

$$\mu_{act} = D \frac{X_t}{X_v} + \frac{1}{X_v} \cdot \frac{dX_t}{dt}$$

where X_v is the viable cell concentration, X_t the total cell concentration, i.e. the sum of the viable and dead cell concentration, t the culture time and D the dilution rate, which is logically set at zero in the case of a batch culture.

Given the mass balances, the specific rates of consumption of a nutrient S (glucose or glutamine) (ν_s) and of production of a product P (lactate, ammonium ions or monoclonal antibodies) (π_p), are given by and calculated as:

$$\nu_s = \frac{1}{X_v} \left[D(S_a - S) - \frac{dS}{dt} \right] \text{ and}$$

$$\pi_p = \frac{1}{X_v} \left[D(P - P_a) + \frac{dP}{dt} \right]$$

where S and P are the concentrations of a nutrient and a product respectively in the reactor. The subscript 'a' refers to their respective concentrations in the feed medium.

Flow cytometry

Sampled cells, typically 10^6 , were fixed in 75% (v/v) methanol, after a wash step with PBS, and stored at -20 °C. For intracellular IgG staining, they were incubated with a FITC conjugated goat anti-mouse antibody, specific for heavy and light chains (Sigma ref.

Table 2. Results obtained for batch spinner cultures performed as described in the materials and methods section

	Osmolality (mOsm kg ⁻¹)		
	335	400	425
Maximal viable cell density ($\cdot 10^6$ mL ⁻¹)	1.3	0.93	0.66
Maximal total cell density ($\cdot 10^6$ mL ⁻¹)	1.4	1.2	1.0
Final antibody titer (mg L ⁻¹)	35	35	34
μ_{act} (h ⁻¹) ^a	0.042	0.031	0.024
π_{MAbs} (mg h ⁻¹ $\cdot 10^{10}$ viable cells) ^b	7.0	9.1	12
Mean cell volume (μ m ³) ^c	1177–1578	1258–1910	1300–2032

^a Slope of the neperian logarithm of the total cell concentration versus time.

^b Slope of the antibody concentration versus the integral of the viable cell density with respect to time (Renard et al., 1988).

^c The first value is the mean cell volume obtained at the culture beginning after a few hours incubation time, the second value is the mean cell volume obtained during the growth phase.

F 2012), after a wash step with PBS. These samples were then analyzed as such, after two wash steps with PBS, or stained subsequently for their DNA content. For this purpose, they were treated with RNase (1 mg mL⁻¹) and stained with PI at a concentration of 50 μ g mL⁻¹. Non fixed cells were stained immediately after sampling from the culture for surface IgG as described in Cherlet et al. (1995). The flow cytometer used was an Epics C Coulter (Coultronics, Margency, France) with an argon ion laser (Innova 90.5, Coherent) tuned to 488 nm. The light stabilised beam power was set at 200 mW. Four parameters were collected at a rate of 150–250 cells s⁻¹: the forward angle light scattering (FALS), the right angle light scattering (RALS) and the green and the red fluorescences, all in linear mode. Fluorescences were represented on a 256 channel histogram in relative units on a linear scale. Gates on the RALS vs. FALS cytogram were used to distinguish between viable and dead cells in the case of non fixed cells, and to exclude cellular debris and cell aggregates from the analysis in the case of fixed cells.

Electrophoretic analysis of culture supernatants

SDS-PAGE was performed on a gel consisting of a 5% stacking gel and a 12% resolving gel at 200 V (20 mA) for approximately 45 min. For reference, molecular weight markers from Pharmacia (St.-Quentin-Yvelines, France) were included, and also a reference mouse IgG (Sigma ref. I 5381). 10 μ L per lane was used, corresponding to a dilution 1/3 of the culture supernatant in the load buffer (β -mercaptoethanol 10% (v/v), SDS 3% (w/v), ureum 6 M, Tris-HCl 0.125 M and bromophenol blue 0.1% (w/v)). Af-

ter electrophoresis, gels were stained with Coomassie Brilliant blue or used for blotting on nitrocellulose membrane. This was carried out at 10–15 V during 15–30 min at a current of 5.5 mA cm⁻² of gel. The blot was then developed using respectively a goat anti-mouse antibody (Tago ref. 4150, Immunosource, Varilhes, France) and a phosphatase alkaline conjugated rabbit anti-goat antibody (Sigma ref. A 7650). IEF analysis was performed on gels with a pH gradient from 5.5 to 8.5 (Pharmacia) at 1500 V, 25 mA and 12 W during 3 h, after concentration of the samples with microfiltration devices (Microsep, Filtron Technology Corporation, Northborough, U.S.A.). For reference, proteins with known isoelectric pH (p_I) were included (Pharmacia). The staining of the gels is done with Coomassie Brilliant blue.

Results and discussion

Effect of hyperosmotic stress on cell growth and antibody production in batch culture

The very first preliminary study in T-175 flasks showed that the OKT3 hybridoma cells were unable to proliferate at osmolalities above 500 mOsm kg⁻¹. A more complete study in T-175 flasks in the range of 335 (standard value) to 450 mOsm kg⁻¹ permitted to identify osmolality values interesting to test in subsequent spinner cultures, more precisely 400 and 425 mOsm kg⁻¹. The results of these spinner cultures are summarised in Table 2. It is evident from this table that maximal cell density decreased with increasing osmolality, for similar culture durations, re-

Figure 1. Evolutions of the viable cell density ($\bullet$) and the monoclonal antibody concentration ($\circ$) (a); Evolution of the specific antibody production rate ($\circ$) (b), during the continuous culture at variable osmolality.

flecting in a decrease of the actual specific cell growth rate. The final antibody titers however were comparable for the three cultures, meaning that the specific antibody production rate is increasing at higher osmolality. The increase of the cell volume by 25% at higher osmolality can only partially explain the doubled specific antibody production rate. Overall, these findings correspond well with the ones of Ozturk and Palsson (1991). It is also interesting to note that cell metabolism remained comparable for the three cultures, as does the antibody production: the same quantities of glucose and glutamine were consumed and the same quantities of lactate and ammonium ions were produced, indicating that the higher specific an-

tibody production rate was accompanied by higher metabolic rates.

Effect of hyperosmotic stress on cell growth and antibody production in continuous culture

A continuous culture was then performed with comparable osmolality values as the ones studied in the spinner batch cultures. In the materials and methods section one can find all necessary details about its operation. In Figure 1, the evolutions of the viable cell density, the antibody titer and the specific antibody production rate during this culture are represented. Table 3 synthesizes the most important data of this culture at the different steady states.

Table 3. Results obtained at steady state at the different osmolalities tested during the continuous culture, performed as described in the materials and methods section

	Osmolality (mOsm kg ⁻¹)				
	335	370	400	425	450
Viable cell density ($\cdot 10^6$ mL ⁻¹)	1.6	2.1	2.0	1.8	1.4
Dead cell density ($\cdot 10^6$ mL ⁻¹)	0.5	0.5	0.5	0.4	0.4
Total cell density ($\cdot 10^6$ mL ⁻¹)	2.1	2.6	2.5	2.2	1.8
Viability (%)	77	81	80	80	79
Antibody titer (mg L ⁻¹)	45	83	87	48	30
π_{MAbs} (mg h ⁻¹ $\cdot 10^{10}$ viable cells)	5.5	8.0	8.6	5.5	4.2
Cell volume (μ m ³)	1152	1220	1318	1361	1483
v_{gln} (mmole h ⁻¹ $\cdot 10^{10}$ viable cells)	0.52	0.49	0.58	0.69	0.75
% of cells in G1 phase	33	43	46	51	45
% of cells in S phase	54	43	44	38	42
% of cells in G2/M phase	13	14	10	11	13

The values represented are the mean values for the last six data points before the osmolality change at 370, 400 and 450 mOsm kg⁻¹, for the last seven at 425 mOsm kg⁻¹ and for the last eight at 335 mOsm kg⁻¹.

It is evident from Figure 1 and Table 3 that the continuous culture mode we used permitted to reduce the growth inhibitory effect of hyperosmotic stress on cell growth, observed in batch culture. Indeed, after each osmolality change, a decrease of the cell density was observed, followed by an increase after some adaptation period of the cells. In this way, cell densities comparable to the one observed at the standard osmolality of 335 mOsm kg⁻¹ could be obtained at each higher osmolality tested. At 370 and 400 mOsm kg⁻¹, they were even significantly higher. Even at 425 mOsm kg⁻¹, it was still slightly higher. These higher cell densities were rather surprisingly, since no limiting factor that had disappeared could be identified. Only at 450 mOsm kg⁻¹ a decrease of the cell density to a value lower than the one at the standard osmolality was observed. It is also interesting to notice that the viability was surprisingly relatively constant at approximately 80% all over the culture, indicating that the higher osmolalities have no effect on cell death in the given culture mode. This corresponds besides to the normal value for our hybridoma cell line in continuous culture at the same intermediate dilution rate of 0.020 h⁻¹, and at standard osmolality (results not shown). The only results at our knowledge concerning continuous hybridoma cell culture at hyperosmotic stress in the literature until now can be found in the paper of Reddy and Miller (1994). They increased the osmolality gradually over a short term period at a constant dilution rate of 0.023 h⁻¹, which did not permit to maintain normal cell growth, since viable cell den-

sity and viability dropped all over the culture, at our opinion because the time for adaptation of the cells was too short. Remember that in our culture, osmolality was increased stepwise and over a much longer time period. We want to cite here also the work of Fong et al. (1997) who increased osmolality by adding potassium acetate at 1 g L⁻¹ to a perfusion culture in its plateau phase. They observed a maintenance of cell density, however at a particular low level of 10⁶ cells mL⁻¹ for perfusion culture, and a slight increase of monoclonal antibody concentration in the presence of potassium acetate, over a short time period of about 100 h.

Our alternative strategy to increase the final antibody titer at higher osmolality shows out to be working: at 370 and 400 mOsm kg⁻¹, it was about 85 mg L⁻¹, while it was only 45 mg L⁻¹ at 335 mOsm kg⁻¹. We remember that a similar increase was observed by Oh et al. (1993) and Øyaas et al. (1994a). This corresponds to an increase of the specific antibody production rate from 5.5 to 8.5 mg h⁻¹ $\cdot 10^{10}$ viable cells. Surprisingly, this stimulating effect was no longer observed at 425 and 450 mOsm kg⁻¹. At 425 mOsm kg⁻¹, a similar antibody titer and production rate were observed as for the standard osmolality. The effect seems even inversed at 450 mOsm kg⁻¹, where both were significantly lower. As for the batch cultures, an increase of 25–30% of the cell volume is noticed at high osmolality, as compared to the reference osmolality. It is also worthwhile mentioning that at each step, an osmolality change was initially fol-

lowed by an important increase of the cell volume. The cell volume then gradually decreased to a new steady state value, but remained higher than at the preceeding step at lower osmolality, as can be seen in Table 3. This evolution corresponds probably to the adaptation period, seen in the evolution of the cell density, since both evolutions occurred at the same time (Thomas and Roberts; 1989; Wentz and Schügerl, 1992).

We finally want to remark here that the osmolality in the reactor broth was always lower than the one of the corresponding feed medium. The following values were measured: 331 ± 3 , 361 ± 5 , 388 ± 4 , 413 ± 4 and 437 ± 5 for the feed media of 335, 370, 400, 425 and 450 mOsm kg⁻¹, respectively. The same observation was besides made in batch culture, where osmolality decreased slightly as a function of culture time.

Effect of hyperosmotic stress on the stability of the antibody production in continuous culture

At first sight it seems that the cancellation of the stimulating effect on antibody production at hyperosmotic stress in the continuous culture might be explained by the appearance of a non-producing subpopulation. Indeed, staining of intracellular IgG by an FITC conjugated antibody revealed the appearance of a subpopulation with decreased fluorescence, compared to the rest of the population. An example of such an analysis is shown in Figure 2a for a sample in the phase of the culture at 450 mOsm kg⁻¹, where the peak at lower fluorescence represents about 25% of the total population. We design here this peak as a non-producing subpopulation (Altschuler et al., 1986; Lee et al., 1991; Kromenaker and Srienc, 1994b), although we can not exclude the possibility that these cells lost only partially their antibody production capacity. In Figure 2b, one can also observe that these non-producing cells are present in all phases of the cell cycle. Interestingly, the bimodal distribution was also observed when staining the surface IgG with an FITC conjugated antibody (Figure 2c). The low fluorescence peak represents then 31% of the total population. The separation between the two populations is clearly less pronounced than for the intracellular IgG. The mentioned bimodal distribution was not observed during other continuous cultures, of comparable duration, and at an even higher dilution rate. An example of a normal observed unimodal distribution is given in Figure 3, for a sample after 900 h of continuous culture at $D = 0.040 \text{ h}^{-1}$. This might be indicating a significant effect

of the increase of the osmolality on the appearance of the non-producing subpopulation, although real evidence for this hypothesis is inexistant in the literature at our knowledge.

At first sight, one could think that these low fluorescence cells correspond to the dead cell population. However, this is not the case. First of all, the analysis of surface IgG was performed on non fixed cells, immediately after cell sampling from the culture. Therefore the viable and the dead cells could be distinguished on the RALS vs. FALS cytogram, the first being the population at high FALS and low RALS, and the latter being the one with low FALS and high RALS (Cherlet, 1995). The data on surface IgG presented on Figure 2c concern therefore only the viable cell population, after adequate gating on the RALS vs. FALS cytogram. Secondly, we showed before that our hybridoma cell line dies essentially by apoptosis (Pauly et al., 1995). Therefore, the dead cells appear after staining with PI and analysis by flow cytometry as a sub-G1 population on a red fluorescence histogram. The data on intracellular IgG presented on Figure 2a concern only the cells corresponding to viable G1, S and G2/M cells by exclusion of the sub-G1 cells on the FALS vs. red fluorescence cytogram. Besides on Figure 2b, one can see that the low fluorescence cells are indeed situated at similar positions compared to the high fluorescence cells with respect to their DNA content. Thirdly, if the peak with low fluorescence cells would actually correspond to the dead cell population, one would expect it to be present also at the lower osmolalities, since viability was nearly constant all over the culture, or even during other cultures.

Figure 4 shows the evolutions of the producing and non-producing subpopulations as a function of culture time during the continuous culture presented above, obtained thus by the analysis of the samples stained for intracellular IgG, the non-producing population being the one with low green fluorescence and the producing population the one with normal high fluorescence. The experimental points are represented from 700 h of culture, since from that moment on the percentage of non-producing cells became significant, and its peak distinct from the second one of the normal producing cells. The results clearly indicate a progressive increase of the percentage of non-producing cells as a function of time, reaching a value close to 25% after 50 days of culture. A linear regression on the experimental points from 700 h of culture, represented by the straight lines on Figure 4, allows to evaluate in a theoretical way the evolution of both populations before

Figure 2. Green fluorescence histogram of intracellular IgG, a gating on the FALS vs. red fluorescence cytogram was used to exclude dead apoptotic sub-G1 cells (a); Green fluorescence of intracellular IgG versus red fluorescence of DNA cytogram (b); Green fluorescence histogram of surface IgG, a gating on the RALS vs. FALS cytogram was used to exclude dead cells (c), for a sample after 1180 h of the continuous culture at variable osmolality (step at 450 mOsm kg^{-1}) (all fluorescences in relative units on a linear 256 channel scale).

Figure 3. Green fluorescence histogram of intracellular IgG for a sample after 900 h of a continuous culture at $D = 0.040 \text{ h}^{-1}$ at the standard osmolality of 335 mOsm kg^{-1} (fluorescence in relative units on a linear 256 channel scale).

Figure 4. Evolutions of the producing (○) and non-producing (●) populations, according to the content of intracellular IgG, as analysed by flow cytometry, during the continuous culture at variable osmolality. The straight lines represent a linear regression on the percentage of non-producing cells.

that moment. It localizes the initial appearance of the non-producing cells around 450 h of culture. An exponential regression however, representing probably better what really happens, especially at the moment of appearance of the non-producing cells, when their number might be increasing with an initial 'lag' phase, situates their appearance already at the transition of 335 to 370 mOsm kg⁻¹.

Simulations have been carried out taking into account the three factors influencing the development of such a non-producing subpopulation during a culture: i) the presence of a non-producing population in the inoculum of the culture, which was not the case for this culture; ii) the conversion rate α from producing to non-producing cells, as a consequence of mutations or losses of genetic material; iii) the difference between growth rates of the two subpopulations (Cherlet, 1995). The principal conclusion that can be withdrawn from these simulations is that a difference of 12.5 to 14% between the growth rates of non-producing and producing cells is the principal factor explaining the observed evolutions of both populations, with respect to the range of conversion rates found in the literature. In the literature, values of 7% (Lee et al., 1991), 9% (Kromenaker and Srienc, 1994b) or 20% (Frame and Hu, 1991) can be found. However, a certain conversion rate of producing to non-producing cells is necessary to explain at least the initial appearance of the non-producing cells. It is possible that such non-producing cells might also have appeared during the spinner batch cultures at increased osmolality presented in Table 2. Since these batch cultures are characterised by a growth phase of about 60 h, time is not sufficiently long compared to the continuous culture for the non-producing cells to partially grow over the culture. Repeated batch cultures would be necessary to observe any possible influence of the non-producers on the culture behaviour, and especially on antibody production.

It is now possible to calculate the actual specific antibody production rates during this continuous culture at the different steady states taking into account only the producing viable cells. These calculations, which results are given in Table 4, are performed with the percentages obtained with the linear regression of Figure 4. They indicate a higher stimulating effect than the one found before at 400 mOsm kg⁻¹, a little stimulating effect at 425 mOsm kg⁻¹, and a maintenance of the antibody production rate at 450 mOsm kg⁻¹ compared to the reference osmolality, where before a negative effect was announced. However, it is clear

that the inclusion of the non-producing population does not permit to demonstrate a net stimulating effect on antibody production at 425 and 450 mOsm kg⁻¹, contrary to the observations made in batch culture (Table 2).

Electrophoretic analysis of the monoclonal antibodies produced at hyperosmotic stress

The analysis of the culture supernatants at the different osmolalities tested by means of electrophoretic techniques is shown in Figure 5. On the SDS-PAGE gel (result not shown), the positions of the heavy and light chains are estimated to be 55 and 27 kDa respectively and correspond well with the ones found for a standard antibody run on the same gel. However, at 425 and 450 mOsm kg⁻¹, a supplementary band was found, situated slightly superior to the light chain position. These are visible also on the Western blot shown in Figure 5a, especially for the sample at 450 mOsm kg⁻¹. It is not clear for us whether this band corresponds to a degradation of the heavy chains, a very incomplete synthesis of them, or something else such as a stress protein. Pellicciari et al. (1995) for instance report the synthesis of a 35 kDa stress protein at hyperosmolality, which corresponds well with the position of our supplementary band, although such a stress protein should a priori not be detected on the Western blot. It is however remarkable that the appearance of this supplementary band coincides with a significant proportion of non-producing cells, suggesting a link between both phenomena. In this way, one could consider the non-producing cells actually as a population synthesising a very defective antibody. Another hypothesis might be that both phenomena were occurring simultaneously, without any link, except maybe the cause of their appearance, i.e. the increased osmolality: the observed cancellation of the stimulation of the osmolality on antibody production would then be a superposition of two phenomena, namely the appearance of non-producing cells and the appearance of cells producing a defective antibody, not detected by the ELISA test. The fact that the inclusion of the non-producing cells alone in the calculations does not permit to demonstrate the stimulating effect on antibody production for the highest osmolalities of the continuous culture, observed in batch culture, could be some kind of indication in favour of this second hypothesis. However, it is not possible to quantify the population that might synthesise a defective antibody and to include it in the calculations of the antibody

Figure 5. Western blot and IEF analysis of culture supernatants for each steady state of the continuous culture at variable osmolality of Figure 1. H and L indicate for the blot the position of the heavy and the light chains respectively. For the blot: lane 1 supernatant 335 mOsm kg⁻¹, lane 2 supernatant 370 mOsm kg⁻¹, lane 3 supernatant 400 mOsm kg⁻¹, lane 4 supernatant 425 mOsm kg⁻¹, lane 5 supernatant 450 mOsm kg⁻¹ and lane 6 reference antibody. For the IEF gel: lane 1 pI markers, lane 2 supernatant 335 mOsm kg⁻¹, lane 3 supernatant 370 mOsm kg⁻¹, lane 4 supernatant 400 mOsm kg⁻¹, lane 5 supernatant 425 mOsm kg⁻¹ and lane 6 supernatant 450 mOsm kg⁻¹.

Table 4. Comparison of the specific antibody production rates, taking into account or not the non-producing cells, at steady state at the different osmolalities tested during the continuous culture, performed as described in the materials and methods section

	Osmolality (mOsm kg ⁻¹)				
	335	370	400	425	450
π_{MAbs} (mg h ⁻¹ · 10 ¹⁰ viable cells) (total population)	5.5	8.0	8.6	5.5	4.2
π_{MAbs} (mg h ⁻¹ · 10 ¹⁰ viable cells) (producing population only)	5.5	8.3	9.6	6.7	5.5

production, as a supplementary factor for the loss of antibody productivity at high osmolality, observed for the continuous culture.

Analysis of the supernatants by IEF shows also some modifications at the level of the isoelectric pH of the monoclonal antibodies (Figure 5b). At 335 and 370 mOsm kg⁻¹, they are characterised by 7 distinct bands in the range 7.2 to 7.9. At 400 mOsm kg⁻¹, a supplementary band appears, not very well visible on the picture. At 425 mOsm kg⁻¹, this band is very well visible, and even a second supplementary band appears. It furthermore seems that the most intense bands are shifted towards the more acidic region of the gel at higher osmolality. This pattern is conserved at 450 mOsm kg⁻¹, it is however not very clear on the picture, due to the lower antibody concentration. It is remarkable that these modifications coincide once again with the above mentioned ones. It is worthwhile remarking that these modifications have probably an intracellular origin, since no proteolytic activity could be detected in our culture conditions at pH 7.0 and at 5% (v/v) FCS. Only in supernatants of cultures grown at 1% FCS such an activity could be detected, and also after a decrease of the pH of culture supernatants to 3.0–4.0, indicating the presence of an acid protease, however inactive under normal culture conditions.

Mechanism of stimulation of monoclonal antibody production at hyperosmotic stress

We finally want to present and discuss briefly some of our results related to the mechanism that could explain the stimulation of the antibody production at higher osmolality. These results concern essentially the above presented continuous culture, and the stimulating effect of osmolality on antibody production is for the interpretation supposed being confirmed also at 425 and 450 mOsm kg⁻¹, which means that we suppose

that non-producing cells did not appear, as well as the other above presented modifications.

One important factor to consider is the increased higher extracellular Na⁺ ion concentration at hyperosmolality. The steeper gradient of Na⁺ across the cell membrane can have multiple consequences for the cell behaviour: i) A better transport of amino acids into the cell by the transport mechanisms in co-transport with Na⁺, resulting in a possible higher antibody synthesis (Flickinger et al., 1992; Chua et al., 1994). Although, we did not perform a systematic determination of all amino acid concentrations, we can confirm that at least the glutamine specific consumption rate was increasing at higher osmolality (see Table 3), indeed maybe as a consequence of its better transport across the cell membrane. Remember that this was also the case for the spinner batch cultures. This might be an important factor contributing to the observed higher specific antibody production (Flickinger et al., 1992; Oh et al., 1995). The simultaneous increase of the glucose metabolism observed for the batch cultures was however not confirmed for the continuous culture; ii) A stimulation of the activity of the Na⁺/H⁺-exchanger, which in its turn can influence cell volume and intracellular pH (Al-Habori, 1994; Demarex and Grinstein, 1994). We showed already in this paper that the cell volume was up to 30% higher at hyperosmolality, which might have a supplementary positive effect on the cellular antibody synthesis capacity. We showed elsewhere that intracellular pH was significantly higher at hyperosmolality compared to 335 mOsm kg⁻¹, for the continuous culture presented, with respective mean values of 7.35 and 7.45 being measured (Cherlet, 1995). It seems that higher intracellular pH is in some cases associated with important protein synthesis (Grainger et al., 1979), so

also this intracellular pH increase might be of positive influence on antibody production.

Another important factor to consider is the cell cycle distribution at the different osmolalities tested, since evidence has been published that MAbs production might be cell cycle related (Kromenaker and Srienc, 1991; Kromenaker and Srienc, 1994a). Antibody synthesis seems to be particularly important in the G1 phase of the cell cycle, also for our hybridoma cell line OKT3. Addition of butyric acid for instance permitted to double the final antibody titer in batch culture, which seems to be the consequence of an important redistribution in the cell cycle, and more precisely an important accumulation of cells in the G1 phase (Chevalot et al., 1995). A similar redistribution was observed at higher osmolality during the continuous culture (see Table 3): the percentage of cells in the G1 phase was indeed increased significantly by at least 10% at hyperosmolality, while simultaneously a decrease of S phase cells occurred. The percentage of cells in the G2/M phase remained relatively constant. A similar effect has been observed by Pellicciari et al. (1995), but in their case growth rate was also lowered at high osmolality, which did not happen at each steady state of the continuous culture presented here. Oh et al. (1995) observed lower DNA levels at hyperosmolality associated with higher RNA levels, which might indicate higher transcription and possible higher translation rates of proteins in general, and of monoclonal antibodies in particular.

It is important to realize that the above mentioned effects of hyperosmolality at the steady states of the continuous culture were observed independently of the cell specific growth rate, since the dilution rate was fixed at 0.020 h^{-1} all over the culture, contrary to previously published studies in batch culture where the cell specific growth rate was therefore in constant evolution.

Conclusions

We showed in this paper that we were able to take real profit of the stimulating effect of hyperosmolality on antibody production by hybridoma cells, by reducing the negative effect on cell growth of such a stress. This was achieved in continuous culture, where the medium osmolality was increased by successive steps. This permitted the cells to adapt to the higher osmolality at each step, attested by the maintenance of a normal cell growth, characterised by a high viable cell density

and a constant cell viability. The stimulating effect of hyperosmolality on the specific cellular antibody production observed in the batch mode was furthermore conserved during the continuous culture, resulting in doubled antibody titers at 370 and 400 mOsm kg^{-1} , compared to the standard osmolality. It seems to be the result of several subtle changes of the cell behaviour: an acceleration of the amino acid and especially the glutamine metabolism, an increase of the cell volume, an increase of the intracellular pH and an accumulation of cells in the G1 phase of the cell cycle. However, for longer culture times at still higher osmolalities, this stimulating effect was not confirmed. At 450 mOsm kg^{-1} , the observed effect was even negative. The cancellation of the stimulating effect seems to be linked to the appearance of non-producing cells in the cell population, containing inherently no such non-producers at the inoculation and also during all other cultures with this cell line, especially in continuous mode for a comparable duration and at an even higher dilution rate. This suggests a significant effect of the osmolality on their appearance. At the same moment of the culture, a structural change of the synthesised MAbs became apparent. It is not clear for us whether these two observations reflect the same phenomenon or not. These last observations indicate that such an attractive factor as the osmolality to increase monoclonal antibody production, being besides the subject of a worldwide patent (Maiorella et al., 1989), can change drastically cell behaviour with respect to antibody synthesis after long term exposure, as well on a quantitative as on a qualitative level, which must be taken into account when a culture process including such a stress will be designed.

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