Stimulation of monoclonal antibody production of hybridoma cells by butyrate: evaluation of a feeding strategy and characterization of cell behaviour

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Received 22 April 1998; accepted 15 March 1999

Key words: bioreactor, butyrate, cell cycle, hybridoma cells, intracellular IgG, monoclonal antibody production

Abstract

Experiments, earlier performed in our laboratory, showed the stimulating effect of butyric acid on monoclonal antibody production by hybridoma cells. Its simultaneous inhibitory effect on cell growth can however compensate for this, so that no increase of monoclonal antibody titer might be obtained. We show in this article an experiment with addition of butyric acid in the middle of the growth phase of a batch culture, as a strategy to take real profit of such an addition by a significant increase of final monoclonal antibody concentration. Indeed, in this way a significant cell density could be obtained before the addition of butyric acid, while the remaining culture time was still sufficiently long for its action, resulting in a two fold increase of final monoclonal antibody titer. The experiment was carried out in a 2 L bioreactor, showing the real practical interest of such an addition for the large scale production of proteins. Furthermore, analysis of the produced IgG by SDS-PAGE and Western blot did not reveal structural changes after stimulation by butyric acid. An original point of our study is the characterization of the cell behaviour, by flow cytometry and other related techniques, leading to a better insight in the effect of the butyric acid addition on cell growth and monoclonal antibody production. Although there exists a lot of knowledge about the effects of butyrate on cells in the field of molecular biology, our article is at our knowledge one of the first to show some of its effects on cell behaviour in bioreactor culture, carried out under perfectly defined and controlled conditions, and with the aim to stimulate monoclonal antibody production.

Introduction

It is well known for some time that butyrate has a stimulating effect on protein production by animal cells (Kruh, 1982), despite its growth inhibitory effect. Ganne et al. (1991) obtained an increase of factor VIII production by CHO cells in the presence of 3 mM butyrate by a factor 2, by an increase of the specific production of the cells by a factor 3. Gebert and Gray (1991) showed that the production of FSH by CHO cells was two times higher in the presence of 1 mM butyrate, while cell density was halved. The t-PA production of CHO cells was also increased by a factor 2 to 9 in the presence of 5 mM of butyrate (Palermo et

al., 1991). In the range of 0.5 to 5 mM of butyrate, the GGT production by CHO and V79 cells increased by a factor 3 to 5 (Oster et al., 1993). Also for hybridoma cells, such an effect was observed by some authors. Oh et al. (1993) showed that an increase of the medium osmolality to 350 msm/kg, in combination with an addition of 0.1 mM of butyrate, resulted in doubled antibody titers. Chen et al. (1993) showed an increase of monoclonal antibody concentration in the presence of 1 to 5 μ M of butyrate. In our laboratory, it was shown that the specific antibody production of our hybridoma cell line OKT3 increased by a factor 1.5 to 2.5 in the presence of 1 to 2 mM of butyrate (results not shown).

Figure 1. Evolutions with time of the viable cell density (diamonds), the dead cell density (triangles) and the total cell density (circles) (a); Evolution with time of the monoclonal antibody concentration (b), during a batch culture without (open symbols, thin lines) and with (closed symbols, normal lines) butyric acid addition.

The apparent effects of butyrate on cell growth and protein production seem to be linked at a molecular level. Butyrate induces a hyperacetylation of the histones, probably by inhibiting in a non-competitive manner a deacetylase (Kruh, 1982), and also a dephosphorylation of the histones (Boffa et al., 1981). Therefore, the links of these histones with DNA become weaker, increasing the accessibility of DNases to the DNA, resulting in its turn in a higher transcription rate. Several authors found indeed an increase of the mRNA synthesis of their protein of interest (Dorner et al., 1989; Ganne et al., 1991; Palermo et al., 1991; Oster et al., 1993). These hyperacetylation and dephosphorylation of the histones are probably also implicated in the inhibitory effect of butyrate on cell growth. A hyperacetylation favors the condensation of the chromatin as a consequence of the weaker

histone-DNA links. But now, at the end of the mitosis, a deacetylation is necessary to permit the cells to progress through the G1 cell cycle phase. Butyrate, being an inhibitor of such a deacetylation, will therefore block the cells at a point in early G1 phase (Kruh, 1982). The dephosphorylation has an opposite effect. A phosphorylation of the histones is associated with the condensation of the chromatin during mitosis. Butyrate, inducing a dephosphorylation of the histones, blocks in this case the cells at the transition point from the G1 to S phase, since a phosphorylation is necessary to initiate the S phase (Boffa et al., 1981).

Kruh (1982) noticed that the increased production of a protein in the presence of butyrate is not always, or at least not only, a result of a specific induction, especially if it occurs only after several hours. More precisely, in the case of a protein with a more

Figure 2. Evolution with time of the neperian logarithm of the total cell density during a batch culture without (○, dashed line) and with butyric acid addition (●, full line).

Figure 3. Evolution of the monoclonal antibody concentration with the integral of the viable cell density versus time during a batch culture without (○, dashed line) and with butyric acid addition (●, full line).

Figure 4. SDS-PAGE and Western blot analysis of culture supernatants at the end of a batch culture without and with butyric acid addition (see Figure 1). H and L indicate the position of the heavy and the light chains, respectively. For the gel: lane 1 molecular weight markers, lane 2 supernatant reference batch culture, lane 3 supernatant batch culture with butyrate addition and lane 4 reference antibody. For the blot: lane 5 supernatant reference batch culture (same as lane 2), lane 6 supernatant batch culture with butyrate addition (same as lane 3), lane 7 reference antibody (same as lane 4).

Figure 5. IEF analysis at different time points for a batch culture without and with butyric acid addition. Batch culture with butyric acid addition: lane 1 time 91 h, lane 2 time 47 h, lane 3 time 168 h. Batch culture without butyric acid addition: lane 4 time 74 h, lane 5 time 49 h. Lane 6: p_I markers.

important synthesis during the G1 cell cycle phase compared to the other ones, as some authors report for monoclonal antibody production by hybridoma cells (Kromenaker and Srienc, 1991), its increased produc-

tion might reflect rather the effect of butyrate on cell cycle distribution by the accumulation of cells in the G1 phase, than a specific induction.

We present in this article an interesting experiment with butyric acid addition, and this for two main reasons. Firstly, the butyric acid is added in such a way that its growth inhibitory effect was minimized, resulting in a significant increase of monoclonal antibody titer, showing a real practical interest of such a butyric acid addition to improve protein production, especially since at least the structural properties of the protein seem to remain unaffected. Therefore, it was added only after 41 h of culture, in the middle of the rapid growth phase, when cell density had increased already significantly before, and when the remaining culture time was still sufficiently long for the butyric acid to affect the cell and therefore the culture behaviour. In most of the previously published studies cited before, butyrate was added from the culture beginning, so that its growth inhibitory and production stimulating effects might compensate for each other, despite the indeed observed increase of the specific production. Also, when butyrate was added in a later culture phase when growth rate was already significantly reduced, for example at the plateau phase of a fed-batch culture, no positive effect on protein production could be detected (Chevalot et al., 1995; Dardenne, 1995). Secondly, besides the evaluation of the effect of the

butyric acid addition on cell growth and monoclonal antibody production, a detailed study of its effect on cell behaviour has been carried out, mainly by flow cytometry techniques, leading to a better insight in how its effect on cell behaviour determines the culture performances. At our knowledge, our study is one of the first to show such for a large scale bioreactor culture performed under perfectly defined and controlled culture conditions, with the aim to stimulate monoclonal antibody production, completing in this way the already previously published results on this matter based on T-flask experiments, especially in the field of molecular biology.

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, 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 penicillin and 10,000 µg/ml 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 bioreactor cultures.

Bioreactor cultivation

The batch bioreactor cultures were performed in a 2 L stirred tank reactor (SGI, Toulouse, France) with a working volume of 1.2 L. Cells were inoculated at a density of 3×10^5 cells/mL. 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. One culture was carried out as a control batch culture. A second culture received an addition of butyric acid in

the mid exponential phase, more precisely after 41 h of culture, when the reactor volume was about 1 L. At this purpose a volume of 25 mL was added, being a dilution of the pure (at 99.5%) liquid butyric acid (Fluka, St. Quentin Fallavier, France) at a concentration of 40 mM in DMEM medium, to obtain a final concentration in the reactor broth of about 1 mM butyric acid. The dilution effect of this addition was considered negligible.

Analytical methods

Cell concentration was determined with a hemacytometer and viability by trypan blue exclusion. MAb concentration was determined using an ELISA technique. Butyric acid concentration was determined by ion exchange HPLC on a Polypore H column of Brownlee Laboratories. The elution solvent used was sulfuric acid of 0.04 N, at a flow of 1.5 mL/min and at a temperature of 65 °C. The eluted organic acids were quantified at the column outlet by an UV detector (Waters, St. Quentin en Yvelines, France) tuned at 210 nm.

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 Acucomp software (Windows).

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), 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) and stained with PI at a concentration of 50 µg/mL. 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

Figure 6. Evolution with time of the butyric acid concentration in the reactor broth for the culture with butyric acid addition.

light stabilised beam power was set at 200 mW. Four parameters were collected at a rate of 150–250 cells/s: the forward angle light scattering (FALS), the right angle light scattering (RALS) and the green and the red fluorescences. Fluorescences were represented on a 256 channel histogram in relative units. Gates on the RALS vs. FALS cytogram were used to exclude cellular debris and cell aggregates from the analysis. All analysis were carried out with the software available on the flow cytometer. Cell cycle distribution analysis was carried out with the Multicycle software of Phoenix Flow Systems (San Diego, CA, U.S.A.).

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 en Yvelines, France) were included, and also a reference mouse IgG (Sigma). 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)). After 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/cm² of gel. The blot was then developed using respectively a goat anti-mouse antibody (Tago, Immunosource, Varilhes, France) and a phosphatase alkaline conjugated rabbit anti-goat antibody (Sigma). 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 the butyric acid addition on cell growth and monoclonal antibody production

The evolutions with time of the cell density and the monoclonal antibody titer for both the control culture and the culture with butyric acid addition are shown in Figure 1. The viable cell density increased after inoculation to a maximum of 2.5×10^6 cells/mL after 56 h for the control culture. The addition of butyric acid reduced this maximum to 2.2×10^6 cells/mL, reached

Figure 7. Evolutions with time of the percentages of cells in the G1 (diamonds), the S (circles) and the G2/M (triangles) cell cycle phases, during a batch culture without (open symbols, thin lines) and with (closed symbols, normal lines) butyric acid addition.

later after 75 h of culture. However, while the total cell density remained stable at 2.7×10^6 cells/mL when the maximum viable cell density was reached for the control culture, it increased steadily for the culture with butyric acid addition up to 3.2×10^6 cells/mL at the end of the culture. For both cultures, maximal viable cell density coincided with a depletion of glutamine from the culture medium. An interesting behaviour was observed for monoclonal antibody production. MAb's concentration increased to 50 mg/L during the rapid growth phase for the control culture, and remained stable thereafter. However, for the culture with butyric acid addition it increased all over the culture time to reach a final value of 100 mg/L, being thus a two fold increase of final monoclonal antibody titer.

Figure 2, representing the evolution with culture time of the neperian logarithm of the total cell density for both cultures, permits to appreciate more precisely the effect of the butyric acid addition on cell growth. For the standard batch culture, two phases can be distinguished: a rapid growth phase, with a relatively constant growth rate of 0.041 h^{-1} , and a decline phase, where growth was completely stopped. For the culture with butyric acid addition, an additional phase can be observed. Before the addition of butyric acid, a rapid growth phase is observed, with comparable growth rate as for the reference culture. After the addition of butyric acid however, a phase of slower growth can be observed, which must be a consequence of this

addition, since it was not observed for the control culture. The lower growth rate of 0.015 h^{-1} , associated to this phase, explains the above mentioned longer growth phase, and in a less extent also the lower maximal viable cell density reached. During the final decline phase, growth rate never really dropped to zero, reflecting the continuous increase of the total cell density.

In the same way, Figure 3 permits to evaluate more precisely the effect of the butyric acid addition on monoclonal antibody production. It represents the evolution of the monoclonal antibody concentration with the integral of the viable cell density versus time for both cultures. The slope on such a representation is then a measure of the specific monoclonal antibody production rate (Renard et al., 1988). As for the growth rate on Figure 2, two phases can be distinguished for the reference culture: the first one corresponds to the growth phase and is characterized by an apparently constant specific monoclonal antibody production rate of $7.5 \text{ mg}/10^{10}$ viable cells/h. During the second phase, the decay phase of the culture, no antibody production could be detected. Again, a supplementary third phase can be observed for the culture with butyric acid addition. The specific monoclonal antibody production rate was also relatively constant before the addition of butyric acid, but somewhat lower than for the reference culture, probably due to some inherent variability between cultures. It increased to a new constant value close to $10 \text{ mg}/10^{10}$

Figure 8. Normalized cell volume distributions obtained by analysis of samples on a Coulter counter at different time moments of a standard batch culture. At each distribution, the one obtained after 22.5 h, where the mean cell volume reached a maximal value, is shown as a reference by a bold line.

viable cells. h after the addition of butyric acid, which was maintained during the rest of the growth phase, showing a stimulating effect of butyric acid on monoclonal antibody production, by a factor 1.3 to 1.7, comparing respectively to the reference culture and to the culture phase before its addition. Once the maximal viable cell density was reached, the specific monoclonal antibody production rate decreased significantly, and was for the rest of the culture maintained at a constant value.

It is interesting to notice that the structural properties of the monoclonal antibodies, produced at a higher level by our hybridoma cell line in the presence of butyric acid, remain unchanged, at least at our level of analysis. This can be seen on Figure 4, where the monoclonal antibodies produced during the standard batch culture and the batch culture with butyric acid addition are compared by SDS-PAGE and Western blot techniques. Also after IEF analysis, no significant changes in isoelectric pH were observed (see Figure 5): the produced antibodies are in the absence and in the presence of butyric acid characterized by 6 to 7 distinct bands in the pH range 7.2 to 7.9, typically observed for our hybridoma cell line (Cherlet and Marc, 1999). Several other authors also observed no such changes of the secreted protein (Palermo et al., 1991; Oster et al., 1993; Chevalot et al., 1995). It is worthwhile mentioning here the work of Dorner et al. (1989) who compared the expression of three different proteins (factor VIII, factor von Willebrand and erythropoietin) by CHO cells. In the three cases, butyrate had an inducing effect revealed by mRNA determination. However, factor VIII was not detected in the culture medium. The authors could show the presence of GRP78 and GRP94 (glucose-regulated protein) associated to the endoplasmatic reticulum. These proteins form stable complexes with incorrectly folded or incorrectly glycosylated proteins and block in this way their secretion, as it was the case for factor VIII. They avoid thus a priori that altered proteins could be secreted in the culture supernatant and it is interesting to notice that their expression was increased in the presence of butyrate. The GRP proteins are besides also implicated in the assembly of the heavy and light chains of IgG.

Figure 6 shows the evolution of the butyric acid concentration in the culture medium for the culture with addition of it. It decreased after its addition and seems to be consumed by the cells. A minimum concentration of 0.56 mM was reached after 75 h of culture, corresponding to the maximum viable cell

density. After that time, it increased again slightly, corresponding probably to a passive release of non metabolized product by dying cells with a permeabilized cell membrane. The butyric acid consumption can be explained by the β -oxydation pathway of fatty acids. The formed acetyl-CoA can be a supplementary energy source for the cells by its oxydation in the Krebs cycle and can therefore contribute in some way to the increased culture duration. It is worthwhile noticing that Kruh (1982) states that the observed effects of butyric acid must be due to the butyric acid itself, and not to a by-product of its metabolism, since other fatty acids, metabolized by the same pathway, and thus resulting in the same metabolites, do not induce, or only little, these effects.

Effect of the butyric acid addition on cell behaviour

The most evident parameter to study at this level is probably the cell cycle distribution for the obvious reasons mentioned in the Introduction section. For the control culture, the distribution of the cells in the cell cycle was little variable during the rapid growth phase: on average, about 30% of the cells were situated in the G1 phase, 55% in the S phase and 15% in the G2/M phase (Figure 7). At 56 h, where maximal viable cell density was reached, a drastic change of this distribution occurred, with a diminution of 20 and 5% of the percentages of cells in the S and G2/M phases respectively. Logically, these cells accumulated in the other phase, the G1 phase, which increased thus by 25% in importance. This evolution reflects here actually the fact that the cells stop to cycle and to divide, since total cell density remained stable thereafter. It is further accentuated during the decay phase of the culture, when cells, not able any more to progress in the cell cycle and arrested in the G1 cell cycle phase, finish to die. The same distribution as the one observed during the rapid growth phase of the control culture was observed before the addition of butyric acid for the culture with addition (Figure 7). Only 10 h after this addition, cell cycle distribution had already significantly changed. 24 h after the addition, the percentage of G1 cells had increased with about 20% from 33 to 54%, and the percentage of S cells decreased from 55 to 37%. One must realize that this change occurred before reaching the maximal viable cell density, thus at a moment when cells were still actively dividing, contrary to what was observed for the control culture. It reflects therefore not the fact that the cells stop to cycle and to divide, as for the reference culture, but rather a change in cell

Figure 9. Normalized cell volume distributions obtained by analysis of samples on a Coulter counter at different time moments of a batch culture with a 1 mM butyric acid addition after 41 h. At each distribution, the one obtained after 41 h, just before this addition, is shown as a reference by a bold line.

cycle behaviour between two cell divisions, after and induced by the addition of butyric acid. The fact that the percentage of G2/M cells increased initially following the butyric addition reflects probably the shift of cells from the S to the G1 phase: the cells being in the S phase must pass the G2/M phase before being blocked in the G1 phase (Boffa et al., 1981; Kruh, 1982). Thereafter, the percentage of G2/M cells decreased also progressively. Our results indicate thus clearly an effect of the butyric acid on the distribu-

tion of the cells in the cell cycle, and more precisely a block of the cells in the G1 phase. This observation explains certainly the slower cell growth observed after the addition (see Figure 2).

Another revealing parameter to evaluate the cell behaviour in the presence of butyric acid showed out to be the cell volume. For the control culture, the cell volume distribution was unimodal during the rapid growth phase (left panel of Figure 8), with a slight increase of the mean cell volume after inoculation

Figure 10. Evolutions with time of the intracellular IgG contents, determined by ELISA on lysed cell samples (circles), and by flow cytometry after fixation and staining of the cells as detailed in the Materials and Methods section (diamonds), during a batch culture without (open symbols, thin lines) and with (closed symbols, normal lines) butyric acid addition.

during the first day of the culture. After that time, it decreased progressively until all cells became non viable. At 70.5 h of culture, where viability was about 50%, the unimodal distribution became bimodal, reflecting the difference in cell volume between the dead and viable cells: a first pic with lower cell volume corresponds then to the dead cells, while a second pic with higher cell volume contains the remaining viable cells. Logically, the importance of the second pic decreased for longer culture times until the distribution became again unimodal at the end of the culture, when all cells became non viable (right panel of Figure 8). For the culture with butyric acid addition, a similar behaviour was observed before the addition, i.e. a unimodal cell volume distribution with similar mean values as for the reference culture (Figure 9). Ten hours after the butyric acid addition, a significant decrease of the cell volume of $100 \mu\text{m}^3$ was observed. For longer culture times, the cell volume distribution clearly became bimodal, with the mean cell volume decreasing progressively, while the modulus or the maximum of the distribution remained stable at $1030 \mu\text{m}^3$, reflecting to our opinion the shift of S and G2/M cells to the G1 phase. The bimodal distribution changed then indeed by a progressive accumulation of cells in the population around the pic of $1030 \mu\text{m}^3$, corresponding to the accumulation of cells in the G1 phase. In the mean time, cells with higher cell volumes, typically S and G2/M phase cells, disappeared. From 91 h of culture, an apparition of dead cells became visible corresponding to the first pic of Figure 8. At the end of the culture,

logically the distribution became also unimodal as for the reference culture, representing the only remaining population at that time, namely the dead cells. One must realize that the observed bimodal distribution for the culture with butyric acid addition is from a completely different nature than the one observed for the reference culture: it is an evolution at the inside of the viable cell population reflecting their drastically changing cell cycle distribution, while for the reference culture, it reflected the transition of viable to dead cells during the decay phase of the culture.

The intracellular IgG content of the cells is a third interesting parameter to evaluate cell behaviour after butyric acid addition. This quantity has been determined as well by flow cytometry as by ELISA on lysed cell preparations. Both techniques gave essentially the same results as can be seen on Figure 10. For the reference culture, the intracellular IgG content of the cells was fairly stable all over the growth phase and close to $1 \mu\text{g}/10^6$ cells. It decreased rapidly when maximal viable cell density was reached. For the culture with butyric acid addition, again a similar behaviour was observed before the addition. 10 h after the addition, no difference was observed. 24 h after the addition, intracellular IgG content was significantly increased. At 75 h of culture, cells contained even two times more antibody than before the butyric acid addition. This coincided with maximal viable cell density for this culture, and intracellular IgG content decreased thereafter, as for the reference culture. At the end of the culture however, cells contained still a quantity of

IgG corresponding to their basic level before the addition of butyric acid. Remember that for the control culture, much lower levels were observed at the end of the culture. This difference might explain the stop of monoclonal antibody production for the reference culture when maximal viable cell density was reached, and the maintenance of it at a lower level for the culture with butyric acid addition.

The observed profile of intracellular IgG content after the addition of butyric acid, compared to the reference culture, provides important information about the monoclonal antibody production process of our hybridoma cell line. First of all, it seems that the antibody synthesis rate exceeds the antibody secretion rate after the butyric acid addition, resulting in some intracellular accumulation revealed by our analysis. The maximal difference, corresponding to the maximum in intracellular IgG content, is about 20%. Before the addition, and also for the reference culture, the synthesis and secretion rates seem to balance each other, resulting in a constant intracellular IgG content. Comparison of Figures 7 and 10 shows that the intracellular antibody accumulation after the butyric acid addition coincides with an accumulation of cells in the G1 phase. This might explain why secretion of monoclonal antibody is in some way slower than its synthesis, since it has been shown that antibody secretion rates of hybridoma cells vary as a function of cell cycle position, with lowest values for G1 and S phase cells and higher values for G2/M cells (Lucocq and Warren, 1987; Takizawa et al., 1993; Cherlet et al., 1995). Secondly, the coincidence of the intracellular IgG accumulation and the accumulation of G1 cells mentioned before also leads to another important conclusion. Since both intracellular IgG content and specific antibody secretion rate increased after butyrate addition, such an addition clearly stimulated antibody synthesis rate. Clearly, this happened at a moment where cells were blocked in the G1 cell cycle phase. This could confirm the theory and/or the results of other authors that the G1 cell cycle phase is the most productive one for monoclonal antibody synthesis by hybridoma cells (Suzuki and Ollis, 1990; Kromenaker and Srien, 1991; Al-Rubeai et al., 1992), especially since the fact that only a slight effect of the addition is visible after 10 h, and that it takes about 24 h to observe a significant effect, indicate that the stimulating effect might be rather a consequence of the cell cycle redistribution capacity of the butyric acid, than of a real specific induction effect (Kruh, 1982).

Conclusions

Earlier experiments in T-flasks in our laboratory had shown that butyric acid addition in the range of 1–2 mM increased specific antibody production by a factor 1.5 to 2.5. However, final monoclonal antibody concentration remained the same, or was even lower, compared to normal cultures, as a consequence of the significant effect of this component on cell growth. For these cultures, the butyric acid was added to the medium immediately after inoculation. In this article, we show a strategy of butyric acid addition, which takes profit of the stimulating effect of this component on antibody production, while minimizing its negative effect on cell growth. This is achieved by adding the butyric acid in the middle of the rapid growth phase of a batch culture: a significant cell density is then already achieved, and the remaining culture time is sufficient for the action of the butyric acid. This last point is important to consider since addition of butyric acid in a later culture phase when growth rate was already significantly reduced, for example at the plateau phase of a fed-batch culture, did not affect protein production in a positive way, as was shown in our laboratory (Chevalot et al., 1995; Dardenne, 1995). Monoclonal antibody titer was in this way doubled. The increase of the specific antibody production rate was about 1.3 to 1.7, which corresponds to the observed stimulation in T-flask. This culture was furthermore carried out in a 2 L bioreactor, showing the practical use of the addition of a cheap component such as butyric acid to improve the production of proteins in large scale cultures. Electrophoretical analysis of the produced IgG showed that the stimulated production did not result in the synthesis or secretion of structurally altered IgG, at least at our level of analysis.

A study of the cell behaviour, mainly by flow cytometry techniques, revealed how the effect of the butyric acid addition on cell behaviour and culture performances are linked. Butyrate addition induced a redistribution of the cells in the cell cycle, and more precisely blocked viable cells in the G1 phase, as shown by cell cycle analysis, which must explain the observed slower growth. This progressive accumulation of cells in the G1 phase affected by its importance also the cell volume distribution and resulted in a continuous decrease of the mean cell volume of the viable cell population. Intracellular IgG content however increased significantly. The fact that in the mean time specific antibody production rate was equally in-

creased, attests of a real increased specific antibody synthesis rate after the addition of butyric acid. The often supposed or shown relation between G1 cell cycle phase and high antibody synthesis (Suzuki and Ollis, 1990; Kromenaker and Srien, 1991; Al-Rubeai et al., 1992) seems to be confirmed by our experiment. However, the antibody secretion pathway must have been somewhat saturated after the butyrate stimulation, as revealed by the intracellular IgG accumulation, which is not in contradiction with biological data concerning secretion of proteins by animal cells (Lucocq and Warren, 1987; Takizawa et al., 1993). Although some of this information was already available in the field of molecular biology, our study is one of the first to show such data on cells cultured in the controlled environment of a bioreactor, and with the aim to stimulate their protein production.

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