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Growth limitation in hybridoma cell cultures: The role of inhibitory or toxic metabolites

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Abstract

Hybridoma cells usually grow to fairly low cell densities in batch cultures ($1–3 \times 10^6$ cells/ml). The reason for this is either that essential nutritional components of the medium are consumed, or that the cells produce some kind of inhibitory or toxic metabolite. This investigation presents evidence for the latter. Spent medium from cultures of hybridoma cells did not support growth of cells at lower cell densities ($1–3 \times 10^5$ cells/ml). The ability to support cell growth could not be restored by adding additional serum, energy sources (glucose, pyruvate) or L-glutamine. Furthermore, the consumption of amino acids could not account for this growth inhibition. On the contrary, the spent medium contained a substance that inhibited cell growth. This substance or metabolite was found in a fraction eluted from a gel filtration column when spent medium was applied to the column. This substance was found in the spent medium from all hybridoma and myeloma cell lines that were tested. The molecular weight of the substance was about 5 kD. Spent medium from two hybridoma cell lines also contained a substance that was eluted in the same fraction as albumin (67 kD). It is likely that this (or these) substance(s) is responsible for the growth limitation in hybridoma cell cultures.

Abbreviations: PBS – phosphate buffered saline

Introduction

Hybridoma cells are commercially important because they produce monoclonal antibodies. The antibodies are produced either *in vivo* (ascites fluid) or *in vitro* in various types of bioreactors. The concentration of antibody in the ascites fluid is normally much higher than that obtained in simple batch type bioreactors (stirred tanks, airlift etc.). Maximum cell densities in such types of reactors are typically $1–3 \times 10^6$ cells/ml. The reason for this is either that essential nutritional components are consumed or that the cells produce some kind of inhibitory or toxic compound. One way to overcome this problem has been to use perfusion type bioreactors where nutrients are added and undesirable compounds removed, while cells and antibody are retained in the reactor. Microporous membranes have been extensively used for that purpose, and the concentration of antibody in such systems may approach that of ascites fluid (Handa-Corrigan, 1988).

Lactate and ammonia are usually considered to be the most important waste products from cell

cultures (Glacken *et al.*, 1986). However, it is uncertain if accumulation of these products in the medium really is the reason for the growth limitation in hybridoma cell cultures (Thorpe *et al.*, 1987; Schlaeger and Schumpp, 1989; Rønning and Schartum, 1990). The present study was undertaken to investigate if hybridoma cells in batch cultures stop growing and start dying because (i) essential nutritional compounds have been depleted, or (ii) inhibitory or toxic compounds have accumulated in the growth medium.

Materials and methods

Cells and cell culture

The cell lines used in the present study are listed in Table 1. All cell lines were grown in Dulbeccos modified Eagles medium containing 25 mM HEPES buffer and 4.5 g/l glucose (GIBCO/BRL) and further supplemented with 10 or 15% fetal calf serum (Flow Laboratories), 2 mM L-glutamine (GIBCO/BRL), 1 mM Na-pyruvate (GIBCO/BRL) and antibiotics (Penicillin/streptomycin or kanamycin, GIBCO/BRL).

Amino acid analysis

Analysis of amino acid content in fresh and spent medium was performed on a BIOTRONIK LC 5001 amino acid analyser with the physiological fluids program and ninhydrin detection. Samples (80 µl) were deproteinized with 10% 5-sulphosalicylic acid (20 µl), left on ice for 30 minutes, and then centrifuged for 10 minutes in an Eppendorf centrifuge. The supernatant (75 µl) was mixed with sample dilution buffer (75 µl) and analysed.

Dialysis of spent medium

Cells were allowed to grow to maximum cell density as determined by direct cell counting with a haemocytometer. At this stage, the medium was centrifuged to remove the cells. The spent medium was then filled into dialysis tubing (Spectra pore) with MW cut off of 50,000 dalton and dialysed against fresh Dulbeccos medium (containing all components except serum), overnight at 4°C with stirring. The tubings were pretreated according to Sjøgren-Jannson and Jeansson (1985). After dialysis the medium was filtered through a sterile filter (0.2 µm).

Gel filtration of spent medium through Sephacryl S-200 or S-300

The spent medium was separated on a gel filtration column (Pharmacia, 26/40 column) packed with Sephacryl S-200 or S-300 (Pharmacia). The bed volume was 200 ml and the gel height was 40 cm. The column was equilibrated and eluted with Dulbeccos medium containing all components except serum. Ten millilitres of spent medium

Table 1. The cell lines used in the present investigation

Cell line	Reference	Origin	Product
AB 4	Kvalhein *et al.*(1987)	Mouse spleen × mouse myeloma	Monoclonal IgM
6C5	Lund *et al.* (1988)[a]	Mouse spleen × mouse myeloma	Monoclonal IgG
1H1	unpublished[a]	Human spleen × mouse/human heterhybridoma	Monoclonal IgG
3H12	unpublished[a]	Human spleen × mouse/human heterhybridoma	Monoclonal IgM
P3X63-Ag8.653	ATCC CRL 1580	Mouse myeloma	
K6H6/B5	Caroll *et al.* (1986)	Human × mouse heterhybridoma	
DAUDI	ATCC CCL 213	Human lymphoma	
Swiss 3T3	ATCC CCL 92	Mouse embryo fibroblasts	

[a] The cell line was established at SI (Center for Industrial Research).

was applied to the column and the flow rate was 2 ml/min. Fractions of 8 ml were collected from the time the spent medium was applied to the column. Each fraction was filtered through a sterile filter (0.2 μm) and supplemented with fetal calf serum to a final concentration of 10 or 15 % (depending on cell type).

Gel filtration of spent medium through Sephadex G-25

The spent medium from roller bottles (170 ml of a total of 200 ml) was separated on a gel filtration column (Pharmacia, 50/60 column) packed with Sephadex G-25 (Pharmacia). The bed volume was 550 ml and the gel height was 30 cm. The column was equilibrated and eluted with Dulbecco's medium containing all components except serum. Spent medium (170 ml) was applied to the column and the flow rate was 7 ml/min. Fractions of 34 ml were collected from the time the spent medium was applied to the column. The protein peak (170 ml in five fractions), as determined by UV-absorption at 280 nm, was collected, filtered through a sterile filter (0.2 μm) and returned to the roller bottle still containing all the cells in the remaining 30 ml of medium.

Relative cellular activity (MTT-test)

The relative cellular activity was measured with a colorimetric assay (Mosmann, 1983). Briefly, 0.1 ml of cell culture (in triplicate) was transferred to a microtiter plate containing 96 wells (Costar), added 10 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) solution (5 mg/ml in PBS) and incubated for 4 h at 37°C in a humidified CO_2-incubator. Thereafter, 0.2 ml of 0.04 N HCl in isopropanol was added to the wells to dissolve the blue crystals made by the living cells. The plates were centrifuged to remove protein precipitates and 200 μl of the supernatant was transferred to other wells and read at 590 nm in an "ELISA-reader" (ImmunoReader NJ-2000).

Quantification of monoclonal antibody content (ELISA)

The amount of monoclonal antibody in the supernatant was determined with a sandwich ELISA-technique using magnetic polystyrene particle as solid phase (Rønning and Christophersen, in press).

Results

Cell growth on spent medium

Figure 1 shows the growth curve for a mouse hybridoma cell line (AB 4), which is typical for these kind of cells: A rapid decline in viability occurs after maximum cell density is reached. In order to investigate this phenomenon further, the spent medium was taken from the AB 4 cell cultures at the time when cell density had passed maximum (day 3 in Fig. 1) and added to rapidly growing AB 4 cells at a lower cell density (1×10^5 cells/ml). Similarly, spent medium was taken from other cell cultures that had reached maximum cell density and added to rapidly growing AB 4 cells.

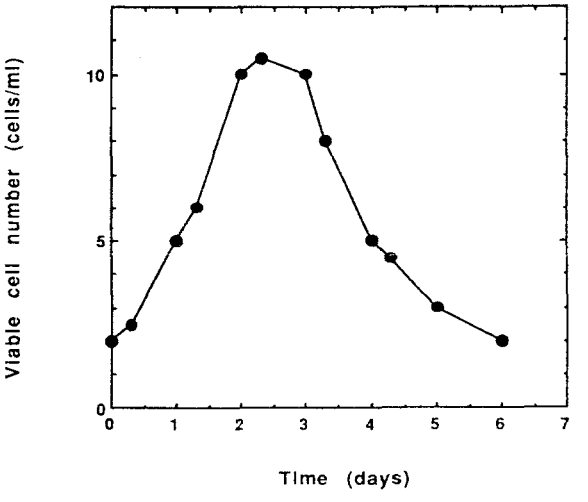

Fig. 1. Growth curve of AB 4 hybridoma cells. Cells were seeded at an initial density of 2×10^5 cells/ml in a tissue culture flask. Number of viable cells were counted in a haemocytometer after viable staining with trypan blue. Ordinate is $\times 10^5$.

Figure 2 shows the growth curves of AB 4 cells grown in these different kinds of spent media. When AB 4 cells were grown in fresh medium, cell density reached 1×10^6 cells/ml during a three day period, as in Fig. 1. However, when seeded into spent medium, the cells started dying immediately, even though the cells that were added were in a state of rapid growth and were seeded at a relatively low cell density. Thus, the cessation of cell growth is not the result of high cell density *per se*. The spent medium must either have been depleted of essential nutrients or growth factors, or contain metabolites that induce cell death. AB 4 cells were able to grow to some extent on the spent media from DAUDI or Swiss 3T3 cells, however, this was not the case when grown on the spent medium from myeloma cells, P3X63-Ag8.653 (the fusion partner of AB 4).

Figure 3 shows growth curves of AB 4 cells grown in spent medium dialysed against fresh medium (without serum). This treatment restored the ability of the spent medium to support cell growth. However, the dialysis treatment either

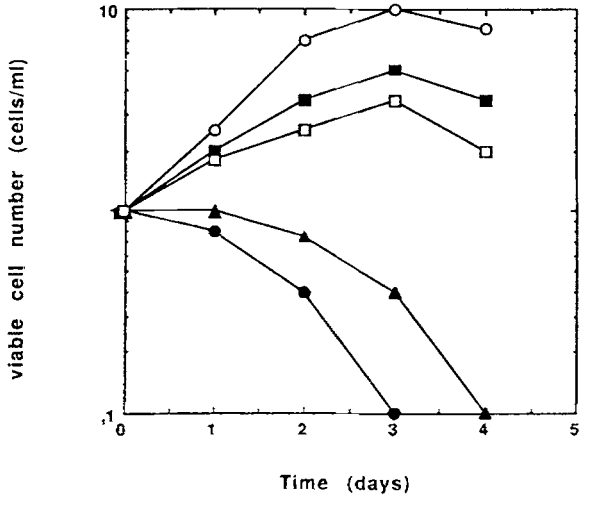

Fig. 2. Growth curves of AB 4 cells grown on spent medium from different cell lines. Cells were taken from the log phase of a culture and seeded at an initial density of 1×10^5 cells/ml in tissue culture flasks. The spent medium was taken from: (●) AB 4 cells, (❑) DAUDI cells, (■) Swiss 3T3 cells or (▲) P3X63-Ag8.653 cells. One flask received fresh medium (○). Number of viable cells were counted in a haemocytometer after viable staining with trypan blue. Ordinate is $\times 10^5$.

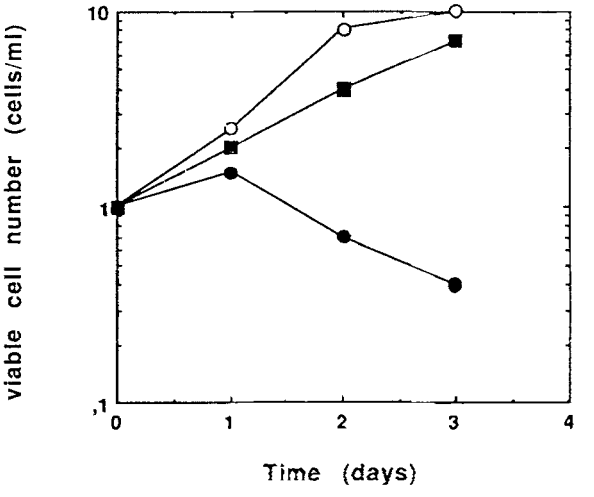

Fig. 3. Growth curves of AB 4 cells grown on different kinds of media. Cells were taken from the log phase of a culture and seeded at an initial density of 1×10^5 cells/ml in tissue culture flasks with different types of media: (●) spent medium, (■) spent medium dialysed against fresh medium, (○) fresh medium. Number of viable cells were counted in a haemocytometer after viable staining with trypan blue. Ordinate is $\times 10^5$.

provided potential depleted nutrients, or removed toxic metabolites, giving no answer to the question raised in the introduction. If the hybridoma cells (AB 4) died as a result of depletion of essential nutrients or growth factors, it should be possible to restore the growth supporting property by adding this (or these) substance(s) to the spent medium. However, neither additional serum, L-glutamine, Na-pyruvate or glucose did so (data not shown).

In order to investigate if some amino acids were depleted, the amino acid content was analysed both in fresh and spent medium. Table 2 shows the result of this analysis. There was no reduction of any amino acid that would account for the dramatic cell death in the culture. Two amino acids showed a significant increase, i.e. alanine and proline. In order to investigate if these amino acids caused inhibition of cell growth they were added to fresh medium (1.3 and 0.16 mM, respectively). However, no inhibition of cell growth was observed (data not shown).

The level of NH_4^+ increased by only 50% during the growth period, and it is therefore

Table 2. Amino acid content in fresh Dulbecco's medium and in spent medium from AB 4 cells

Compound	Fresh medium (μmol/l)	Spent medium (μmol/l)	% difference
ALA	160	1300	710
ARG	350	260	−25
ASP	11	19	72
CYS	130	82	−40
GLN	2000	650	−68
GLU	130	190	19
GLY	440	480	9.0
HIS	170	150	−12
ILE	730	570	−22
LEU	730	550	−25
LYS	750	650	−13
MET	170	120	−30
PHE	320	310	−3.1
PRO	33	155	370
SER	390	190	−51
THR	710	680	−4.2
TRP	67	43	−36
TYR	360	350	−2.8
VAL	680	580	−15
NH_4+	650	970	49

unlikely that the ammonium ions are responsible for the cell death (Table 2).

The above experiments were performed in tissue culture flasks without control of e.g. pH. The pH in the spent medium was 6.5. In order to investigate the effect of the lowered pH *per se*, experiments were performed where: a) The pH of the spent medium was increased to pH 7.2 by addition of NaOH or $NaHCO_3$ or; b) the pH of fresh medium was decreased to pH 6.5 by addition of HCl. The results of these experiments (Table 3) show that adjusting the pH of the spent medium to pH 7.2 resulted in some improved cellular activity after three days in culture. However, the activity was still far from that of fresh medium. On the contrary, decreasing the pH of fresh medium to pH 6.5 did not affect cellular activity significantly.

Fractionation of spent medium

Since there was no indication of severe depletion of any nutrients or growth factors, that would account for the toxic property of the spent medium, the medium was fractionated on a Sephacryl S-200 gel filtration column, to see if it was

Table 3. Cellular activity in cultures of P3X63-Ag8.653 cells in different types of media. 1×10^5 cells/ml were seeded into 1 ml of the different types of media in tissue culture wells (24 wells/plate). Cellular activity was measured with the MTT-test after three days in culture. The values are the mean from duplicate wells and did not deviate by more than 15%

Type of medium	Cellular activity (OD at 590 nm)
Dulbeccos, 15% serum, (pH 7.2)	0.61
Dulbeccos, 15% serum, pH 6.5[a]	0.57
Spent medium (pH 6.5)	0.21
Spent medium, pH 7.2 (NaOH)[b]	0.36
Spent medium, pH 7.2 ($NaHCO_3$)[c]	0.31
Spent medium, pH 6.5 (NaCl)[d]	0.19

[a]pH of medium adjusted to pH 6.5 by addition of 1M HCl.
[b]pH of spent medium adjusted to pH 7.2 by addition of 1M NaOH.
[c]pH of spent medium adjusted to pH 7.2 by addition of 1M $NaHCO_3$.
[d]1M NaCl added to the spent medium to give the same molarity as in [c].

possible to identify any fraction that contained inhibitory or toxic material. The fractions that were eluted from the column were used directly as growth medium for AB 4 cells after the addition of serum (additional 10%). The cells were seeded onto tissue culture plates (24 wells/plate, Costar). Figure 4a shows the cell number and viability of AB 4 cells after three days on the eluted fractions. The results show that the spent medium contained two inhibitory fractions (18 + 19 and 26).

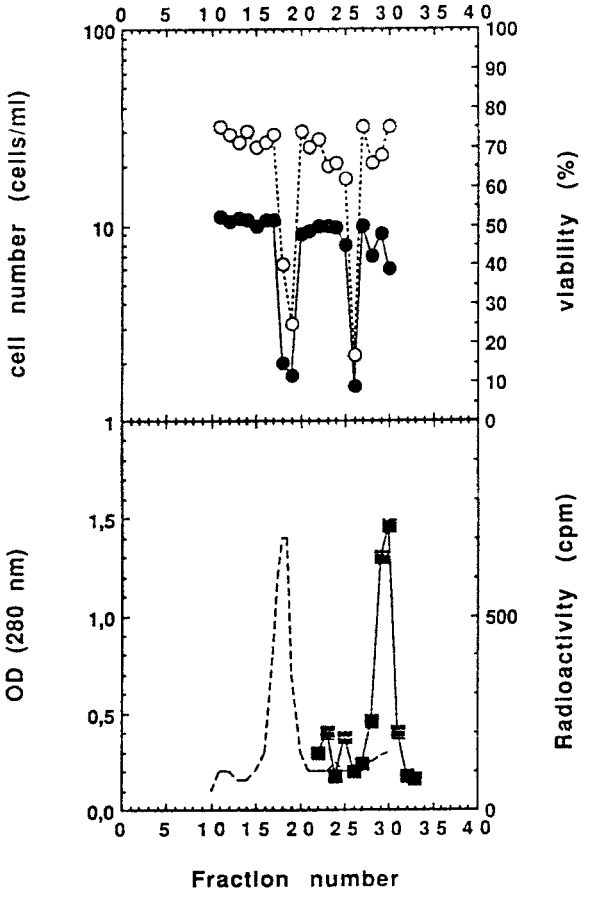

Fig. 4. (a; upper panel): Cell number and viability of AB 4 cells grown on spent medium fractionated on a sephacryl S-200 column. Each fraction, supplemented with 10% serum, was transferred to a tissue culture flask and inoculated with rapidly growing AB 4 cells to an initial density of 1×10^5 cells/ml. Cell number (●) and viability (○) was measured after three days by use of a haemocytometer after viable staining with trypan blue. Ordinate (left) is $\times 10^5$. (b; lower panel): Optical density (broken line) as continuously recorded in the eluted material, and radioactivity from [^{3}H]-thymidine (■) that was mixed with the spent medium prior to application to the column.

Sephacryl S-200 separates proteins in the range $5 \times 10^3 - 2.5 \times 10^5$ D. To keep track of the small molecular weight fraction ($< 5 \times 10^3$ D), trace amount of [^{3}H]-thymidine was added to the spent medium prior to addition to the column, and the radioactivity was counted in each fraction with a β-scintillation counter. Figure 4b show that the radioactivity was eluted in fractions 29 and 30, indicating that the small molecular compounds were found in these fractions. In addition, figure 4b show the optical density (OD) at 280 nm as continuously monitored in the eluted material. The highest absorbance was found in fraction 18. Thus, this fraction represent the molecular weight of albumin (67 kD) the main protein component of serum. The molecular weight of the two inhibitory fractions shown in Fig. 4a are thus 67 kD (as albumin) and close to the "salt fraction" (<5 kD).

In order to exclude the possibility that the inhibitory metabolites were produced only by the hybridoma cell line used above (AB 4), the spent medium from some other hybridoma and myeloma cell lines were fractionated in the same manner, except that Sephacryl S-300 was used. In these cases, the MTT-test was used to measure the cellular activity in each fraction. Figure 5 shows the results of these experiments. Spent medium from all the cell lines tested contained the low molecular weight inhibitory fraction (Figs. 5a and 5b), while only medium from the myeloma line, K6H6/B5, and the hybridoma cell lines, 3H12, (Fig. 5b), and AB 4, (Fig. 4a), contained the high molecular weight inhibitory fraction in addition.

Implication for large scale production

The observation that it was possible to separate the inhibitory or toxic metabolites from spent medium by gel filtration, led to the idea that perhaps a gel filtration column could be used in a production situation. In that case all the high molecular weight components had to be recirculated (growth factors, product, etc.), while the

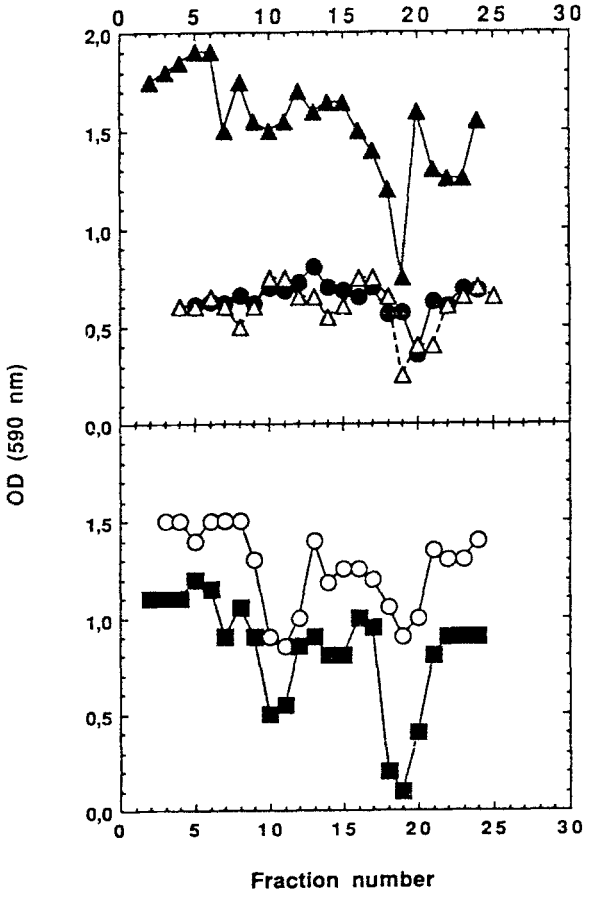

Fig. 5. Relative cellular activity (OD at 590 nm) in cultures of different cell types grown on spent medium (from the same cell type) fractionated on sephacryl S-300. One millilitre of each fraction, supplemented with serum, was transferred to a well of a tissue culture plate and inoculated with 1–2 × 10^5 (depending on cell type) cells/ml. The cellular activity was measured with the MTT-test after three days. (5a; upper panel): (●) 6C5, (▲) 1H1, (Δ) P3X63-Ag8.653; (5b; lower panel): (■) 3H12, (○) K6H6/B5.

small molecular weight components (containing most of the toxic metabolites) were excluded. Experiments were performed to mimic what might happen if a gel filtration column were to be used in conjunction with a large scale batch culture. In this case, the Sephacryl was exchanged for a gel used for group separations, e.g. Sephadex G-25 (a gel extensively used for desalting, buffer exchanges, etc). The purpose would be to discard the "salt fraction" containing inhibitory metabolites and recirculate the macromolecular fraction containing the antibodies and the growth factors in fresh basal medium.

Roller bottles were used as a model system, and mouse hybridoma cells (6C5) were allowed to grow to maximum cell density (2–3 × 10^6 cells/ml), which is usually achieved after 3 days if the starting concentration is 2 × 10^5 cells/ml. At this time four cell cultures (200 ml each) were centrifuged to concentrate the cells in 30 ml of medium (in the real case of a large scale culture, the cells have to be retained in the reactor by other means, e.g. immobilized in polymer beads, by continuous centrifugation, etc.). The remaining medium (170 ml) was: a) run through a Sephadex G-25 column as described in materials and methods and the macromolecular weight fraction returned to the cells; b) returned to the cells (untreated spent medium); c) replaced with fresh Dulbeccos medium, or; d) replaced with fresh Dulbeccos medium containing 15% serum.

Figure 6 shows the growth curves, of these

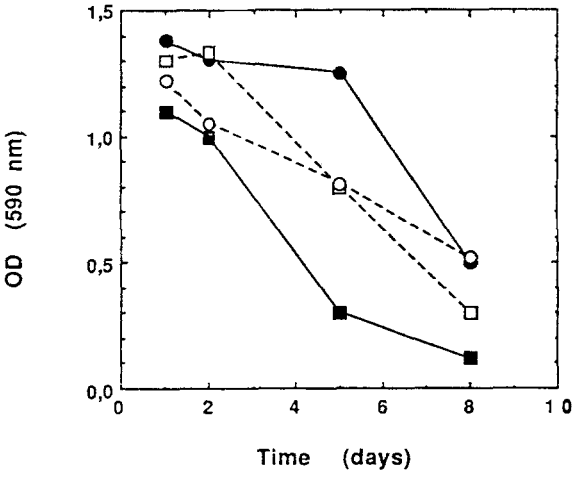

Fig. 6. Relative cellular activity (OD at 590 nm) in cultures of 6C5 cells grown in different kinds of media. Cells at maximum cell density (3 × 10^6 cells/ml) were harvested and reseeded at the same cell density into (■) the same medium (spent medium), (●) spent medium that had been run through Sephadex G-25, (❑) fresh medium with 15% serum, or (○) fresh medium without serum. Samples (0.1 ml in triplicate) were removed from the cultures at the times indicated and transferred to a tissue culture plate, and the cellular activity was measured with the MTT-test.

Table 4. Concentration of monoclonal antibody in cultures of 6c5 cells grown in different types of media. Day 0 is the day of treatment. The results are shown as mean ± SD of three different experiments

Type of medium	Concentration of antibody (μg/ml)	
	Day 0	Day 8
a. Treated by gel filtration	48 ± 9	146 ± 64
b. Untreated	62 ± 12	73 ± 9
c. Dulbeccos	0	67 ± 25
d. Dulbeccos + 15% FCS	0	81 ± 17

four cultures starting from the day of the above treatments (day 0). The culture with the untreated spent medium showed an immediate decline in cell viability, while the culture with the spent medium that had been passed through the Sephadex G-25 column showed an almost steady state value for 4 days before the decline started. The cultures given fresh medium showed values between these extremes. The concentration of antibody in the medium was measured when the cultures were made (on day 0) and at the end of the experiment (on day 8). Table 4 shows these values for the four cultures. In the culture with the untreated spent medium (b) there was an insignificant increase in antibody concentration, while in the culture with the spent medium treated by gel filtration (a) the concentration at day 8 was about twice that of the other cultures.

In the culture where the inhibitory factors had been removed by gel filtration, there was no further increase in cell number; however, the viability of the culture was prolonged for several days. This is illustrated in Fig. 7 where the growth curve, of the culture (a) and (b) in Fig. 6 are superimposed onto the curve representing a normal untreated culture (like Fig. 1). The curve for culture (b) (spent untreated medium) followed the declining part of the growth curve completely, while for culture (a) (medium treated by gel filtration) maximum cell viability was attained for a longer time period.

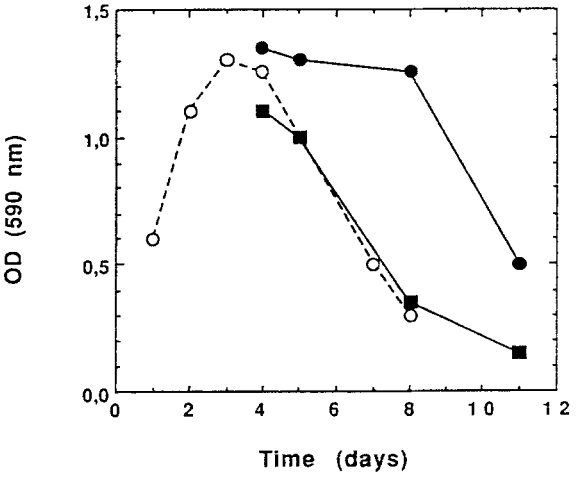

Fig. 7. Two curves from Fig. 6 are superimposed to a normal growth curve of 6C5 cells (○) to illustrate the prolonged lifespan of the culture grown on spent medium that had been run through a Sephadex G-25 column (●), compared to cultures grown on untreated, spent medium (■). For further details see legend to Fig. 6.

Discussion

It is well known that hybridoma cells fail to grow to higher cell densities than the order of 10^6 cells/ml. Despite considerable effort to understand the nutritional aspects of hybridoma cell growth (see e.g. recent reviews by Butler and Jenkins, 1989; Newland *et al.*, 1990), it is still not clear why the cells stop growing at this cell density. This investigation shows that it was not possible to restore the growth supporting property of the spent medium by supplying additional serum, L-glutamine, glucose or Na-pyruvate.

Furthermore, none of the amino acids were reduced to such low levels that it was likely they would be limiting to cell growth. In a study by Wagner *et al.* (1988) the amino acids were calculated to last for about three days when various animal cell lines were grown in Dulbeccos medium at a cell density of 10^6 cells/ml. Thus, it is likely that growth limitation of hybridoma cells is induced by inhibitory or toxic metabolites produced by the cells themselves. However, spent medium from the other cell lines tested (DAUDI and Swiss 3T3) did not inhibit growth of hybridoma cells to the same extent. This may indicate that the spent medium from myeloma or hybridoma cells are more toxic than media from cells of other origin, and that this may be one reason for the general observation that hybridoma cells are more sensitive to high cell densities than other cell lines.

The observation that it is possible to remove the toxic metabolites by dialysis is thus probably the reason for obtaining up to 10^7 cells/ml when the cells are grown inside dialysis tubing (Sjøgren-Jansson and Jeansson, 1985; Schumpp and Schlaeger, 1989) and also explains the success of dialysis based bioreactors (e.g. hollow fibers; micro encapsulation; and Comer *et al.*, 1990). While hollow fibers are well suited for removing toxic metabolites from continuous cultures, a gel filtration column can be used in a "batch" mode of growth. One passage of the spent medium through such a column may prolong the life span of the culture, doubling the antibody yield of the culture. Sterility in such a system has to be maintained by *in situ* sterilization prior to inoculation of medium and cells. The column can be sterilized separately and coupled to the reactor at the time of treatment. After treatment it may be uncoupled and cleaned immediately.

In the spent medium from all the hybridoma and myeloma cell lines that were tested, a substance of relatively low molecular weight that inhibited cell growth was identified. Three cell lines produced growth inhibitory substance that was eluted together with albumin. Either these cell lines also produce a high molecular weight inhibitory substance, or the low molecular weight substance in some cases may be conjugated or attached to albumin which often functions as a carrier molecule for waste products.

Lactate and ammonia are normally considered to be the most important growth inhibitors in hybridoma cell cultures (Glacken *et al.*, 1986; Newland *et al.*, 1990). However, lactate is normally not regarded as being responsible for cell death as long as pH is controlled, (Newland *et al.*, 1990). This is in line with the present investigation where the spent medium contained inhibitory activity even if the pH was adjusted to pH 7.2. In general, ammonia becomes inhibitory to cell growth at a concentration of >2 mM (Newland *et al.*, 1990).The level of NH_4^+ increased by only 50% in the spent medium (< 1 mM final concentration) making it unlikely that it accounted for the inhibitory effect.

Also Thorpe *et al.*, (1987) and Schlaeger and Schumpp (1989) have raised the question of whether lactate and ammonia alone account for the growth limitation and suggest that some other substance may be involved. Kidwell (1989) propose $TGF\beta_1$ as a potential inhibitory substance produced by some hybridoma cells. However, closer examination has to take place in order to resolve the nature of the growth inhibiting metabolite(s).

Acknowledgements

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