Effects on growth behavior in continuous hybridoma cell cultures: The role of viral contamination

Andrea Hawerkamp*, Dirk Lütkemeyer, Frank Gudermann, Anna Falkenhain,
Heino Büntemeyer & Jürgen Lehmann

Institute of Cell Culture Technology, University of Bielefeld, P.O. Box 100131, 33501 Bielefeld, Germany

E-mail: aha@zellkult.techfak.uni-bielefeld.de

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Abstract

This article describes the retrovirus expression with optimal nutrient supply and its potential growth inhibition effects in continuous hybridoma cell cultivation. A special reactor setup with total cell retention was developed to examine growth inhibition effects. Using this fermentation strategy we observed a decrease of viability cell rate which occurred at a defined state of the process despite sufficient nutrient supply. Therefore we assume that inhibitory substances are responsible for these effects. The molecular weight range of the inhibitory substances and the possible retrovirus cooperation of these growth inhibition effects were examined. To determine the molecular weight range we used the following methods: ultrafiltration, gel filtration, ultracentrifugation and gel electrophoresis. Furthermore, RT-PCR and western-/immunoblot are used to detect retrovirus particles in the supernatant and to show a retrovirus participation on growth inhibition effects. The possible growth modulation was tested in a biological assay (MTT-assay).

Abbreviations: BSA: Bovine Serum Albumine; E-MuLV: Ecotrope Murine Leukemia Virus; H₂O₂: Hydrogen Peroxide; IAP: Intracisternal A-type particle; K_S: Monod constant [mol L⁻¹] NaCl: Sodium Chloride; PBS: Phosphat Buffered Saline; TAE: Tris-Acetate-EDTA; TBS: Tris Buffered Saline; Tris: [Tris(hydroxymethyl)aminomethane]; Tris-HCl: [Tris(hydroxymethyl)aminomethane hydrochloride]; TTBS: Tris Buffered Saline + Tween 20.

Introduction

Hybridoma cells are commercially used for the production of monoclonal antibodies for diagnostic and therapeutic purposes. Typical cell densities in continuous perfusion fermentation processes are about 1×10^7 cells mL⁻¹. During this type of fermentation strategy a decrease of the viability can often be observed. Some plausible reasons for this are either the consumption of essential medium components or the production of some kind of inhibitory substances. Rønning et al. (1991) described two potential inhibitors with molecular weights of 67 and <5 kDa.

Another kind of potential inhibitory substances are IAP's (Intracisternal A-type Particles). Johnson et al. (1997) examined the relationship between IAP production, cell growth in culture and antibody production in murine hybridoma cultivation. They suggested that high levels of retrovirus particle production might inhibit cell growth and antibody yield significantly. Most of the commercially used murine hybridoma cell lines are known to contain retrovirus-like particles and in many cases infectious c-type murine leukemia viruses (MLV) which can be either ecotropic (infectious for cell lines of murine origin or closely related species) or xenotropic (infectious for species other than murine (Froud et al., 1997)). These particles are derived from MuLV-related sequences inserted into the mouse genome. Sequences belonging

* Author for all correspondence.

to the ecotropic and xenotropic group can produce replication-competent viral particles (Irving et al., 1993). The level of those murine retroviruses particles during batch fermentation (Froud et al., 1997) followed the viable cell density. It appeared that maximum titres of virus particles were found in the exponential growth phase of the cells at optimum nutrient supply. Reduction of nutrient supply caused a decrease of virus titres.

In accordance with these results we examined the virus expression and a potential growth inhibition effect of retrovirus-like particles in a continuous perfusion fermentation process. Therefore a special reactor setup (Figure 1) was developed which guaranteed a sufficient nutrient supply and the continuous removal of waste products.

Materials and methods

Cell cultures

The cell line used in the present study was the rat-mouse hybridoma 187.1 (ATCC, HB 58) cultured in a serum free medium (Jäger et al., 1988) based on DMEM/F12 (1:1) with human transferrin (5 mg L^{-1}) and bovine insulin (5 mg L^{-1}). The amino acids were supplemented to necessary concentrations (see also Figure 3).

Bioreactor

The cultivation was performed in a 2 litre reactor (Figure 1), Biostat BF 2 (B. Braun Biotech International) with bubble free membrane aeration and internal perfusion membrane (Lehmann et al., 1988). This perfusion membrane enables the medium feed and to harvest the cell free supernatant. The bioreactor was equipped with separate cooled reservoirs for glutamine and basal medium. Glutamine (100 mmol L^{-1}) was added according to its consumption by the cells and kept on a concentration above $250 \mu\text{mol L}^{-1}$ ($K_s = 30\text{--}70 \mu\text{mol L}^{-1}$; Heidemann et al., 1998).

Fermentation process

After inoculation the perfusion fermentation process (Figure 2) was started with a dilution rate of $D = 1.75 \text{ d}^{-1}$. When reaching a viable cell density of $1 \times 10^6 \text{ cells mL}^{-1}$ (24–48 hr after inoculation) the dilution rate was reduced ($D = 0.26 \text{ d}^{-1}$) and the pulsed glutamine feed was started. Cell free supernatant was

collected over 24 hr and harvested (HAR1-HAR8, 500 mL per day). Furthermore, 10 mL of cell containing sample were taken once a day, centrifugated and the supernatant was used for RT-PCR analysis (P1-P8). Throughout the complete fermentation process optimal nutrient supply was maintained by daily monitoring of substrates and concentration of metabolic products. Free amino acids were analysed by means of an automated reversed phase HPLC system DS 450 (Kontron GmbH) with precolumn derivatization using the OPA method (Büntemeyer et al., 1991) to determine all of the amino acids except for proline, cysteine and the dipeptide cystine. Inhibitory effects of ammonia were avoided by external consumption-dependent glutamine feed.

Purification

The harvests were concentrated by ultrafiltration (Ultra-sette, cut off: 1 kDa, Pall). The molecular weight range of the growth inhibitory substances were determined by separation of the concentrated harvest (8 mL) on a Superdex 30 (separation range: $<10 \text{ kDa}$) gelfiltration column C 16/100 with 120 mL gel volume and on a Sephacryl S-1000 (separation range: $>600 \text{ kDa}$) C 16/100 with also 120 mL gel volume (both Amersham Pharmacia Biotech). The determination of the void volume (V_0) of Superdex 30 was follows by Blue Dextran (molecular weight: 2000 Da, Sigma). Determination of the void volume of Sephacryl S-1000 was not possible due to the separation range of 10^8 Da . The total volume of these two columns were specified by using acetone (molecular weight: 54 Da). Each column was equilibrated with 20 mM phosphate and 150 mM NaCl, pH 7.2. Fractions of 5 mL were collected starting with the beginning of sample application. All separations were performed at 7°C with a BioPilot-System (Amersham Pharmacia Biotech).

Native PAGE

The inhibitory fractions were separated by native PAGE, PhastGel Gradient 4–15 (Amersham Pharmacia Biotech). Separation and the following silver staining (Heukeshoven and Dernick, 1988) were performed with the PhastSystem (Amersham Pharmacia Biotech). A high molecular weight marker (Thyroglobulin: 669 kDa, Ferritin: 440 kDa, Catalase 232 kDa, Lactate Dehydrogenase: 140 kDa and BSA: 67 kDa from Amersham Pharmacia Biotech) was used

Figure 1. Setup of a 2 L perfusion bioreactor BIOSTAT BF 2 (B. Braun Biotech International). The reactor is equipped with a cooled medium reservoir and an additional consumption-dependent glutamine feed. The medium feed and the harvest of cell free supernatant are made possible by an internal perfusion membrane.

as a reference to determine the molecular weight of the inhibitory substances.

Western blot

The western blot was performed with Trans-Blot Electrophoretic Transfer Cell (Biorad). As blot membrane we used nitrocellulose (Schleicher and Schüll) with a pore size of $0.2 \mu\text{m}$. The transfer-buffer was: 25 mM Tris, 192 mM Glycine and 20% Methanol (pH 8.3). The blotting conditions were 120–140 V (0.8–0.9 A) for 3 hr.

Immunoblot

After the western blot procedure the membrane was first washed for 3×10 min with wash solution (2). In next step the membrane was blocked for 1 hr by using solution 3. Afterwards a washing step (2×10 min, solution 2) was followed. The incubation with the first antibody was performed for 2 hr. The washing step was repeated (3×10 min, solution 2) and the incubation (1 hr) with the second antibody followed. Another

washing step (3×10 min with solution 2 and 10 min with solution 1) was performed. At least the membrane was treated with the developer.

Solutions:

1. 20 mM Tris-HCl, 500 mM NaCl, pH 7.5 (TBS)
2. Wash Solution: 20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20 (TTBS)
3. Blocking Solution: TBS and 1% BSA
4. Antibody Buffer: TTBS and 1% BSA
5. Developer: 10 mg 4-chloro-1-naphtol, 3.3 mL methanol, 16.7 mL TBS and $10 \mu\text{L}$ 30% H_2O_2

Antibody-Dilutions:

1. Polyclonal-Antibody: Goat-Anti-Mouse-E-MuLV (1:500)
2. Monoclonal-Antibody: Anti-Goat-IgG-Peroxidase-Conjugate from mouse (Sigma), (1:1000)

Figure 2. Time course of a continuous perfusion fermentation process with glutamine feed. After inoculation the perfusion process was started with a dilution rate of $D = 1.75 \text{ d}^{-1}$. When reaching a viable cell density of $1 \times 10^6 \text{ cells mL}^{-1}$ (24–48 hr after inoculation) the dilution rate was reduced ($D = 0.26 \text{ d}^{-1}$) and the pulsed glutamine feed was started. Cell free supernatant was collected over 24 hr and harvested (HAR1-HAR8, 500 mL per day). Furthermore, 10 mL cell containing sample were taken once a day, centrifuged and the supernatant used for RT-PCR analysis (P1-P8). The figure shows, that the viability decreased at day 5. Preliminary analysis demonstrated an inhibitory effect in HAR 5 to HAR 8.

Bioassay

To analyse inhibitory effects two test systems, the MTT-assay and the $[^3\text{H}]$ Thymidinincorporation (Andersson et al., 1972) are established in our institute. For our examinations we chose the MTT-assay which determines the activity of dehydrogenases by colorimetric reaction (Mossman, 1983; Hansen et al., 1989) and therefore, it is an indirect measure of cell growth activity. This measured activity is independent from the DNA-Synthesis (the S-Phase of cell cycle) in contrast to $[^3\text{H}]$ Thymidinincorporation. The gelfiltration fractions ($0.2 \mu\text{m}$ filtered) were diluted 1:1 with fresh medium and inoculated with cells ($1 \times 10^6 \text{ cells mL}^{-1}$) on microtiter plates (Nunc). After a proliferation period of two days, $10 \mu\text{L}$ MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) solution (5 mg mL^{-1} in PBS) was added and the plate was incubated for 3 hr at 37°C in a CO_2 -incubator. Then $100 \mu\text{L}$ of 40 mL N,N-dimethylformamid, 40 mL dest. H_2O and 20 g SDS (Sigma) were added to each well to dissolve the blue crystals formed by the living cells. The intensity of the tinge was measured at 570 nm in an microplate-reader (Modul Biotek).

Isolation of RNA

First the cell supernatant (10 mL) samples P1 to P7 were ultracentrifugated ($40000 \times g$, 20 min, 4°C) and the pellets (1 mL) were used for RNA-Isolation with QIAamp Viral RNA kit (Qiagen GmbH). The inhibitory fractions 11 (Superdex 30) and 21 (Sephacryl S-1000) were used for RNA-Isolation without pre-preparation (no concentration with ultracentrifugation). Additional supernatant samples from Mink lung (ATCC: CCL64), Mus dunni (ATCC: CRL 2017) and a medium sample were used as negative controls.

RT-PCR

Specific primer (Life Technologies) for detection of murine leukemia viruses (E-MuLV and MCF) were used (Irving et al., 1993) in RT-PCR (PCR kit, Perkin Elmer). For visualization, $4 \mu\text{L}$ of the amplification products were separated by gel electrophoresis on 3% agarose (Sigma) in a TAE buffer containing ethidium bromide (Figures 9a and b).

Ultracentrifugation

The fractions 5 (control) and 12 (inhibitory) were ultracentrifugated ($198000 \times g$, 5 hr, 4°C) by using

Figure 3. Amino acid concentrations in the 2-Liter bioreactor during perfusion fermentation with HB58 over a 17 days cultivation process. No limitation could be observed. Glutamine was always kept on a concentration above $250 \mu\text{mol L}^{-1}$.

Figure 4. Purification scheme to determine the molecular weight range of the inhibitory substances.

Figure 5. Results of the MTT-assay with Superdex 30 fractions of HAR 6-concentrate. This test determines the metabolic state of the cells. Therefore, it is an indirect measure of cell growth activity in % relating to fresh medium and buffer. A drastic inhibitory effect was observed by the fractions 10–14. These fractions were collected from the void volume ($V_0 = 60$ mL) of the Superdex 30 column ($V_t =$ total volume, 130 mL) which indicates a molecular weight of the inhibitory substances above 10 kDa.

Figure 6. Results of the MTT-assay with Sephacryl S-1000 fractions of HAR 7-concentrate. A Sephacryl S-1000 column was used to determine the molecular weight range of the inhibiting substances. The fractions 20–23 showed an cell growth inhibiting effect relating to fresh medium and buffer. The range between 100 and 125 mL is the same for the elution of inhibitory substances and Blue Dextran. The possible molecular weight could therefore be reduced to the range between 600 and 2000 kDa.

Figure 7. Native PAGE, PhastGel Gradient (4–15) of the inhibitory fractions 19 and 20 from the Sephacryl S-1000 column. The fractions show a approx. 600 kDa complex and DNA.

Figure 8. Activities of Superdex 30 fractions 5 and 12 before and after ultracentrifugation. Fraction 5 showed a constant activity of approx. 100%. At first the activity of fraction 12 was 65% of the control. After ultracentrifugation the supernatant showed an activity of 120%, whereas the pellet caused a significant inhibitory effect (65% activity).

Figure 9. RT-PCR of fermentation samples P1 to P7 and the inhibitory fractions 11 (Superdex 30) and 21 (Sephacryl S-1000) for detection of E-MuLV and MCF. The figures show that all supernatant samples, P1 to P7 and the inhibitory fractions (F 11 and F 21) were E-MuLV-(9b) and MCF-(9a) positive. From P1 to P7 an increasing signal could be observed, particularly for the E-MuLV. The samples from Mink lung, Mus dunni and the medium sample are retrovirus negative.

Sorvall OTD 50 (Rotor: TH 641). The resulting supernatants and pellets were analysed in the MTT-assay.

Results

Cell growth during perfusion fermentation

To examine growth inhibition effects and the expression of retrovirus-like particles we use HB 58 in

continuous perfusion fermentation process. A typical growth curve (Figure 2) for this hybridoma cell line shows a viability rate of more than 90% during the first 4 days. After day 4 the viability decreased rapidly to 33% at day 8. The maximal viable cell density is reached at 6×10^6 cells mL^{-1} between day 4 and 5. From day 5 to day 8 the viable cell density is relatively constant between $4\text{--}5 \times 10^6$ cells mL^{-1} , whereas the viability decreases. All substrates were monitored throughout the complete fermentation

Figure 10. Results of the western blot and the following immunoblot with polyclonal serum against E-MuLV. The left lane of the figure shows the marker after western blot and coomassie blue staining. The marked approx. 600 kDa complex is shown on the right line.

process and no growth inhibition level of ammonia ($<6 \text{ mmol L}^{-1}$ at the end of the fermentation) and lactate ($<30 \text{ mmol L}^{-1}$ at the end of the fermentation) was measured. A growth inhibition effect caused by glucose (data not shown) and amino acid (Figure 3) limitations was not observed.

Determination of the molecular weight of the inhibitory substances

The growth inhibitors already described in literature belong to the class of peptides (Paukovits and Laerum, 1982; Elgjo and Reichelt, 1984) and proteins (Hsu and Wang, 1986; Wells and Mallucci, 1983) like TGF- β (Transforming growth factor- β) (Tucker et al., 1984; Sporn and Roberts, 1992) with a native molecular weight of 25 kDa. In accordance with these references we developed a purification strategy (Figure 4) to determine the molecular weight range of the inhibitory substances. Using this strategy the daily cell free supernatants (HAR5-HAR8) were first concentrated (1:5) by ultrafiltration and then separated on Superdex 30.

The resulting fractions of the Superdex 30 column (separation range: $<10 \text{ kDa}$) were used in the MTT-assay to measure the cellular activity in each fraction. Figure 5 shows the results of this MTT-assay. A drastic inhibitory effect was observed in the fractions 10–14, collected from the void volume (V_0) of the Superdex 30 column.

This indicates that the inhibitory substance has a molecular weight above 10 kDa. For more exact determination we used a Sephacryl S-1000 column with a separation range of 5×10^5 – 10^8 Da . Figure 6 shows the activity of the Sephacryl S-1000 fractions of HAR 7-concentrate in the MTT-assay. The fractions 20–23 showed an inhibitory effect. The elution of the inhibitory substances and Blue Dextran are simultaneously between 100 and 125 mL. Therefore we can reduced to the molecular weight range between 600 and 2000 kDa. Apart from other biomolecules, the gel electrophoresis (native PAGE, PhastGel Gradient 4–15, Amersham Pharmacia Biotech) of the inhibitory fractions demonstrated (Figure 7) the existence of a complex with a molecular weight of approx. 600 kDa.

Further analysis (DNase treatment) show that these fractions also contain DNA (data not shown).

Further examination demonstrated the possibility of concentrating the inhibitory substances by using ultracentrifugation. The fractions 5 (control) and 12 (inhibitory) were ultracentrifugated. Afterwards the resulting supernatants and pellets were analysed in the MTT-assay. The results are shown in Figure 8. The fraction 5 had a constant activity of approx. 100%. The activity of fraction 12 was 65% of the control. After ultracentrifugation the supernatant showed an activity of 120%, whereas the pellet caused a significant inhibitory effect (65% activity). These results document the high molecular weight of the inhibitory substances and the possibility of concentrating by forced sedimentation.

Participation of retroviruses on growth inhibition effects

The cell line HB58 with the myeloma part P3X63Ag8.653 is known to contain E-MuLV, MCF and IAP's. To determine whether there are these type of particles present in the supernatant, fermentation samples P1 to P7 (see Figure 2) and the inhibitory fractions 11 (Superdex 30) and 21 (Sephacryl S-1000) were used for the detection of E-MuLV and MCF virus. Additional supernatant samples from Mink lung, Mus dunni and a medium sample were employed as retrovirus-negative references. Figures 9a and b show that all supernatant samples, P1 to P7 and the inhibitory fractions (fraction 11 and 21) were E-MuLV and MCF virus positive. From P1 to P7 an increase of the signal could be observed, particularly for E-MuLV.

To indicate whether the approx. 600 kDa complex (native PAGE, PhastGel Gradient 4–15) is virus origin we use western blot and immunoblot with polyclonal goat-anti-mouse serum against E-MuLV membrane glycoproteins. Figure 10 shows the E-MuLV origin of this complex.

Summary and conclusion

During this type of perfusion fermentation process the viability decreased in spite of optimal nutrient supply. Inhibitory fractions could only be derived from harvests taken after this decrease in viability. Examination of the harvest demonstrated that an isolation of the inhibitory substances is possible. By using gel filtration and gel electrophoresis a complex with a

molecular weight of approximately 600 kDa could be determined. It seems that this complex is E-MuLV origin. Furthermore two types of viruses, E-MuLV and MCF were detected in the supernatant. An increasing E-MuLV and MCF signal (RNA-content) could be observed in the supernatant throughout the whole fermentation. The inhibitory fractions were also MCF- and E-MuLV-positive. Therefore, we assume a correlation between the retrovirus-expression in continuous fermentation processes and the observed growth modulating effect. Further studies will aim to verify the involvement of retroviruses in growth inhibition processes.

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