PRODUCTION OF MONOCLONAL ANTIBODIES CB IFN 2.4 IN THE HOLLOW FIBER BIOREACTORS ACUSYST-R AND SACCEL

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ABSTRACT

Monoclonal antibodies (MAbs) are produced in mice by the intraperitoneal injection of the MAb-producing hybridoma cells. The laws for animal protection and the regulations required for the production of MAbs for human use are more and more strict and have lead to develop new techniques to obtain these molecules. In this paper we compare a new bioreactor system: the cell culture automatic system (SACCEL) (ICID, Havana) with a well known commercial system: ACUSYST-R (Endotronics, Minneappolis, USA). Taking into account quality, subclass and immunopurification performance we did not obtain significant differences regarding the bulk harvest yield and MAb quality when we used the MAb CB IFN 2.4. We reduced the medium pump rate in relation to MAb production and found that even a 50 % reduction of the culture medium consumption did not affect the specific antibody production. This result represents a 13 % reduction of the production cost per unit.

Key words: SACCEL, ACUSYST-R, hybridoma, monoclonal antibody, hollow fiber

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RESUMEN

Los anticuerpos monoclonales (AcMs) se obtienen en ratones mediante la inyección intraperitonial de hibridomas productores de AcMs. Las leyes para la protección de animales, y los elementos regulatorios requeridos para la producción de éstos para uso humano son cada vez más estrictos, por lo que se han desarrollado nuevas técnicas de producción: el cultivo celular en sistema de fibra hueca. En este trabajo comparamos un nuevo biorreactor: SACCEL, (ICID, Habana) con el sistema comercial ACUSYST-R (Endotronics, Minneappolis, USA). La cosecha, subclase, comportamiento en inmunopurificación y calidad del AcM fueron similares cuando se utilizó el AcM CB IFN 2,4. Se redujo el consumo de medio de cultivo estableciendo una estrategia de suministro programado de glucosa. Se comprobó que un 50 % de reducción de este medio no afecta la producción de anticuerpos para la línea celular CB IFN 2.4. Este resultado representa una reducción de los costos de producción por unidad de un 13 % respecto a corridas anteriores utilizando biorreactores ACUSYST-R.

Palabras claves: SACCEL, ACCUSYST-R, hibridoma, anticuerpo monoclonal, fibra hueca

Introduction

The development of large scale cell culture technologies is important for the production of monoclonal antibodies (MAbs) and other therapeutic proteins in genetically modified mammalian cells.

For large scale mammalian cell cultures, conventional fermentors are used, however, other types of reactors have been developed. The selection of an appropriate bioreactor to be used in the manufacturing process is essential to establish an economical production of the protein. Some researchers have examined certain factors that affect the choice of bioreactors: its dimension, characteristics of the selected cell lines and optimum fiber spacing (1).

Hollow fiber systems were described by Knazek RA (2), and are applied for large scale MAb production from culture supernatants of hybridoma cells (3-6). This technology imitates more closely the mammalian *in vivo* environment (with its circulatory system of capillaries and lymphatic vessels) than the static cell culture systems or fermentation technologies. Besides, it offers several advantages for the large scale cell culture:

- space efficient instrumentation
- potentiality to obtain high cell densities (10^8 cells/mL)
- separation of inexpensive feed medium from expensive serum components and the secreted product
- constant harvest of the concentrated product
- ability to reduce the viral source of contamination (it is possible to harvest MAbs in one system without laboratory animals and, in

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some cases, eliminate the fetal bovine serum as a growth factor)

- CB IFN 2.4 MAbs were produced using a new bioreactor system: cell culture automatic system (SACCEL) (ICID, Havana), and compared with ACUSYST-R (Endotronics, Minneappolis, USA). Both bioreactors were used at a laboratory scale for the production of biomolecules. The MAb yield was not affected when the medium pump rate was reduced keeping the glucose perfusion rate equal to its consumption rate, based on a previous report where cultivation of cells on microcarriers was used (7).

Materials and Methods

Cell line

The mouse hybridoma cell line CB IFN 2.4 (8), secreting IgG1 MAbs against recombinant human alpha 2b interferon was put in the bioreactor to grow in 2.5 % of fetal calf serum. Prior to cell inoculation into bioreactors, the cell line was screened showing no mycoplasma, bacteria or fungi contamination.

Bioreactors

Two standard hollow fiber systems were used, including the ultrafiltrate fiber set (Endotronics), with a molecular weight cutoff of 10'000 Da. One unit was placed in ACUSYST-R (9) and the other in SACCEL (10). In both cases the operation procedure, installation, cell inoculation and process control were performed according to the manufacturer's instructions.

Culture media and cell inoculation

RPMI 1640 (GIBCO), supplemented with 2 mM glutamine, 1 mM sodium piruvate, sodium bicarbonate 2.5 g/L, 2.8×10^3 g/L 2-mercaptoetanol, 100 mg/mL streptomycin and 10^4 UI/mL penicillin (all from Sigma, St. Louis) was used for the intracapillary space (ICS). The same medium, including 2.5 % of fetal calf serum (Tecnomara) was employed for the extracapillary space (ECS). The 0.67 m^2 bioreactors were inoculated with 290×10^6 cells and their viability was > 92 %.

Metabolic parameters

Samples were drawn periodically and analyzed for pH, glucose concentration, lactate and IgG. An external pH monitor (Corning) was used and pH was adjusted daily.

The circulation rate was initiated at 100 mL/min and increased up to 300 mL/min. Harvest began at 2 mL/hr on the second day using the same flowrate of ECS factor pump. This harvest rate was increased up to 4 mL/hr.

Measurement of MAbs concentration

An ELISA was performed (8). Microtiter plates were coated with 5 mg/mL of recombinant alpha 2b interferon in an appropriate buffer (0.1 M $NaHCO_3$ pH 9.6) and incubated for 20 min at 50 °C in a wet chamber. After washing with phosphate buffered saline plus 0.05 % Tween 20 (PBS-Tween), samples were added to the dilution from 1/8000 to 1/16000 in the blocking solution (PBS + 0.5 % milk + 0.5 % Tween 20) and incubated at room temperature (RT) for 2 h in a wet chamber. The samples were washed ten times as recommended. An affinity purified antimouse IgG conjugate with horseradish peroxidase (HRP) (Sigma) was diluted 1:1500 in the blocking solution, incubated at RT for 1 h in a wet chamber, and washed ten times again. Reactions were developed using H_2O_2 and orthophenylendiamine in phosphate/citrate buffer at pH 5.0. The reaction was stopped after incubation at RT for 15 min by adding a 50 mL/well of 2.5 M H_2SO_4. The reaction was measured at 492 nm in a microplate reader Multiskan (Flow).

Detection of glucose and lactate

Glucose and lactate concentrations were detected using appropriate diagnostic kits (from Sigma Diagnostics) according to the supplier's indications.

Reference run

The same cell line, culture medium conditions and cell inoculation procedures were used in the reference experiment using ACUSYST-R bioreactors. The metabolic parameter was analyzed using a similar test. The perfusion rate was increased according to the manufacturer's recommendations.

MAb purification from bioreactor supernatants

MAbs were purified from the bulk harvest of each bioreactor. Bioreactor supernatants were centrifuged (Hitachi 7RS) at low speed (7 000 g for 10 min). Two ammonium sulfate (45 % sat), (MERCK) precipitations were obtained by low stirring for 30 min. The pellet was recovered by centrifugation at 9 200 g for 20 min at 4 °C. The pellet was dissolved in 500 mL of phosphate buffered saline (PBS) 150 mM pH 8 and, after that, it was desalted by gel filtration in Sephadex G-25 coarse (Pharmacia) in a column (Pharmacia) at a linear flow of 130 cm/h. After semipurification Protein A affinity chromatography was used using glycin 1.5 M/NaCl 3 M pH 8.9 (MERCK) as the adsorption buffer and citric acid 0.2 M pH 6 (MERCK) as the elution buffer in column (BP 113/15) (Pharmacia) at 27 cm/h. The eluted fraction of the protein A affinity chromatography after previous desalting by gel filtration was purified by ionic exchange using DE 52 Cellulose

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(Whatman International Ltd, Madison, UK) in order to reduce the mouse DNA level in the final preparation in a column (XK 26/40) (Pharmacia) using tris-hydroxi-aminomethane, (MERCK) buffer 20 mM pH 7.6 as adsorption buffer and the immunoglobulins and DNA were eluted using a NaCl (MERCK) discontinuous gradient at 70 mm and 500 mM respectively.

Determination of mouse DNA content

Mouse DNA content was determined by hybridization analysis using as P^{32} a labeled mouse DNA probe in a range from 4 pg to 1 ng as specified by Sambrook J (11).

Determination of total protein concentration

Protein concentration was determined according to Lowry O (12).

SDS-PAGE

SDS-PAGE was performed according to the established procedure (13).

Immunoadsorption

The antibodies purified from both bioreactors were coupled to sepharose Cl-4B (Pharmacia) (14). The immunosorbents were spiked with recombinant human alpha 2b interferon to evaluate their adsorption and elution capacity and the release of IgG under the drastic elution condition.

Results and Discussion

In this report we established a strategy to decrease the consumption of glucose, based on the hypothesis that the consumption of this metabolite is affected proportionally by its concentration at any defined run time; also, different reports (7, 15) presume that, in batch cultivation, the glucose concentration can be manipulated at a low level by the programmed feeding of glucose, besides, they demonstrate that the glucose consumption rate and the fraction of glucose converted to lactate can be reduced and oxidation of glutamine increased and that even using hollow fiber bioreactors it is also possible to regulate a culture perfusion system.

To reduce the lactate production and optimize the consumption of glucose in bioreactors, the glucose perfusion rate and its consumption rate were evened to obtain similar results. According to this, we kept a minimal concentration of glucose (below 0.25 mg/mL) during the run time in the ICS and ECS. Figure 1 shows the results when this strategy was used, demonstrating the proportionality between the glucose perfusion rate parameter and the glucose consumption rate.

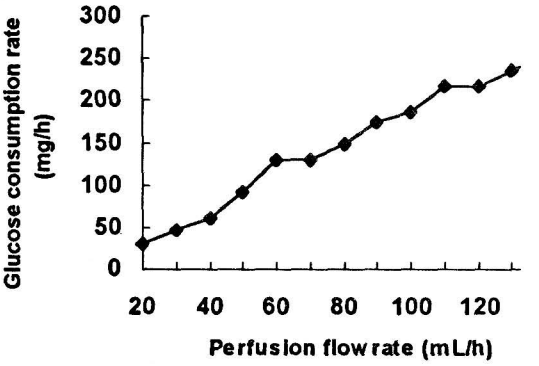

Figure 1. Variation of the perfusion rate according to the glucose consumption rate (SACCEL).

Figure 2 shows the glucose uptake rate (GUR) for both runs. The curves are very similar; nevertheless, in culture day 19th, the ACUSYST-R had a GUR fall, caused by a cultureware system failure. Lactate levels were always kept below 15 mM (Figure 3). This result suggests the possibility of managing this parameter keeping the level of perfusion rate similar to the consumption glucose rate.

Figure 4 shows the accumulative production of specific MAbs for both systems. Initially, the higher amount of IgG was detected in the ACUSYST-R, however, after 30 days in culture, the SACCEL system accumulated a higher IgG level. This performance is probably a consequence of the particularities of each system. The final results for both bioreactors were similar, about 3 g of active IgG.

The strategy to reduce the glucose consumption and lactate production by cells, in 50 %, according to an old reference run (R-I) that implies a significant reduction in the final culture medium consumption, is shown in Figure 5.

A total IgG production in both bioreactors (4.47

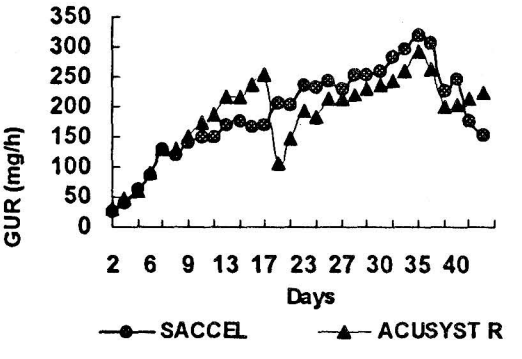

Figure 2. Glucose uptake rate. The profiles were similar for both bioreators except in the 19th culture day when the ACUSYST-R had a fall caused by a cultureware system failure.

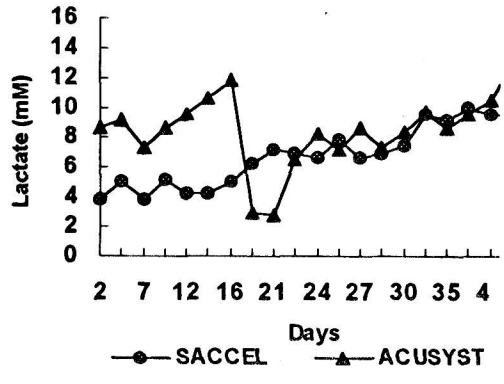

Figure 3. Variation of lactate concentration in ECS. Lactate levels were always kept below a critical value of 15 mM.

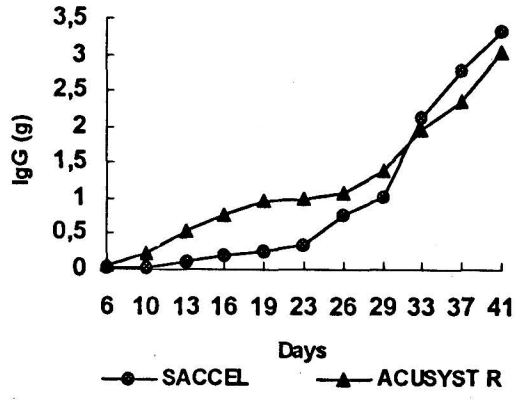

Figure 4. Accumulative productions of MAbs. The final result for both bioreactors was similar (about 3 g of active IgG).

and 3.15 for SACCEL and ACUSYST-R, respectively) correlated quite well with previous experiences with this cell line in an ACUSYST-R bioreactor (4.08, R-I). We found that it was possible to reduce the perfusion rate of the culture medium without affecting production, at least for this cell line.

Cells were cloned by a limited diluted method, and screened twice for specific IgG production: prior to its inoculation into bioreactors and at the end of the run. In both cases, the values were analogous, 98.4 % of positive clones for ACUSYST-R and 100 % for SACCEL at the end of the run; considering 100 % of positiveness for the initial population. This demonstrated that the designed strategy did not affect the specific yield of MAbs production using these hollow fiber bioreactors and this murine hybridoma cell line.

We did not obtain significant differences between the MAbs produced in both bioreactors in comparison with reference data of MAbs purified from ascitic fluid using the same cell line (Table 1). Both antibodies were IgG 1 and maintained a similar performance in the immunopurification process and the yield of the cell line was similar.

Cancer risk may be associated to heterogeneous DNA containing potentially oncogenic coding or regulatory sequences like hybridoma cells. The DNA concentration -determined by dot-blot hybridization- obtained in both bioreactors was less than 10 pg/mg of MAbs (Table 1). These antibodies were used in the immunopurification of recombinant human alpha 2b interferon for human use and the recommendations regarding DNA 100 pg per single dose (16).

The only difference that we found with this cell line was in the production level. When we used ACUSYST-R and SACCEL bioreactors, the hybridoma production level was around 1 g of MAb /L of harvest, and in mice we obtained 4-5 g of MAb/L of ascitis.

For an economic evaluation of both bioreactors we considered the following:

Comparison of the process cost, considering the exploitation of each bioreactor, concluding that they only differ in the raw material expense and their depreciation. The SACCEL bioreactor raw material expense was 66.67 %/month; the depreciation value for the ACUSYST-R bioreactor was 125 %/month.

The final run time was 45 days, having 3.12 g for the SACCEL bioreactor and 3.3 g for the ACUSYST-R model.

The results of the analysis were:

- The ACUSYST-R bioreactor process cost was 11 % higher than the SACCEL bioreactor.
- The SACCEL bioreactor productivity was about 5 % higher than the ACUSYST-R model.

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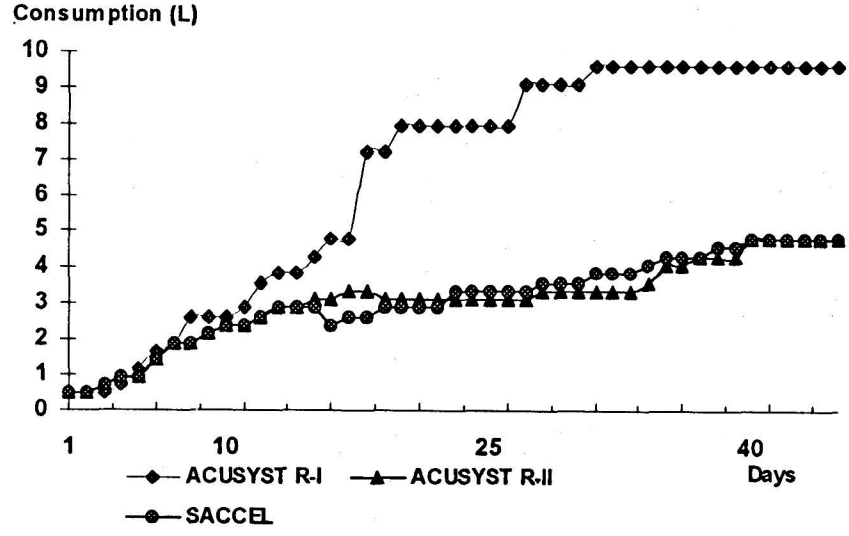

Figure 5. Consumption of RPMI/Bioreactor. The strategy to reduce the excess glucose supply was effective by reducing the culture medium consumption without affecting yield.

- The unitary cost for production in the SACCEL bioreactor was 15 % less than in the ACUSYST-R bioreactor.
- Considering mass production, the cost in the SACCEL bioreactor is 15 % less than in the ACUSYST-R.

Conclusions

The achievement of a considerable reduction of the medium consumption without affecting yield was obtained in this study. It was demonstrated that even a 50 % reduction of the culture medium consumption does not affect the specific antibody production, at least in the cell line described.

This result represents a 13 % reduction of the total production cost per unit for both bioreactors in relation to previous runs using the ACUSYST-R bioreactor. Besides, the use of hollow fiber bioreactors simplify the validation studies of the production process and support the reduction of production cost.

The production of 6.00 g of CB IFN 2.4 MAbs was achieved, including 3.3 g manipulating a new, nationally produced device (SACCEL) designed for this purpose.

The MAbs produced in both bioreactors did not have significant quality differences when they were compared with the MAbs produced in mice using the same cell line. The main difference was in the productivity of both systems (Table 1) but the importance of the production in hollow fiber bioreactors refers to the cost, considering the regulations for the use of laboratory animals.

Table 1. Characterization of monoclonal antibody CB IFN 2.4 produced *in vivo* and *in vitro* using hollow fiber bioreactors.

Characteristic	ACUSYST-R	SACCEL	Reference data*
mg MAb/mL harvest	0.84	0.64	4-5
Purity of MAb	92.4 %	96.7 %	> 90
Mouse DNA pg DNA/mg MAb	< 4	< 4	< 10
Specific Activity	75	80	> 70
MAb Immobilized	99 %	99 %	> 95
µg Ag eluted/mg MAb immobilized	91.5	93	80

**In vivo* production at the Division of MAbs Production, Genetic Engineering and Biotechnology Center (unpublished data).

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