RESEARCH

Production of an anti-HBsAg mouse IgG-2bk monoclonal antibody in hollow fiber bioreactors using different cell culture media and operation modes

✉ Rodolfo Valdés, Marcos González, Yanely González, Hector Gómez, Janet García, Tatiana Alvarez, Leonardo Gómez, Jorge V Gavilondo

Monoclonal Antibody Production Department and Cancer Department,
Center for Genetic Engineering and Biotechnology (CIGB),
PO Box 6162, Cubanacán, Playa, Havana 10 600, Cuba
E-mail: rodolfo.valdes@cigb.edu.cu

ABSTRACT

Serum-and protein-free media IMDM/SSR, RPMI/SSR, PFHM, TurboDoma™HP-I, and TurboDoma™HP-II were evaluated for anti-HBsAg CB.Hep-1 Mab production in hollow fiber bioreactors. RPMI 1640 and IMDM, supplemented with FCS, were used as controls. Initial testing of media in 75 cm^2 T flasks demonstrated that achievable cell densities (0.13-2.39 x 10^6 cells/mL) and IgG concentrations (5.2-18.7 mg/mL), were very much media-dependent. Performance with PFHM and TurboDoma™HP-I were best, and comparable with IMDM/5% fetal calf serum (FCS) in terms of maximum IgG concentration, while the usage of RPMI 1640 or TurboDoma™HP-II as base media was not favorable for this hybridoma. TurboDoma™HP-I was selected for further studies in Cell-Pharm™1000 bioreactor, due to its optimization posibilities. IMDM/SSR was excluded from these experiments due to its high cost. Mab concentration increases in Cell-Pharm™1000 began after 10 days and reached maximum values between 16 and 31 days, in correspondence with the medium and operation mode used. The only disadvantage observed with TurboDoma™HP-I was the long cell latency phase. The latter led to the addition of 1% of FCS and 500 μM ferric citrate to this medium for scaling up studies in the Cell-Pharm™2500 bioreactor. This medium fortification led to a drop in latency of 1.6 and 1.4 times, relative to values seen for TurboDoma™HP-I and IMDM/5% FCS in Cell-Pharm™1000 and also allowed the reclonning of cells extracted from the bioreactor extracapillary space without adaptation to another culture medium formulation. Our results indicate that Cell-Pharm™2500 bioreactor could produce about 14.4 grams of this antibody per month, harvesting 420 mL every 48 hours if the plateau phase could be maintained for 30 days.

Key words: Mammalian cell culture, mouse hybridoma, hollow fiber bioreactor, monoclonal antibody, Hepatitis B surface antigen

Biotecnología Aplicada 2005;22:112-116

RESUMEN

Producción de un anticuerpo monoclonal de raton IgG-2B anti-HBsAg en bioreactores de fibra hueca empleando diferentes medios de cultivos y modos de operación. Se evaluaron los medios libres de suero y de proteínas IMDM/SSR, RPMI/SSR, PFHM, TurboDoma™HP-I y TurboDoma™HP-II para producir el Mab CB.Hep-1 anti-HBsAg en reactores de fibra hueca. Se emplearon como control los medios RPMI 1640 y IMDM suplementados con suero bovino fetal. La prueba inicial realizada en frascos T de 75 cm^2 demostró que la densidad celular (entre 0.13 y 2,39 x 10^6 células/mL) y la concentración de IgG (entre 5.2 y 18.78 mg/mL) fueron muy dependientes del medio de cultivo empleado. Los medios libres de proteínas PFHM y TurboDoma™HP-I resultaron ser los mejores, y comparables con el medio IMDM/5% FCS en cuanto al valor máximo de concentración de IgG, mientras que el empleo del RPMI 1640 o TurboDoma™HP-II como medio base para el cultivo de este hibridoma no fue aconsejable. Para los estudios en el bioreactor Cell-Pharm™1000 se seleccionó el medio TurboDoma™HP-I, debido a sus posibilidades de optimización posterior. Se excluyó de los mismos la formulación de IMDM/SSR debido a su alto costo. El incremento en la concentración del Mab en el bioreactor Cell-Pharm™1000 comenzó a los 10 días, alcanzando el valor máximo entre 16 y 31 días, en correspondencia con el medio de cultivo y la forma de operación. La única desventaja detectada con el TurboDoma™HP-I fue la larga fase de latencia celular. Ello llevó a que el TurboDoma™HP-I se enriqueciera con 1% of FCS y 500 μM citrato férrico para los experimentos en el bioreactor Cell-Pharm™2500. Este suplemento hizo que el tiempo de latencia disminuyera en 1,6 y 1,4 veces, en comparación con lo observado en el bioreactor Cell-Pharm™1000 con los medios TurboDoma™HP-I y IMDM/5% FCS, y además permitó el clonaje de las células extraídas del espacio extracapilar del bioreactor sin la necesidad del uso de otra formulación de medio de cultivo. Nuestros resultados indicaron que el bioreactor Cell-Pharm™2500 podría producir 14.4 gramos de este anticuerpo por mes, cosechando 420 mL cada 48 horas si la fase estacionaria se prolonga por 30 dias.

Palabras claves: Cultivo de células de mamíferos, hibridomas de ratón, bioreactor de fibra hueca, anticuerpo monoclonal, antígeno de superfice del virus de la Hepatitis B

*I*ntroduction

Monoclonal antibodies (Mab) have evolved into a platform technology with important applications in the field of biomedical research, and in the development of new diagnostic reagents and therapeutic drugs for a wide variety of human diseases [1]. While for years the ascitic fluid generated by the growth of hybridoma cells in the peritoneum of live mice has been a routine source of Mab production, animal protection laws in several

1. Gavilondo J, JW Larrick. Antibody engineering at the millenium. Biotechniques 2000;29(1):128-36.

✉ Corresponding author

countries have either banned or restricted its use [2-3], creating a serious limitation for the availability of these useful reagents. As a consequence, research has been boosted on new *in vitro* production techniques and for the development of automated cell culture systems.

Among the latter, batch operated stirred tank reactors, fed batch and perfusion modes are now commonly employed [4]. Nevertheless, in this culture method cell growth is limited by the accumulation of water soluble non-gaseous metabolic products [5]. Hollow fiber culture systems, which are more sophisticated and efficient, have appeared as an attractive alternative to stirred tank reactors [6]. This technology mimics the capillary type circulatory system found *in vivo*. Hundreds of thousands of hair-like cellulose acetate or polypropylene permeable hollow fibers are bundled together into glass or plastic cylinders to form a cartridge called a bioreactor. The cartridge is then attached to a perfusion system, which circulates vital nutrients for cell growth through the fibers in a continuous flow of the medium. Cells are inoculated outside the fiber in the cartridge. Nutrients such as glucose and aminoacids, which have a low molecular weight, diffuse through the membrane of each fiber and nourish the cells. At the same time, metabolic waste products are transported through the membrane in the opposite direction into the circulating medium to be eliminated from the cell compartment. Proteins with high molecular weights, such as antibodies, are retained and concentrated in the extracapillary space and then collected and submitted to purification [7-9].

The production of Mab for human applications in hollow fiber bioreactors came true after Cytogen Corporation (Princeton, NJ) launched its Mab-based imaging product ProstaScint™, the first injectable substance approved by the FDA that was produced using the hollow fiber technology [10]. The advantages [11-13] of hollow fiber technology for Mab production are: (a) high Mab concentration (equivalent to ascites) and reduced extraneous or cellular proteins in the harvested fluid, which simplifies the downstream processing, (b) reduction of total operation volume (large number of cells grow in a very small volume), determining a smaller facility size, and (c) scalability to multi gram production.

CB.Hep-1 is a IgG-2bκ mouse antibody that is specific for the Hepatitis B surface antigen [14]. This Mab is produced in multi-gram quantities in bioreactors and employed as the basic purification ligand for the industrial production of the Cuban recombinant Hepatitis B vaccine Heber Biovac [15]. This report describes the results of a study we conducted on the growth and antibody secretion characteristics of the CB.Hep-1 hybridoma, using hollow fiber culture technology and protein free medium. The study focuses on the issue of culture media optimization, one of the most critical aspects in the production of mammalian cell derived products [16-18].

Materials and methods

Cell line

The cell line used in this study was a mouse hybridoma secreting an IgG 2bκ monoclonal antibody against the HBsAg [14].

Cell culture media and additives

The cell culture media and additives used were: *RPMI1640* (Gibco, Grand Island, USA), 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg/L gentamicin (Gibco, Grand Island, USA), 8% FCS (Hyclone, USA). *RPMI1640* (Gibco, Grand Island, USA), 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg/L gentamicin (Gibco, Grand Island, USA), 2, 5% FCS (Hyclone, USA). *RPMI1640* (Gibco, Grand Island, USA), 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg/L gentamicin (Gibco, Grand Island, USA), synthetic serum replacement (Mediculture, Copenhagen, Denmark). *IMDM* (Gibco, Grand Island, USA), 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg/L gentamicin (Gibco, Grand Island, USA), 5% FCS (Hyclone, USA). *IMDM* (Gibco, Grand Island, USA), 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg/L gentamicin (Gibco, Grand Island, USA), synthetic serum replacement (Mediculture, Copenhagen, Denmark). *PFHM* (Life Cell Culture Technology, Zürich, Switzerlan 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg/L gentamicin (Gibco, Grand Island, USA). *TurboDoma™HP-I* (Life Cell Culture Technology, Zürich, Switzerland), 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg / L gentamicin (Gibco, Grand Island, USA). *TurboDoma™HP-I* (Life Cell Culture Technology, Zürich, Switzerland), 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg/L gentamicin (Gibco, Grand Island, USA)), 1% FCS (Hyclone, USA), 500 µM ferric citrate (Sigma, USA). *Turbo Doma™HP-II* (Life Cell Culture Technology, Zürich, Switzerland), 1% v/v glutaMAX™ (Gibco, Grand Island, USA), 25 mg/L gentamicin (Gibco, Grand Island, USA).

Static batch cutlture

The cell line was mantained in T-75 cm^2 flasks (Costar, Cambridge, USA) in a CO_2-incubator under an atmosphere of 95% air and 5% CO_2 at 37 °C. The cell culture medium adaptation was carried out by diluting the cell suspension 1:4, adding the fresh medium every two days. After 21 days the cells were considered adapted and used for stationary and hollow fiber experiments. In this stationary culture experiment, three T-75 cm^2 flasks were used to evaluate each cell culture medium.

Bioreactors

(A) The Hollow fiber bioreactor Cell-Pharm™1000 produced by Unisyn technologies, Hoptkinton MA, USA was used with a BR22010664 bioreactor (1.5 $foot^2$ and 30 KDa of molecular weight cut-off) Unisyn technologies, Hoptkinton MA, USA, and an OXY1 (Unisyn technologies, Hoptkinton MA, USA) oxigenator with an oxygenation capacity of biological fluids of 10 L/min. After the start of the plateau phase, 80% of the cell culture supernatant was regulary harvested every 48 hours from the extracapillary space.

(B) The Hollow fiber system Cell-Pharm™2500 produced by Unisyn technologies, Hoptkinton MA, USA was used with a BR22011077 bioreactor (35 $foot^2$ 30 KDa of molecular weight cut-off) Unisyn Technologies, Hoptkinton MA, USA and an OXY25A oxygenator (Unisyn technologies, Hoptkinton MA, USA) with an oxygenation capacity of biological fluids of 1000 L/min. In this case, the flow rate of the medium

2. Hendriksen CFM. A call for a European prohibition of monoclonal antibody (MAb) production by the ascites procedure in laboratory animals. ATLA 1998;26:523-40.

3. Hendriksen C, Rozing J, van der Kamp M, de Leeuw W. The production of monoclonal antibodies: are animal still needed. ATLA 1996;24:109-10.

4. Yang J-D, Angelillo Y, Chaudhry M, Goldenberg C. Acheivement of high cell density and high antibody productivity by a controlled-fed perfusion bioreactor process. Biotechnology and Bioengineering 2000;69;1:74-82.

5. Falkenberg F. Monoclonal antibody production: problems and solutions. Research in Immunology 1998;149;6:529-620.

6. Lowry D, Murphy S, Goffe RA. A comparison of monoclonal antibody productivity in different hollow fiber bioreactors. J Biotechnology 1994;36:35-8.

7. Lynn R, Jackson D.V.M, Laura J, Trudel B.A, Neil S, Lipman V.M.D. Small-scale monoclonal antibody production *in vitro*: Methods and Resources. Lab Animal 1999;28:3:38-50.

8. Czirbik JR, Rosen MS, Trunfio MD, Fishberg-Bender WE, Palmer MS. Factros affecting antibody production effciency in hollow fiber bioeractors. IVD technology Magazine 1996;1-9.

9. Jackson LR, Trudel LJ, Fox JG, Lipman NS. Evaluation of hollow fiber bioreactors as an alternative to murine ascites production for small-scale monoclonal antibody production. Journal of Immunol Methods 1996;189:217-31.

10. Chu L, Robinson D. Industrial choices for protein production by large-sacle cell culture. Current Opinion in Biotechnology 2001;12:180-7.

11. Lipman NS, Jackson LR. Hollow fiber bioreactors: an alternative to murine ascites for small scale (<1gram). Monoclonal antibody production. *In vivo* and *in vitro* production of mAbs 1998;10:571-5.

12. Pérez M, Valdés I, Pierrat R, García C, González M, De la Torre D, Alemán R, Valdés R. Production of monoclonal antibody in the hollow fiber bioreactors Acusyst-R and Saccel. Biotecnología Aplicada 1997;14:106-10.

13. Valdés R, Ibarra N, González M, Alvarez T, García J, Llambias R, Pérez A.C, Quintero O, Fischer R. CB.Hep-1 hybridoma growth and antibody production using protein free medium in a hollow fiber bioreactor. Cytotechnology 2001;35:145-54.

14. Fontirrochi G, Dueñas M, Fernández de Cossio M.E, Fuentes P, Pérez M, Mainét D, Ayala M, Gavilondo J, Duarte C. A mouse hybridoma cell secreting IgG and IgM antibodies with specificity for the hepatitis B surface antigen. Biotecnología Aplicaca 1993;10:24-30.

15. Pentón E, Muzio V, González GM. The Hepatitis B virus (HBV) infection y its prevention by a recombinant DNA viral surface antigen (rec-HBsAg) vaccine, Biotecnol. Aplicada 1994;11:1–11.

16. Shi Y, Correira A. Performance of hybridoma cell culture in a computer controlled research- scale to pilot-scale hollow fiber bioreactors system. Application Note 1998; November:56-9.

was fixed daily according to glucose consumption. The system is controlled and monitored in real time from a single PC-based controller. All critical bio-processing functions including basal media, base and factor feed, recirculation rate, temperature, pH, reservoir level, and automated harvesting protocols are controlled by the UniNet software system.

Inoculation

(A). Hollow fiber system Cell-Pharm™1000 technologies, Hoptkinton MA, USA . Cells were cultivated in T-150 cm^2 flasks. The cultures were maintained in an incubator at 37 °C and in a 5-6% CO_2 atmosphere. About 2 x 10^8 cells were inoculated into the bioreactor by means of 10 mL syringes.

(B). Hollow fiber system Cell Pharm™2500 Unisyn technologies, Hoptkinton MA, USA. Cells were cultivated in 1L spinner-flasks. The cultures were shaken at 20 rpm and maintained in an incubator at 37 °C and in a 5-6% CO_2 atmosphere. About 3.8 x 10^9 cells were inoculated into the bioreactor using 50 mL syringes at 10 mL per minute.

Monoclonal antibody assay

Mab concentrations in Cell-Pharm™1000 bioreactor's samples were quantified using a chromatographic method based on protein G [18] and also by ELISA according to the procedure described by Valdés [19] et al. while Mab concentrations in Cell-Pharm™2500 bioreactor samples were only quantified by ELISA. Briefly, a direct ELISA test with a rHBsAg as the coating reagent and a peroxidase-labelled sheep anti-mouse IgG antibody (Sigma, St Luis, USA) for antibody detection were used. A purified IgG 2bκ CB.Hep-1 was used as a standard for determining the antibody concentration in each sample [20].

Glucose concentration and viability

Glucose concentration was determined enzymatically using an appropriate diagnostic kit (Sigma, St Luis, USA) according to the manufacture's instructions. The number and viabilty of the cells were determined using Trypan blue dye exclusion [21].

Results and discussion

Despite important recent efforts in the development of synthetic media for hybridoma cell culture [22-23], information concerning the optimal media is something that has to be produced almost on a case-by-case manner. Particular growth characteristics of the cell line, antibody secretion, and the employed culture system (together with its operational parameters during culture), are all important factors that ultimately determine the limitation of specific nutrient components, the accumulation of toxic and inhibitor substances, and the physiologic state of the cells. The aim of this report is share our experience with the CB-Hep.1 hybridoma. Our work has been extended in order to compare synthetic media supplemented with foetal calf serum, serum-free medium containing synthetic serum replacement substances, and protein-free media, for the production of this Mab in two hollow fiber bioreactor systems from Unysin technologies, Hoptkinton MA, USA.

Table 1 describes the hollow fiber bioreactors and media used to evaluate the growth and antibody production of hybridoma CB.Hep-1. The media included IMDM and RPMI1640 supplemented with FCS, protein free media such as PFHM, TurboDoma™HP-1 and TurboDoma™HP-II, and combinations of IMDM and RPMI1640 with synthetic serum replacement components. Preliminary unpublished experiments with this hybridoma had indicated that RPMI1640 supplemented with 8-10% of FCS is inadequate to produce consistent quantities of the anti-HBsAg Mab in the ACUSYST-R hollow fiber system (Endotronics, Inc. Mineapolis, Minnesota, USA). However, those experiments had not been designed to accurately indicate if this could be attributed to the particular bioreactor system employed, or to the medium formulation.

Table 1. List of cell culture media, supplements, and culture systems employed.

Basal medium	Supplements	Cell culture system	Number of experiments
IMDM	5% FCS/glutaMAX™/Gentamicin	T-75 cm^2 flask	3
		Cell-Pharm™ 100	2
IMDM	SSR/glutaMAXTM/Gentamicin	T-75 cm^2 flask	3
RPMI1640	2.5% FCS/glutaMAX™/Gentamicin	T-75 cm^2 flask	3
RPMI1640	8% FCS/glutaMAX™/Gentamicin	Cell-Pharm™100	2
RPMI1640	SSR/glutaMAX™/Gentamicin	T-75 cm^2 flask	3
PFHM	glutaMAX™ /Gentamicin	T-75 cm^2 flask	3
TurboDoma™HP-I	glutaMAX™ /Gentamicin	T-75 cm^2 flask	3
		Cell-Pharm™100	3
TurboDoma™HP-I	1% FCS/glutaMAX™/500 μM ferric citrate/Gentamicin	Cell-Pharm™2500	1
TurboDoma™HP-II	glutaMAX™/Gentamicin	T-75 cm^2 flask	3

Initial comparisons were done in static cultures, based on previous results of our group that indicated that 75 cm^2 T-flasks can be used as a reference system for a first screening of growth performance of hybridoma CB-Hep.1 under different culture conditions. Table 2 shows that in these flasks densities ranged from 0.13 to 2.39 x 10^6 cells/mL, and IgG concentration values from 5.20 to 18.78 mg/mL. The maximum cell densities achieved in serum free media (IMDM/SSR and RPMI/SSR) were 1.4- and 4.87-fold lower, respectively, when compared to IMDM/5% FCS. Similar results were observed for supernatant IgG concentration. With regards to protein-free media, PFHM and particularly TurboDoma™HP-I produced high cell densities and IgG concentration, well above the control medium IMDM/5% FCS. RPMI 1640 and

17. Valdés R. Alternative Techniques to Obtain Monoclona Antibodies at a small scale: Current Status and Future Goals. Biotecnología Aplicada 2002;19:119-31.

18. Mizrahi A, Lazar A. Media for cultivation of animal cells: an overview. Cytotechnology 1988;1:199-214.

19. Bjorck L, Kronvall G. Purification and some properties of streptococcal Protein G: a novel IgG binding reagent. J Immunol 1984;133:969-74.

20. Valdés R, Leyva J, González E, Mainet D, Costa L. Caracterización de anticuerpos monoclonales contra el antígeno de superficie del virus de la hepatitis B. Biotecnología Aplicada 1994;11:219-24.

21. Patterson MK. Measurement of growth and viability of cells in culture in Jacoby WB, Pastan IH eds. Method of Enzymology. Academic Press, London 1973:58.

Table 2. CB.Hep-1 behavior in T-75 cm^2 flasks using different cell culture media.

Cell culture media	IMDM 5% FCS	IMDM SSR	RPMI 2.5% FCS	RPMI SSR	PFHM	THP-I	THP-II
Maximum cell density, (cells x10^6/mL)	2.39	1.70	0.88	0.49	1.96	1.77	0.13
Maximum Mab concentration, (μg/mL)	15.4	10.12	9.6	5.2	13.2	18.78	-
Maximum Mab production, (pg/cell)	13.2	6.9	12.19	10.6	7.58	16.92	-
Time needed to reach maximum cell density, (days)	6	6	9	10	9	9	7
Time needed to reach maximum Mab concentration, (days)	7	8	7	10	7	7	-
Time needed to reach maximum Mab production, (days)	2	7	7	10	10	7	-
Maximum cell viability, (%)	>95	>90	>95	>90	>90	>90	>98

TurboDoma™HP-II were clearly undesirable for the production of this antibody, indicating that most probably our results with the ACUSYST-R were due to the medium used. The use of IMDM/SSR was also eliminated, in spite of its regulatory advantages over IMDM/5% FCS, because of the high cost.

Our experiments continued in the Cell-Pharm™1000 hollow fiber system. The antibody production efficiency was expressed as milligrams of antibody produced per liter of medium consumed, and of the time needed to reach the maximum IgG concentration. Figures 1A and 1B show the IgG concentration and cumulative IgG production under the following operation modes: (a) increasing the intracapillary flow rate and medium renovation according to glucose consumption (Turbo Doma™HP-I and IMDM/5% FCS), (b) initial high perfusion flow rate and medium renovation from the beginning of the experiment (TurboDoma™HP-I), (c) co-cultivation with murine macrophages during the first fifteen days of the run (TurboDoma™HP-I) [13], and (d) RPMI 1640 supplemented with 8% FCS.

The IgG quantification indicated maximum Mab concentrations of 1.5-2 g/L for all experiments (Figure 1A). The increase in IgG concentration began at 10 days with 100 mg/mL and reached the maximum values between 16 and 31 days depending on the medium and operation mode used. IgG production was 92.7 mg in 30 days for TurboDoma™HP-I at high perfusion and high medium renovation from the begining of the experiment, 77.38 mg in 36 days for Turbo Doma™HP-I plus macrophages, 75.27 mg in 32 days for IMDM 5% FCS, 64.30 mg in 35 days for TurboDoma™HP-I perfused and renovated according to glucose consumption, and 0.72 mg in 37 days for RPMI 1640/8% FCS (Figure 1B), with only 15 mg/mL of the antibody. Cumulative IgG production reported by the manufacturers for the Cell-Pharm™1000 system ranges from 100 to 500 mg per month, always depending on the cell line and culture conditions.

In general, once the Cell-Pharm™1000 bioreactor was inoculated, cells grew rapidly and accumulation was visible in one week, especially in IMDM/5% FCS and in TurboDoma™HP-I (high perfusion and high renovation from the beginning of the experiment). No overgrowth of the culture was observed in the bioreactor when the protein free medium was used, except for the high perfusion and high renovation experiment. Glucose consumption was the main criterion for assessing the metabolic activity of the cultures, and as an indicator for media feed and perfusion. We always kept the glucose concentration above 2 g/L, as lower values could stimulate apoptosis and a rapid decrease of the cell viability in this hybridoma (data not shown).

The consistency of antibody production and medium consumption in the Cell-Pharm™1000 bioreactors were compared for different media formulations and operation procedures. Three arbitrary criteria were used for between-run comparisons: number of days required to reach maximum IgG concentration, volume of the medium consumed per day, and antibody production per consumed medium. Table 3 shows that production efficiency (milligrams of IgG produced per liter of consumed medium) was higher for the TurboDoma™HP-I medium operated under controlled perfusion and medium renovation. However, this protein free medium had a long lag phase (30 days) before reaching the maximum antibody concentration, versus 18 and 25 days for the TurboDoma™HP-I operated under high perfusion and high medium renovation, and IMDM/5% FCS, respectively.

The results obtained with the TurboDoma™HP-I medium in the bioreactors operated with high perfusion and high medium renovation throughout the experiment could probably be explained by an increased gas diffusion, and the achievement of optimal levels of all nutrients needed for growth and Mab production. However, this operation mode directly affects the cost of the product, due to the very high medium consumption. The use of TurboDoma™HP-I under high perfusion and medium renovation consumed 2.06 times more medium than when the bioreactor was operated under controlled perfusion and medium renovation, and 1.38 times more when compared to the conditions established for IMDM 5% FCS.

Clonal stability is always an issue with hybridoma cell lines, as many eventually stop producing antibodies [24]. Stability of Ig production may be affected not only by the properties of the culture medium, but also by the manner in which cells are handled. For example, schedules that generate overgrowth conditions may create selective pressures, which favor survival of non-Ig producers. To study this phenomenum in our conditions, we extracted

22. Jayme W. D, Gruber F. D. Development of serum free media and methods for optimization of Nutrient compostion. Cell Biology: A Laboratory Handbook, Second Edition 1998;1:19-25.

23. Jayme W.D, Smith S. Media formulation options and manufacturing process controls to safeguard against introduction of animal origin contaminants in animal cell culture. Cytotechnology 2000;33;27.

24. Ozturk SS, Palsson B. Loss of antibody productivity during long term cultivationof a hybridoma cell line in low serum and serum free media. Hybridoma 1990;9:167-75.

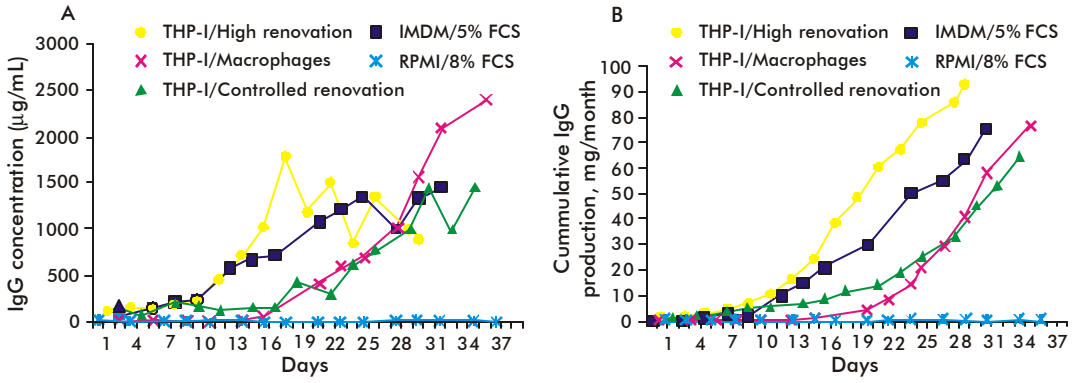

Figure 1. IgG concentration (A) and (B) cumulative IgG production in the hollow fiber system Cell-Pharm™1000.

cells from the extracapillary space at the end of the experiments, and cloned them by limiting dilution. Unpredictably, cells cultivated in the protein-free medium were unable to grow at an average of one cell per well in 96-well tissue culture plates, or in stationary culture 75 cm^2 T-flasks. Therefore, all cloning experiments had to be repeated in the medium supplemented with 1% FCS and 500 µM ferric citrate. Our results verified that all clones continued to produce antibo-dies that detect the HBsAg (data not shown in detail).

The following phase was to scale up hybridoma growth and Mab production in the hollow fiber Cell-Pharm™2500 system. In this case, we added 1% FCS and 500 mM ferric citrate to the medium in both compartments in order to reduce the time needed to reach maximum antibody concentration. Figure 1A shows a profile of a run in which 3.8 x 10^9 cells were seeded in 50 mL of TurboDoma™HP-I supplemented as described above, and with a high content of glucose to avoid glucose starvation during the inoculation. The cells showed a rapid growth throughout the experiment, and the extracapillary space of the bioreactor was saturated after the first 17-19 days of culture. Antibody production, glucose utilization, and the high viability of the cells extracted from the bioreactor suggested that most were metabolically active throughout this time. Glucose levels dropped from 4.5 g/L to 2.9 g/L at day 19 (Figure 1B). Mab quantification by ELISA (Figures 2A and 2B) indicated that more than 1 gram of anti-HBsAg antibody was produced during the first 19 days of the experiment, when the cells should have been in the exponential growth phase. Mab concentration ranged from 145 mg/mL initally to 2.3 mg/mL by day 18, reaching a value similar to that produced in the previous experiments made in the Cell-Pharm™1000 system (32 days).

Our results demonstrate that the combination of the Cell-Pharm™2500 hollow fiber system and the TurboDoma™HP-I protein free medium fortified with 1% FCS and 500 µM ferric citrate meets the needs for our production demands of the anti-HBsAg monoclonal antibody, while maintaining flexibility for further scaling. Studies are now in course to determine how long the high antibody production can be maintained in these conditions. If the IgG concentration achieved can be maintained until the end of the first 30 days of exploitation, the Cell-Pharm™ 2500 system will most probably be able to produce about 14.4 grams per month (harvesting 420 mL every 48 hours).

Table 3. Monoclonal antibody production efficiency in the hollow fiber bioreactor Cell-Pharm™1000 using different cell culture media and operation modes. These values represent the best results for each experimental variant.

Cell culture media/ Operation mode	IMDM 5% FCS Controlled perfusion and renovation	THP-I High perfusion and renovation	THP-I and macrophages Controlled perfusion and renovation	THP-I Controlled perfusion and renovation	RPMI 8% FCS Controlled perfusion and renovation
Total antibody production (mg)	75.27	92.70	77.38	64.30	0.72
Length of the experiments (days)	32	30	36	35	37
Time to reach maximum Mab concentration (days)	25	18	32	31	30
Antibody production (mg Mab/day)	2.35	3.09	2.14	1.83	0.02
Medium consumption (L/day)	0.49	0.68	0.30	0.33	0.39
Antibody production per medium consumed (mg Mab/L)	4.80	4.54	2.14	5.59	0.05

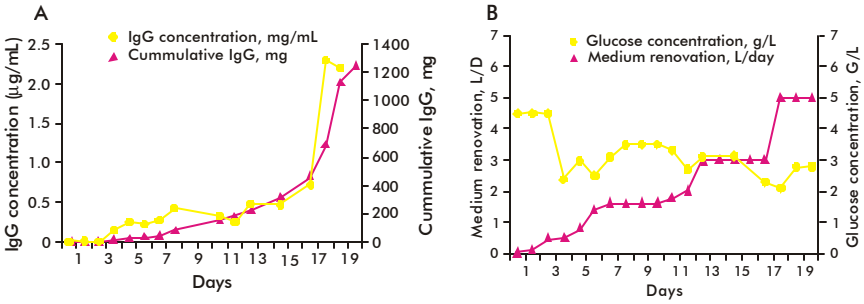

Figure 2. Hybridoma CB.Hep-1 behaviour in the Cell-Pharm™2500 culture system, using TurboDoma™HP-I, supplemented with 1% FCS and 500 µM ferric citrate. A. IgG concentration and cumulative IgG production. B. Extracapillary glucose concentration and intracapillary medium renovation.

Received in may, 2003. Accepted for publication in january, 2005.