CB.Hep-1 hybridoma growth and antibody production using protein-free medium in a hollow fiber bioreactor

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Abstract

The protein-free medium TurboDoma HP.1 (THP.1) was used to produce the CB.Hep-1 monoclonal antibody (mAb) in a CP-1000 hollow fiber bioreactor (HFB). This mAb is used for the immunopurification of recombinant hepatitis B surface antigen (rHBsAg), which is included in a vaccine preparation against the Hepatitis B Virus. By using the experimental conditions tested in this work we were able to generate more than 433 mg of IgG in 43 days. The maximum antibody concentration obtained was about 2.4 mg ml^{-1} and the IgG production per day was approximately 11 mg of monoclonal antibody, which constitutes a good concentration value in comparison to the results obtained in ascitic fluid, where concentration for this hybridoma was around 3 mg ml^{-1}. We used different analytical methods to control the quality of mAbs, obtained from the *in vitro* system. They included affinity constant determination, analysis of N-glycan structures, immunoaffinity chromatography and antigen binding properties. The results obtained suggest that no significant changes occurred in the mean characteristics of the mAb harvested from the bioreactor during the 43 days of cultivation.

Introduction

Since their initial development in the early 70s (Knazek et al., 1972) hollow fiber bioreactors (HFB) gained in popularity and several items of commercial equipment have been developed. In this system, cells attach on the outer surface of semi-permeable fibers, growing out in the extracapillary space (ECS) while culture medium is circulating in the lumen of the fibers, the so-called intracapillary space (ICS). This technology imitates more closely the mammalian *in vivo* environment than the static cell culture systems or the fermentation technologies. One of the most sensitive points of this type of technology is culture media; for instance, the addition of animal serum to culture medium gives rise to certain disadvantages in terms of product purification and cost of the medium (Glassy and Tharakan, 1988). This problem can be solved using protein-free medium (PFM), the efficacy of these media for cell culture has been widely demonstrated (Merten et al., 1991; Bertheussen, 1993; Franek and Dolnikova, 1991), and has the following advantages among others: (1) low protein content and therefore easy downstream purification schedule, (2) no proteolytic activity at neutral pH, that means no IgG degradation, (3) no influence of different serum components and batches (4) reduced risk of contamination, (5) lower production and purification costs, and (6) for some hybridoma cell lines: increase in productivity (Merten et al., 1989).

This article describes the production of an anti-rHBsAg hybridoma, cultivated in the TurboDoma HP.1 protein-free medium using a HFB.
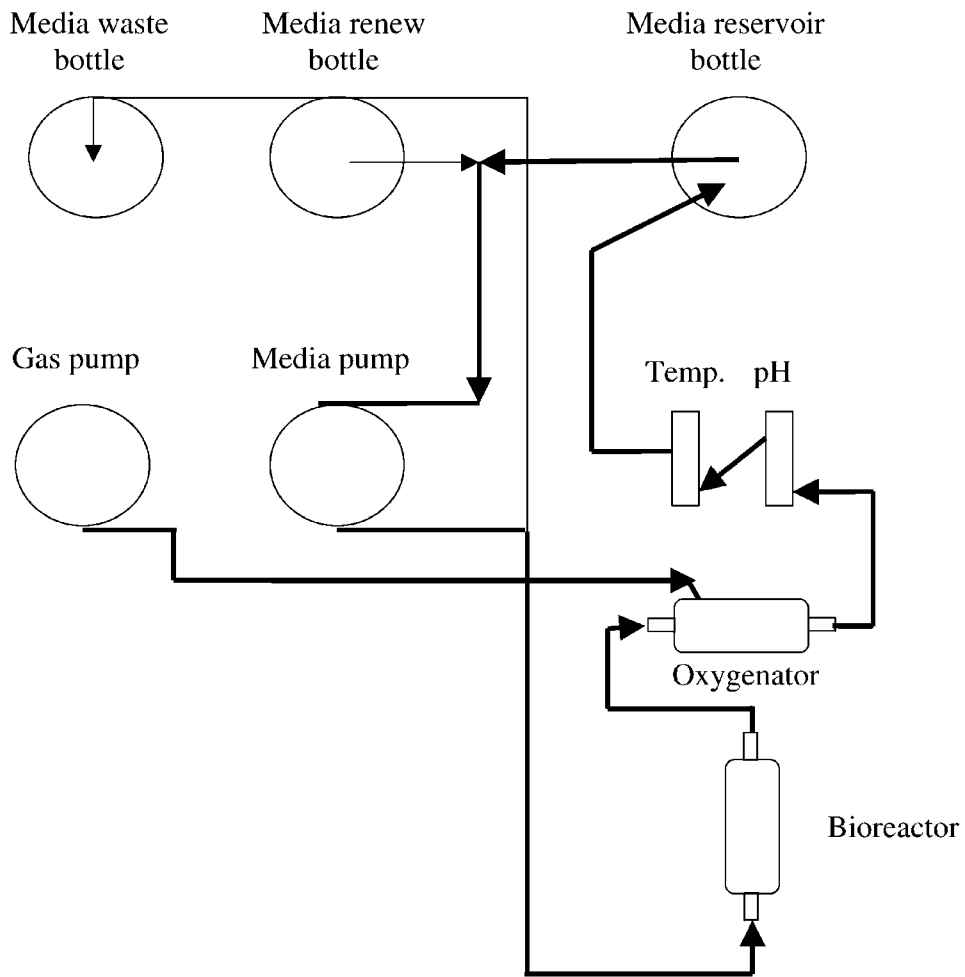
Media waste
bottle

Media renew
bottle

Media reservoir
bottle

Gas pump

Media pump

Temp. pH

Oxygenator

Bioreactor

Figure 1. Schematic representation of the CP1000 hollow fiber bioreactor system.

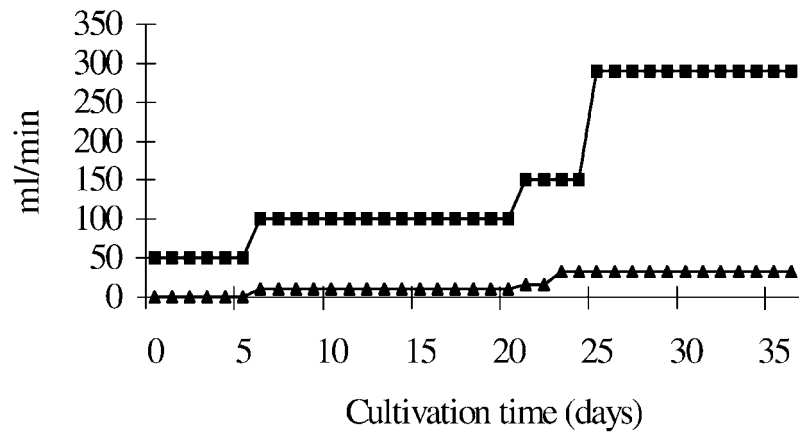*Figure 2.* Flow-rates profile, (▲) medium renewal flow-rate (ml min^{-1}), (■) intracapillary space flow-rate (ml min^{-1}). After the 7th day the medium circulation flow was increased from 50 to 100 ml min^{-1}, and up to 290 ml min^{-1} after the 25th day of the experiment. The medium renewal started on the 7th day at 50 ml min^{-1}.

Table 1. Comparison of CB.Hep-1 mAb glycosylation patterns using *in vivo* and *in vitro* culture systems

Carbohydrate type	Carbohydrate structure	*In vivo*(%)	*In vitro*(%)
Neutral G0	GlcNAcβ1—2Manα1 ⁶₃Manβ1—4GlcNAcβ1—4GlcNAc—ANTS GlcNAcβ1—2Manα1	2.4	1.2
Neutral G0F	GlcNAcβ1—2Manα1 Fucα1 / 6 ⁶₃Manβ1—4GlcNAcβ1—4GlcNAc—ANTS GlcNAcβ1—2Manα1	59.8	55.5
Neutral G1F	Galβ1—4 { GlcNAcβ1—2Manα1 Fucα1 / 6 ⁶₃Manβ1—4GlcNAcβ1—4GlcNAc—ANTS GlcNAcβ1—2Manα1 }	26.6	26.2
Neutral G2F	Galβ1—4GlcNAcβ1—2Manα1 Fucα1 / 6 ⁶₃Manβ1—4GlcNAcβ1—4GlcNAc—ANTS Galβ1—4GlcNAcβ1—2Manα1	9.1	2.4
Monosialylated	NeuNAcα2—6 { Galβ1—4GlcNAcβ1—2Manα1 Fucα1 / 6 ⁶₃Manβ1—4GlcNAcβ1—4GlcNAc— Galβ1—4GlcNAcβ1—2Manα1 }	1.8	14.8
Disialylated	NeuNAcα2—6Galβ1—4GlcNAcβ1—2Manα1 Fucα1 / 6 ⁶₃Manβ1—4GlcNAcβ1—4GlcNAc—ANTS NeuNAcα2—6Galβ1—4GlcNAcβ1—2Manα1	0.4	0.0

Materials and methods

Cell line and medium

The murine B-lymphocyte hybridoma cell line (CB.Hep-1) was previously generated (Fontirrochi et al., 1993). Briefly, it was obtained by fusion of Sp2/0-Ag14 myeloma cells and spleen cells of a BALB/c mouse immunized with natural HBsAg. The murine B-lymphocyte cell line hybridoma CB.Hep-1 secretes mAb IgG_{2b}, specific for the 'a' determinant of the rHBsAg (Fernández de Cossío et al., 1997). This Mab is used as immunoligand in the downstream purification process of rHBsAg employed for a commercially available recombinant hepatitis B virus vaccine (Heberbiovac HB^{TM}, Heber Biotec S.A., Cuba) (Pentón et al., 1992, 1994).

We employed TurboDoma HP-1 (THP.1) medium (Cell culture Technologies, Zurich, Switzerland), supplemented with Gentamycin at 25 mg l^{-1} for the cell culture in a CP1000 bioreactor.
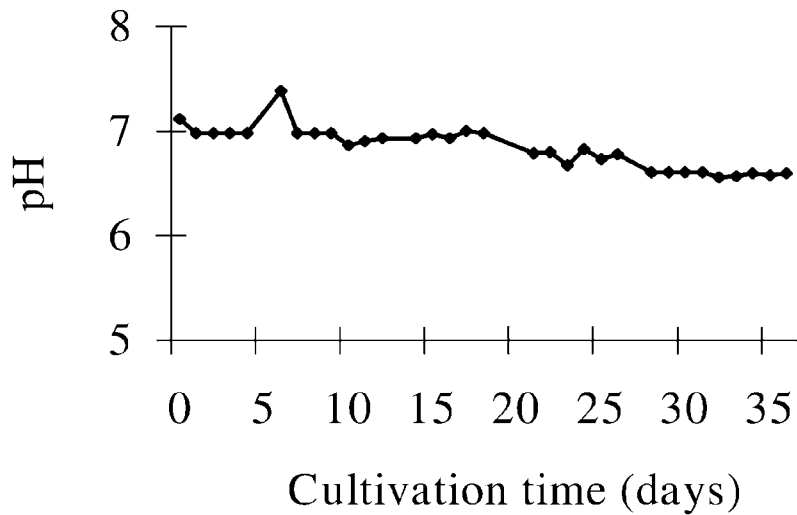
Figure 3. Monitoring of pH.

Static batch culture

The cell line was maintained in T-25 cm^2 flasks (Costar, Cambridge, U.S.A.) in a CO$_2$-incubator under an atmosphere of 95% air and 5% CO$_2$ at 37 °C. The THP.1 adaptation was carried out by diluting the cell suspension 1:4, adding fresh medium every two days. After a period of fifteen days of adaptation 1.6×10^8 of cells were inoculated into the bioreactor extracapillary space.

Hollow fiber bioreactor

A CP1000 HFB (Unisyn Technologies, U.S.A.) (Figure 1) was used with a cartridge BR110 (10 kDa of molecular weight cut-off), with a lumen volume of 13 mL. The membrane material was cellulose acetate and the membrane surface area was 1.5 ft^2 (O.D). We also used an oxygenator (OXY 1, Unisyn Technologies, U.S.A.), with an oxygenation capacity of biological fluids up to 1 l min^{-1}.

The flow-rate of the medium was daily fixed to maintain a glucose concentration of 3 g l^{-1}. As the product was removed, it was simultaneously replaced with the same volume of fresh medium. The system provides several automated features such as gas, pH and temperature control, and oxygen supplementation. Nutrient medium circulated through the lumen of the hollow fibers by means of a peristaltic pump, which allows a diffusive exchange of low molecular weight nutrients gases and metabolic wastes.

Table 2. Immunopurification performance. Immunoaffinity columns 1 and 2 were obtained using CB.Hep-1 mAb purified from CP1000 hollow fiber bioreactor and ascitic fluid, respectively

Run	Immunoaffinity column 1	Immunoaffinity column 2
	(μg rHBsAg ml^{-1} gel)	
1	1050	1060
2	1000	1025
3	980	990
Mean	1010±32.82	1025±32.82
P(T<=t; two-tail)	0.6324	

Glucose determination

Glucose consumption was used as a metabolic guide for medium supplementation and/or replacement. The level of glucose in the medium was used as an indirect method for measuring growth. The glucose concentration was enzymatically determined using an appropriate diagnostic kit (Sigma, St. Louis, U.S.A.) according to the manufacturer's instructions.

Macrophages source

Five BALB/c mice were carefully intraperitoneally inoculated with 15 ml of 10 mM Phosphate-buffered saline (PBS) per animal, the PBS was extracted and centrifuged twice at 100 g. The pellet was diluted in THP.1 and inoculated into the bioreactor in the ECS.
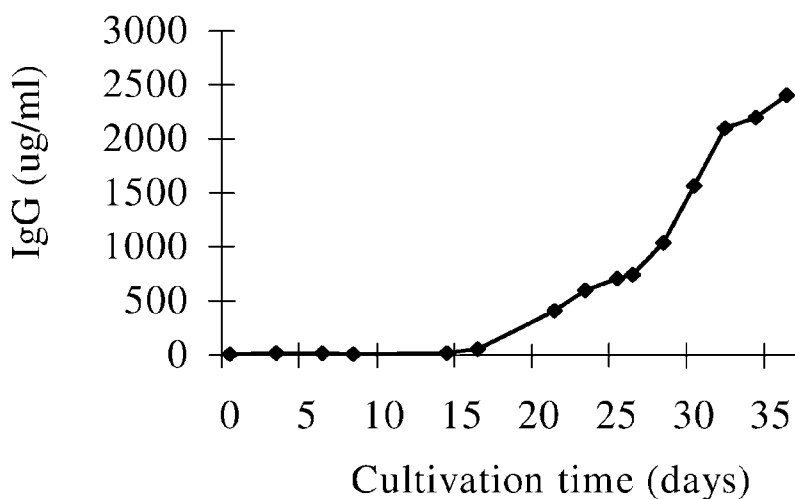
Figure 4. Monoclonal antibody concentration determined by ELISA (see the Section Materials and Methods).

Production of ascitic fluid

The hybridoma CB.Hep-1 was produced by the ascites method according to the method suggested by Hendriksen et al. (1998). Each BALB/c mouse was inoculated at 10^6 cells/animal.

Antibody assays

(1) Quantification of mAbs

An ELISA was developed to establish the antigen specificity of the mAbs in reactor samples secreted by the CB.Hep-1 hybridoma (Valdés et al., 1994). Briefly, a direct ELISA was performed, with rHBsAg as coating reagent and peroxidase-labelled sheep anti-mouse IgG antibody (Sigma, St. Louis, U.S.A.) for antibody detection.

(2) Purification of mAbs

Crude bioreactor supernatants and ascitic fluid were centrifuged and filtered (0.2 μm membranes) to eliminate cell debris. The purification was carried out in a single step using Protein A affinity chromatography (Pharmacia-LKB, Uppsala, Sweden) (Danielson et al., 1988). PBS pH 8.0 and 0.1 M citric acid pH 4.0 were used for adsorption and elution respectively with a flow-rate of 100 cm hr^{-1}. The eluted fractions were immediately adjusted to pH 7.0 using 3 M Tris and tested for specific anti-rHBsAg antibodies by the ELISA described above.

(3) Measurement of DNA content

DNA content of the purified mAb was determined according to the method reported by Brown et al. (1990).

(4) Measurement of Protein A content

Polystyrene plates (Costar, Cambridge, U.S.A.) were coated with 10 μg ml^{-1} of anti-Protein A polyclonal antibody in coating buffer (0.1 M NaHCO$_3$, pH 9.6) and incubated for 3 hr at 37 °C. Plates were washed with 0.15 M PBS/Tween 20 at 1% pH 8.0 solution. After washing steps, plates were blocked with 100 μl/well of 0.15 M PBS/non fat milk at 1% pH 8.0 solution for 1 hr at 37 °C and samples were applied into the plates for 2 hr at 37 °C. Washing steps were repeated again, and Fab fragments of the polyclonal antibodies conjugated with horseradish peroxidase were applied to each well. The reaction was revealed using 100 μl/well of 0.05% orto-phenylenediamine and 0.015% hydrogen peroxide in citrate buffer (pH 5.0). After 20 min the reaction was stopped with 1.25 M H$_2$SO$_4$. The absorbance was measured in a Multiskan ELISA reader (Labsystems, Helsinki, Finland) using a 492 nm filter.

(5) Immunoaffinity chromatography

Sepharose CL-4B (Pharmacia-LKB, Uppsala, Sweden) was activated by the CNBr method (Kohn et al., 1984). The CB.Hep-1 mAb was coupled as recommended by Pharmacia. The amount of coupled antibody was
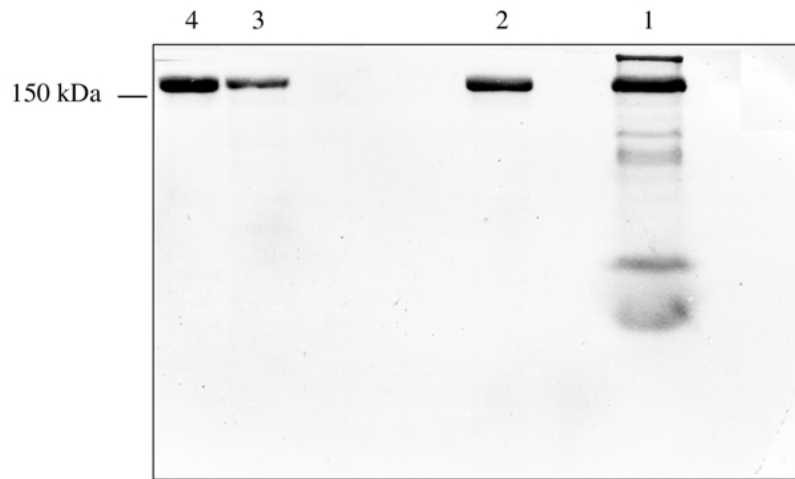
Figure 5. Non-reducing SDS-PAGE of the mAb obtained from the CP1000 bioreactor. Line 1: Ascitic fluid derived CB.Hep-1 mAb. Line 2: Hollow fiber bioreactor harvested mAb. The sample was purified by Protein A. Line 3: Hollow fiber harvested material. Line 4: Ascites derived mAb. The sample was highly purified by Protein A (95% purity, positive control). Protein samples were stained with Coomassie Brilliant Blue according to standard methods.

determined by measuring the total protein before and after the coupling reaction. The gel was packed into an analytical column (5 × 0.7 cm I.D., Pierce) and equilibrated with 20 mM Tris-HCl/3 mM EDTA pH 7.8. Adsorption and elution flow-rates were 20 and 35 cm hr^{-1}, respectively.

The column was loaded with an excess of a partially purified rHBsAg preparation in the equilibrium buffer containing 1 M NaCl. After washing, the bound antigens were eluted with 20 mM Tris/3 M KSCN/3 mM EDTA pH 7.0. Eluted antigens were monitored at 280 nm.

(6) SDS-PAGE

Purity of CB.Hep-1 mAb and rHBsAg, were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% mini-gels) (Laemmli, 1970). The CB.Hep-1 mAb was analyzed under non-reducing conditions and the rHBsAg was analyzed under reducing conditions; 20 μg of protein was applied in each case. Protein samples were stained with Coomassie Brilliant Blue according to standard methods. The percentage of purity was determined by densitometric evaluation, by using the Molecular Analyst statistic processing Software.

We used highly purified samples as positive controls: the mAb was obtained from Protein A and the rHBsAg was purified by immunoaffinity chromatography, the purity was checked by HPLC in both cases and the samples were supplied by Quality Control Department (Center for Gent. Eng. and Biotechnology, Havana, Cuba).

(7) Affinity constant determination

Affinity constant of CB.Hep-1 mAb was determined according to the method suggested by Beatty et al. (1987) and employing the ELISA described in Section 1.

(8) Glycosylation pattern

A two-dimensional mapping technique was applied to determine the profile of IgG derived oligosaccharides after enzymatic deglycosylation of the glycoproteins and labeling oligosaccharides with the fluorophore 8-amine-1, 3, 6-naphthalene trisulfonic acid (ANTS) (Quintero et al., 1998). In a first step ANTS-oligosaccharides were separated on an amine-bounded phase nucleosil 5NH$_2$ HPLC column (4.5 × 250mm, Knauer, Germany) under ion suppression conditions, in a gradient form using a C18 cartridge pre-column. Buffer A: glacial acetic acid (6% v/v) in a 70:30 (v/v) mixture of acetonitrile-water, titrated to pH 5.5 with triethylamine. Buffer B: glacial acetic acid (6% v/v) in water, titrated to pH 5.5 with triethylamine. Gradient: 5% B to 50% B in 90 min at a flow rate of 0.7 ml min^{-1}. Fluorecence was measured using a RF 530 detector (Shimatzu, Japan) λexc 353 nm and λem 535 nm.
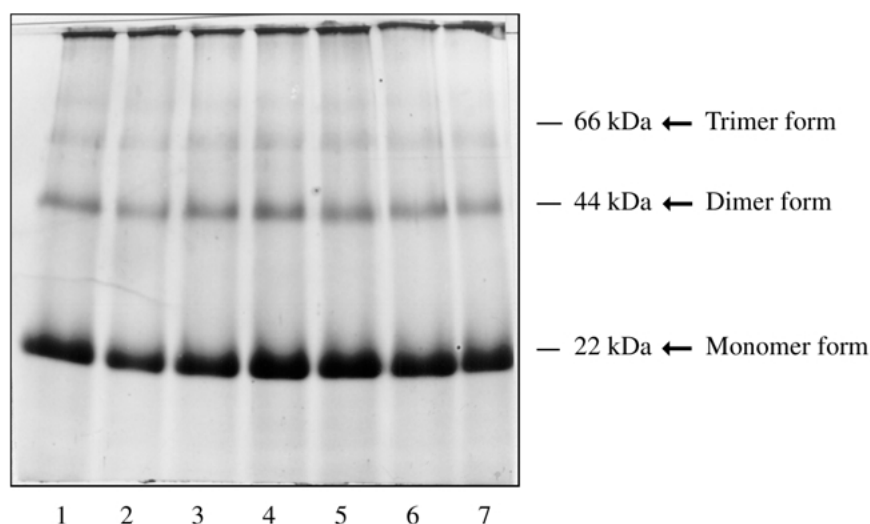
Figure 6. Reducing SDS-PAGE of the immunopurified rHBsAg. Line 1: Positive control. (rHBsAg highly purified: 97% purity). Line 2, 3 and 4: Immunoaffinity column 1, runs 1, 2 and 3. Line 5, 6 and 7: Immunoaffinity column 2, runs 1, 2 and 3. Immunoaffinity columns 1 and 2 are coming from mAbs produced under different conditions: Immunoaffinity column 1. Hollow fiber bioreactor derived mAb, Immunoaffinity column 2: ascites derived Mab. Protein samples were stained with Coomassie Brilliant Blue according to standard methods.

Table 3. Comparison of different scales of hollow fiber systems with ascites production

System	ECS[a] area (ft^2)	Bioreactor amount	IgG concentration (mg ml^{-1})	IgG(mg)/system[b]	Mice equivalence
CP1000	1.1	1	2.5	433	43
CP1500	10	1	2.5	4330	433
CP2500	32.5	2	2.5	28145	2814
CP3500	32.5	4	2.5	56290	5629
Ascites	–	–	3.0	10 mg/mouse	1

[a] Extracapillary space.
[b] Assuming 40 days.

Each of the resulting HPLC fractions was further analyzed on polyacrylamide gel electrophoresis of fluorophore-labeled saccharides (PAGE-FACE). The elution volume in HPLC and electrophoretic mobility of separated oligosaccharides were recorded as glucose units, compared to a ladder of maltooligosaccharides-ANTS series obtained by α-amylase enzymatic hydrolysis of amylose. Glucose unit values were plotted on a two-dimensional map and structures could be proposed after comparison with the behavior of a set of oligosaccharide-ANTS standards. Quantification was performed from HPLC chromatograms since the strict stoichiometry of derivatization make fluorescence nearly independent of oligosaccharide structure.

Results and Discussion

We studied the behavior of the hybridoma CB.Hep-1 in a HFB because of the advantages of this technology, in terms of the obtention of high cellular densities, and high mAb concentrations (Bertheussen et al., 1993). Hollow fiber systems also offer a viable alternative to *in vivo* antibody production. A certain scale up is easily accomplished without the need to modify the method.

At fifteen days of adaptation to THP.1, 1.6×10^8 of cells were inoculated in the bioreactor ECS. The medium circulation through the intracapillary space started on the first day at 50 ml min^{-1} without medium renewal in the extracapillary space (Figure 2).

At the eighth day of experiments cellular growth was almost not detectable. In order to stimulate growth

of the CB.Hep-1 hybridoma, mouse macrophages were added (Sugasawara et al., 1985) and medium circulation was increased to 100 ml min^{-1}, and up to 290 ml min^{-1} after the 25th day of the experiment. Cells started to grow after 10 days of experiment. Medium circulation, medium renewal and harvest, were performed according to the variations in the antibody concentration and the glucose consumption in the bioreactor.

When the cartridge was completely full of cells, pH decreased to values below 6.5 (Figure 3). At this point, in order to eliminate the accumulated lactate and ammonium, the maximum circulation medium flow-rate was set to 290 ml min^{-1} and the medium was renewed every 24 hr, however it was not possible to achieve physiological pH values (7.2).

The maximum antibody concentration obtained was about 2.4 mg ml^{-1} (Figure 4), which is in accordance with the concentration levels normally obtained for this hybridoma in ascitic fluid (Cosme et al., 1993, 1999). This result seems to depend on the characteristics of the medium and of the cell line used. Similar antibody concentrations were found using Iscove minimal essential medium supplemented with 5% of Foetal Calf Serum (FCS) (R. Valdés, unpubl.), but in RPMI supplemented with 10% of FCS we were not been able to obtain mAb concentrations higher than 15 μg ml^{-1}.

We harvested 433 mg of CB.Hep-1 mAb during 43 days, this mAb was purified and it was compared with the same purified mAb obtained from *in vivo* ascites system.

The harvested mAb showed 70% of purity (Figure 5), which was 5-fold higher than using ascitic fluid as starting material. This means that besides the ethical and cost advantages we were able to carry out an easy and effective purification schedule.

Because of its potentially tumorigenic characteristics, murine DNA content in the final bulk is one of the principal drawbacks of using hybridomas for obtaining biopharmaceutical products. Therefore, we determined the murine DNA content for purified mAb. In all cases it was less than 10 pg DNA/mg mAb, which is an acceptable result when compared to the reports that DNA quantities of lower than 100 pg per single human dose do not cause harmful effects (WHO, 1992). The level of Protein A contaminant was also measured in samples of purified mAb and it was less than 10 ppm in all cases.

Moreover, it is known that during culture, proteins can be modified in the degradative environment through several processes involving degradation of sugar moiety, proteolysis and amidation/deamidation (Reisfeld, 1967; Abel et al., 1968; Painter and Freedman, 1971). As a consequence, regulatory authorities recommend that particular attention should be paid to the integrity of the CB.Hep-1 mAb obtained during continuous cultivation. The analytical methods we used to control the quality of purified mAbs obtained from mice and HFB included affinity constant, analysis of glycosylation structures and immunoaffinity chromatography.

We did not find substantial differences between the affinity constant of the CB.Hep-1 mAb at the end of the experiments as compared to the first day. The values were 3.0×10^8 M^{-1} and 3.6×10^8 M^{-1}, respectively.

Determination of N-glycan structures is a requirement to ensure uniform quality of natural glycoprotein intended for pharmaceutical uses. The CB.Hep-1 mAb glycosylation analysis was monitored using a two-dimensional mapping technique. It is known that IgG class antibodies have oligosaccharides attached to a conserved *N*-glycosylation site present in the heavy chains of immunoglobulin molecules. Table 1 summarizes a comparison between the oligosaccharide composition for the CB.Hep-1 mAb, obtained in the CP1000 bioreactor with that obtained using *in vivo* conditions. Our results show only rather small differences in the relative amounts of each oligosaccharide component of IgG produced in ascites fluid or HFB, which is in accordance with previous studies on the influence of different bioreactors in IgG glycosylation (Maiorella et al., 1993). Nevertheless, our results show a higher abundance of less galactosylated oligosaccharide components (G0 and G0F), in contrast to other reports on murine IgG glycosylation under similar cell culture systems (Rothman et al., 1989; Kunkel et al., 1998).

We prepared two immunoaffinity columns with the purified CB.Hep-1 mAb obtained from the bioreactor (immunoaffinity column 1) and from ascitic fluid (immunoaffinity column 2). As shown in Table 2, we compared the elution efficiency of the immunoaffinity columns using the t-test for two-samples assuming equal variances. The P(T<=t; two-tail) was 0.63. This indicates that there were no significant differences in the elution efficiency of the immunoaffinity columns in both cases. The purity of the immunopurified rHBsAg was higher than 95% (Figure 6) and the electrophoretic profile was according to the one suggested for this antigen under reducing conditions (monomer,

dimer and trimer forms) (Fernández de Cossío et al., 1997). This suggests that the new culture system does not affect the immunoaffinity ability of the CB.Hep-1 mAb.

The results obtained for affinity constant, analysis of carbohydrate structures, and immunoaffinity chromatography suggest that there were no significant differences between the mAbs harvested from the bioreactor and from mouse ascites.

Finally, we compared different scales of the HFB system (Table 3), indicating that it is possible to obtain 56.3 g of the CB.Hep-1 mAb in 40 days using the largest scale (CP3500). This suggests that it is possible to obtain the hybridoma CB.Hep-1 derived mAb at larger scales.

As a conclusion, we have shown here that the use of the CP1000 HFB using protein-free media represents a suitable method to cultivate the hybridoma CB.Hep-1.

Acknowledgement

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