TECHNIQUE

Replacement of serum supplemented medium for CB.Hep-1 hybridoma cell freezing and monoclonal antibody production

Hasel Aragón, Marcos González, ✍ Rodolfo Valdés, Tatiana Álvarez, Enma Brown, Yadira Rodríguez, Tatiana González, Regla Somoza, Mayra Wood

Departamento de Producción de Anticuerpos Monoclonales,
Centro de Ingeniería Genética y Biotecnología, CIGB
Ave. 31 e/ 158 y 190, Cubanacán, Playa, Habana 16, CP 11600, La Habana, Cuba
E-mail: rodolfo.valdes@cigb.edu.cu

ABSTRACT

CB.Hep-1 monoclonal antibody (mAb) has been used in Hepatitis B vaccine manufacture. The mAb production was performed by ascites method inoculating cells previously frozen and cultivated in fetal bovine serum supplemented medium (SSM). It is expensive at risk and can be contaminated with adventitious pathogens. Therefore, the main objective of this study was to replace the SSM by the chemically defined medium Filoceth^{plus} (cell freezing) and the protein free medium HyQ®SFM4CHO™ (cell growing). As results, cells cryo-preserved in Filoceth^{plus} for 180 days and cultivated in HyQ®SFM4CHO™ showed no significant differences in regards to viability, maximum viable cell density and growth conditions with the same cells cultivated and frozen in SSM. In addition, CB.Hep-1 cells that were frozen in Filoceth^{plus} and cultivated in HyQ®SFM4CHO™ exhibited similar performance to those frozen and cultivated in SSM in agitated culture, which point out the application of these media for CB.Hep-1 mAb and Hepatitis B vaccine production.

Keywords: Cell freezing medium, monoclonal antibody, protein free medium, serum-free medium

Biotecnología Aplicada 2013;30:57-62

RESUMEN

Sustitución del medio suplementado con suero en la congelación de células del hibridoma CB.Hep-1 y la producción del anticuerpo monoclonal. El anticuerpo monoclonal (AcM) CB.Hep-1 se utiliza en la fabricación de la vacuna contra la hepatitis B. Se produce por el método de ascitis: inoculando células previamente congeladas y cultivadas en medios suplementados con suero fetal bovino (MSS). Este AcM es caro y puede contaminarse con agentes patógenos adventicios. Por ello, el objetivo de este estudio fue la sustitución del MSS por el medio químicamente definido Filoceth^{plus} durante la congelación de células y por el medio libre de proteínas HyQ®SFM4CHO™ durante el crecimiento celular. Los resultados con las células crioconservadas en Filoceth^{plus} durante 180 días y cultivadas en HyQ®SFM4CHO™ no mostraron diferencias significativas en cuanto a la viabilidad, la densidad máxima de células viables y las condiciones de crecimiento con respecto a las células cultivadas y congeladas en MSS. Las células CB.Hep-1, congeladas en Filoceth^{plus} y cultivadas en HyQ®SFM4CHO™, también mostraron resultados similares a los de las células congeladas y cultivadas en MSS en cultivo agitado. Ello indica que estos otros dos medios se pueden emplear para la producción del AcM CB.Hep-1 y la vacuna contra la hepatitis B.

Palabras clave: Medio de congelación de células, anticuerpo monoclonal, medio libre de proteína, medio libre de suero

Introduction

Since the discovery of the monoclonal antibody (mAb) production method [1], there has been a growing effort for improving mAb production efficiency and solving problems associated with biological safety [2, 3]. In such sense, the hybridoma cell exhibits a complex physiology due to its animal origin, which offers many challenges to process development. In this regard, improvements in mammalian cell production systems are, consequently, like to be achieved by better cell culture media, technology designs, technology operation modes and process controls. However, there are several hybridomas that do not grow and secrete well in serum-free cell culture.

In the field of process optimization, there are two areas that have shown significant advances in the last decades. They are the development of bioreactors and cell culture medium design. Evidently, both areas have had to wait for huge advances in the knowledge of animal cell physiology [4-7].

Currently, several cell culture media for a wide collection of biotechnological products have been reported, especially those that require no serum supplementation [8, 9]. These cell culture media are optimized for biomass production and secretion of specific proteins by adding micronutrients (hormones, growth factors, trace elements and vitamins) and macronutrients (glucose, glutamine, lipids and amino acids). Thus, medium formulation for animal cell cultivation is a complex task because eventually, application of high concentrations of these nutrients could lead to undesired accumulation of lactate and ammonium resulting in cell death [10, 11].

For these reasons, HyQ® SFM4CHO™ is one of the protein-free medium (PFM) successfully used. This medium formulation does not contain animal origin components and has been designed for a high performance in a variety of culture vessels. This medium also contains complexed lipids for enhancing cell stability

✍ Corresponding author

1. Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975; 256(5517):495-7.

2. Cobo F, Talavera P, Concha A. Diagnostic approaches for viruses and prions in stem cell banks. Virology. 2006;347(1): 1-10.

3. Even MS, Sandusky CB, Barnard ND. Serum-free hybridoma culture: ethical, scientific and safety considerations. Trends Biotechnol. 2006;24(3):105-8.

4. Zhang X, Stettler M, Reif O, Kocourek A, Dejesus M, Hacker DL, *et al*. Shaken helical track bioreactors: Providing oxygen to high-density cultures of mammalian cells at volumes up to 1000 L by surface aeration with air. N Biotechnol. 2008;25(1):68-75.

5. Wurm FM. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat Biotechnol. 2004; 22(11):1393-8.

and allowing the direct or sequential adaptation of cells to non-serum supplemented environments [12].

Additionally, another challenge less treated in the literature is the development of long term cryo-preservation fetal bovine serum (FBS) free media for eukaryotic cells. In such sense, the reported Filoceth^{plus} medium is a complete chemically defined medium (CDM), which seems to be a valuable alternative to the use of SSM for this purpose [13].

In the present study, we examined the following aspects: CB.Hep-1 hybridoma cell line weaning to HyQ® SFM4CHO™, stability of cells storage in liquid nitrogen using Filoceth^{plus} as freezing medium, CB.Hep-1 hybridoma cell growth in spinner-flasks using HyQ® SFM4CHO™, and CB.Hep-1 mAb production. The same cells cultivated in RPMI supplemented with 8 % FBS were used as control in all experiments. These findings would be of particular interest for the production of mAb and the hepatitis B vaccine.

Materials and methods

Hybridoma

CB.Hep-1 hybridoma is a mouse hybridoma generated with the myeloma SP2/O-Ag14, raised in RPMI 1640 supplemented with 10 % FBS (Gibco, Grand Island, USA). It produces an IgG_{2bk} mAb directed against the hepatitis B surface antigen (HBsAg) [14].

Serum supplemented media

RPMI-1640 (GIBCO-BRL, Gaithersburg, USA) supplemented with 8 % of FBS (v/v) (GIBCO-BRL, Gaithersburg, USA), 2 mM L-glutamine (GIBCO-BRL, Gaithersburg, USA), 1 mM sodium piruvate (Sigma-Aldrich, St. Louis, USA) and 17 mM sodium bicarbonate (Merck KGaA, Darmstadt, Germany) was used for cell growing. The same medium and supplements were used for cell freezing, but with FBS at 20 %.

Protein-free medium

HyQ® SFM4CHO™ (HyClone, Utah, USA) was used as PFM. It contained Pluronic F68 (Sigma-Aldrich, St. Louis, USA) and 4 mM L-glutamine, and did not contain phenol red, being also tested in T-flasks, shaker-flasks, roller-bottles and bioreactors**.** The osmolality of the medium was 290-330 mOs/kg and pH ranged 7.1-7.5 [12].

Chemically defined medium

Filoceth^{plus} (Fischer procryotect, Merenschwand, Switzerland) was used as CDM, which also contains 1 % of Pluronic F68 as active cell cryo-protectant and 10 % dimethyl sulfoxide [13].

CB.Hep-1 hybridoma cell weaning

CB.Hep-1 cells were initially cultivated in RPMI plus 8 % FBS. The whole weaning experiment was performed in 75-cm^2 T-flasks. Cells cultivated in this SSM were sequentially weaned to growth in PFM through 17 subcultures, prior to the cell freezing experiment and assessment of growth in spinner-flasks and mAb production in ascites. Total cell count and cell viability were estimated by the trypan blue dye exclusion method [15]. Briefly, cells were seeded at 3×10^5 viable cells/mL in RPMI supplemented with 8 % FBS. After several subcultures, the medium was completely replaced by RPMI plus 4 % FBS and cells maintained for at least three subcultures in these conditions. From this state, cells were cultivated in a 75:25 (v/v) mixture of SSM:PFM until SSM was completely replaced. All cultures were monitored until the maximum cell density reached 1×10^6 viable cells/mL. This procedure was repeated until cells were stably cultivated in 100 % PFM. All cultures were incubated in a humidified atmosphere of 5-7 % CO_2 in air.

CB.Hep-1 hybridoma cell freezing and recovery from cryo-preservation conditions

The viable cell density and required volume of cryo-preservation media (Filoceth^{plus} and RPMI supplemented with 20 % FBS (control)) were calculated to give a final cell density of 3×10^6 cells/mL. Cryo-preservation media were hold at 4 °C until use. Subsequently, cells were centrifuged at $250 \times g$ for 5 min and resuspended in the pre-determined volume of each cryo-preservation medium (Figure 1). Aliquots were dispensed into cryovials according to manufacturer's specifications (1.5 mL per each 1.8 mL vial) and placed into an ice box for 10 min. Then, all cryovials were transferred to an automated controlled rate freezing apparatus following standard procedures (decrease of 1 °C per min, down to -70 °C). After 48 h, cryovials were finally transferred to a liquid nitrogen refrigerator to start the cyo-preservation study. To recover cells from cryo-preservation conditions, cryovials were rapidly thawed at 37 °C in a water-bath with shaking. The content of each cryovial was transferred into appropriately-sized vessels and centrifuged at $250 \times g$ for 5 min, and cells were seeded at 3×10^5 cells/mL of SSM and PFM, respectively. All cultures were incubated in a humidified atmosphere

6. Kim DY, Lee JC, Chang HN, Oh DJ. Development of serum-free media for a recombinant CHO cell line producing recombinant antibody. Enzyme Microb Technol. 2006;39(3):426-33.

7. Schroder M, Matischak K, Friedl P. Serum- and protein-free media formulations for the Chinese hamster ovary cell line DUKXB11. J Biotechnol. 2004;108(3):279-92.

8. Chen Z, Ke Y, Chen Y. A serum-free medium for hybridoma cell culture. Cytotechnology. 1993;11(3):169-74.

9. Glassy MC, Tharakan JP, Chau PC. Serum-free media in hybridoma culture and monoclonal antibody production. Biotechnol Bioeng. 1988;32(8):1015-28.

10. Martinelle K, Westlund A, Haggstrom L. Ammonium ion transport-a cause of cell death. Cytotechnology. 1996;22(1-3):251-4.

11. Goergen JL, Marc A, Engasser JM. Determination of cell lysis and death kinetics in continuous hybridoma cultures from the measurement of lactate dehydrogenase release. Cytotechnology. 1993;11(3):189-95.

12. Thermo Scientific- HyClone- SFM4-CHO- Cell Culture Media Thermo Scientific* HyClone* SFM4CHO* Cell Culture Media [Internet]. Waltham, MA: Thermo Fisher Scientific Inc.; c2012 [cited 2012 Feb 12]. Thermo Fisher Scientific Inc.; [about 1 screen]. Available from: http://www.fishersci.com/ecomm/servlet/Search?keyWord=Thermo+Scientific-+HyClone-+SFM4CHO-+Cell+Culture+Media&store=&nav=0&offSet=0&storeId=10652&langId=-1&searchType=PROD

13. González Y, Fischer RW. Serum-free culturing of mammalian cells-adaptation to and cryopreservation in fully defined media. ALTEX. 2007;24(2):110-6.

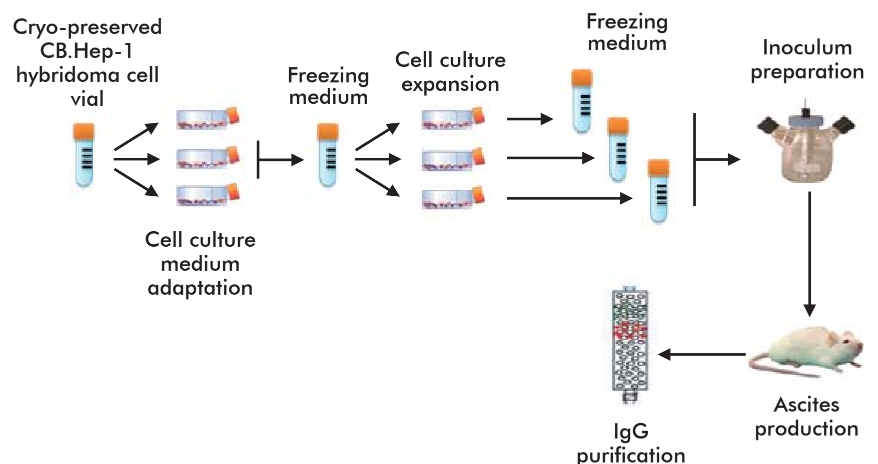

Figure 1. General flow chart of the experimental methodology for mAb production by replacing serum supplementation prior to inoculum preparation. The number of elements represents the number of replicates. The standard RPMI 1640 supplemented with 8 % fetal bovine serum (FBS) at the cell culture medium adaptation step, or with 20 % FBS plus 10 % dimethyl sulfoxide at the freezing step, was replaced by serum-free HyQ® SFM4CHO™ and the chemically-defined Filoceth^{plus} medium, respectively. Prior to inoculum preparation, 30 vials from each freezing replicate were thawed at different times (7, 21, 60 and 180 days) and inoculated in spinner flasks. Subsequently, cells were collected by centrifugation and inoculated in Balb/c mice (n = 20), either from the standard process using SSM or the new one using SFM and –CDM. The IgG mAb was purified by using a Protein A-Sepharose FF affinity chromatography column.

of 5-7 % CO_2, in air, at 37 °C and maintained between 3×10^5 and 1.2×10^6 viable cells/mL for 72 h, to estimate the similitude or differences in viability and maximum cell density. This procedure was repeated after each cryo-preservation time for allowing statistical comparisons.

CB.Hep-1 hybridoma cell cultivation for the inoculation in Balb/c mice

Cells were cultivated in 1-L spinner flasks, starting from 3×10^5 cells/mL in RPMI-1640 supplemented with 8 % of FBS, 2 mM L-glutamine, 1 mM sodium piruvate and 17 mM sodium bicarbonate**.** Cells were always maintained at 37 °C in 5-7 % CO_2 atmosphere and the medium was replaced every 48 h up to reach the maximum viable cell density. A similar procedure was performed for cells cultivated in PFM.

Estimation of the exponential growth rate and doubling time

The exponential growth rate (EGR) was calculated as:

$$EGR\ (h^{-1}) = \frac{\ln X_2 - \ln X_1}{T_2 - T_1}$$

Where:

X_1 and X_2 are the number of viable cells at the time 1 and 2 respectively.

T_1 and T_2 are sampling points 1 and 2 respectively

The doubling time (DT) was estimated as:

$$DT = \frac{\ln 2}{EGR}$$

Ascites production

Mixed groups of Balb/c mice, males and females, of 24 ± 1 and 22 ± 1 g of weigh, respectively, were inoculated for ascitic fluid production. Briefly, animals were maintained at 22 ± 2 °C, 65-80 % of relative humidity and low level of ammonium. Then, animals were primed with 0.5 mL of mineral oil into the peritoneal cavity ten days before cell inoculation. The ascitic fluid was harvested under aseptic conditions by abdominal paracentesis (tapping). After harvests, the ascites was centrifuged at 2000 × *g* for 10 min, to remove cells from the liquid phase. In order to reduce the mouse DNA content in the ascites, this procedure was carefully performed.

CB.Hep-1 mAb purification

The harvested ascitic fluid was filtered and purified by Protein A-Sepharose Fast Flow (PASFF) affinity chromatography [16, 17] using 150 mM phosphate buffered saline solution (PBS); pH 8.0 as adsorption buffer and 100 mM citric acid; pH 3.0 as elution buffer. Columns used were PD10 columns (Amersham-Biosciences, Uppsala, Sweden) loaded with 12 mL of PASFF (Amersham-Biosciences, Uppsala, Sweden) at a linear flow rate of 100 cm/h. Extensive washings with 150 mM PBS, pH 8.0, were performed to remove contaminants.

CB.Hep-1 mAb quantification by an enzyme-linked immunosorbent assay

Microtiter plates (Nunc Maxisorp, Roskilde, Denmark) were coated with 100 µg/mL of the HBsAg in carbonate/bicarbonate buffer, pH 9.6, at 50 °C for 20 min. After incubation, plates were washed. Samples, standard, and control were diluted in 150 mM PBS, pH 7.6, supplemented with 0.2 % bovine serum albumin and 0.005 % Tween 20, and further incubated again at 50 °C for 20 min. Subsequently, wells were washed five times and incubated with 100 µL/well of a goat anti-mouse IgG horseradish peroxidase conjugate at 50 °C for 20 min. Plates were finally washed and the reaction was revealed using O-phenylenediamine as substrate and 0.015 % H_2O_2 in citrate buffer; pH 5.0. Reaction was stopped by adding 50 mL of 2 M H_2SO_4 and immediately measured at 492 nm using an ELISA reader (Labsystem, Helsinki, Finland).

Total protein quantification

Protein quantification was performed following the procedure described by Lowry [18]. The range of the calibration curve was from 10 to 100 µg/mL and absorbance was measured at 730 nm in an UV/visible spectrophotometer Ultrospec (Cambridge, England).

CB.Hep-1 mAb purity estimated by SDS-PAGE

The identity pattern and purity of the CB.Hep-1 mAb was determined following the procedure described by Laemmli [19].

Statistical analysis

All the experiments were done in triplicate. Statgraphics Plus version 5.0 (2000; Statistical Graphics Corporation, USA) and Microsoft Excel programs were used for statistical analysis. One-way ANOVA was used for comparing independent samples. The significance level used for all statistical tests was set at 0.05.

Results and discussion

Several medium formulations are available for hybridoma cell culture [8, 9]. They include SSM, SFM (which contains some proteins and animal origin components), PFM (which contains no proteins but some animal origin components) and CDM (which contain neither proteins nor animal origin components). In addition to cell growth and mAb production, all these non-supplemented medium formulations facilitate recovery and purification of mAbs and greatly reduce regulatory concerns. However, deep assessments should be performed case by case because these SFM are generally highly specific. That's why, in the present study, we weaned the CB.Hep-1 hybridoma cells to PFM to investigate the stability of the CB.Hep-1 hybridoma cell line in absence of serum using a CDM as freezing medium. Next, weaned cells were inoculated into mice to compare the production of CB.Hep-1 mAb with cells cultivated in SSM.

Firstly, the CB.Hep-1 hybridoma cell line was sequentially weaned to grow in PFM in 17 passages. The maximum viable cell density and viability (1.17×10^6 cells/mL, 96.75 % viability) obtained were similar than those observed in cells cultivated in SSM (1.15×10^6 cells/mL, 94.74 % viability). These findings are in agreement with other reports of similar results during the weaning of hybridoma cells to serum-free conditions [20, 21]. However, a slight increase (21.4 %) was observed in the specific secretion rate of CB.Hep-1 hybridoma cells (33.18 vs. 27.31 pg/cell

14. Fontirrochi G, Dueñas M, Fernández de Cossío ME, Fuentes P, Pérez M, Mainet D, et al. A mouse hybridoma cell line secreting IgG and IgM antibodies with specificity for the hepatitis B surface antigen. Biotecnol Apl. 1993;10(1):24-30.

15. Patterson MK, Jr. Measurement of growth and viability of cells in culture. Methods Enzymol. 1979;58:141-52.

16. Valdés R, Álvarez T, Tamayo A, Fernández EG, Montero J, Geada D, *et al*. New Mab CB.Hep-1 Purification Process Eliminates the Need for Pre-Chromatographic Purification. Stability Demonstrated Over 100 Purification Cycles. Chromatographia. 2008;67(11-12):923-7.

17. Harshman JS. Purification of murine monoclonal antibodies by affinity chromatography using protein A-Sepharose. Methods Cell Sci. 2005;9(3):183-6.

18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193(1):265-75.

19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227(5259):680-5.

in PFM and SSM, respectively) (Figure 2). Noteworthy, this parameter demands a particular attention, because it is enhanced in parallel with serum content replacement: 9.2 % (3 %) FBS; 14.2 % (2 % FBS); 27.6 % (1 % FBS) and 21.4 % in PFM.

The increase in the specific secretion under these serum-free conditions was produced without differences in the cell growth promotion because differences in the maximum viable cell density were not detected. Therefore, the inhibitory effect observed on the IgG mAb secretion of this hybridoma cell line could be provoked by the use of FBS. It is well known that most of the IgG mAb secretion-stimulating factors are proteins whose degradation by serum proteases cannot be ruled out [22]. On the other hand, the detected inhibitory effect may also be explained by the fact that protein secretion could be favored by the PFM composition. This explanation is coincident with other reports where the higher peak of IgG secretion was found at serum-free conditions such as the HyQ® SFM4CHO™ medium [12]. Other works have also demonstrated a higher IgG secretion in SFM conditions but with a reduced cell proliferation rate and thus less accumulation of ammonium and lactate [23].

The procedure for mAb production is very tedious and time consuming, with the recovery of cells with high viability after long term storage at low temperatures among its most serious concerns. In this sense, there are several protocols for cell freezing and preservation, but most of them using FBS. However, Filoceth^plus^ was a CDM developed for long term storage of living cells in the absence of FBS [13]. Thus, the stability of the CB.Hep-1 hybridoma cryopreserved in Filoceth^plus^ and stored in liquid nitrogen was assessed during 180 days. Regarding this, frozen cells were thawed after 7, 21, 60 and 180 days, respectively, to measure their maximum viable cell density, cell viability and growth rate until 72 h after thawing. This study revealed that there were no statistically differences concerning the maximum viable cell concentration of cells cultivated and frozen in SSM, to that of cells cultivated in PFM and frozen in CDM ($p = 0.7049$; table 1 and figure 3). Therefore, it validates the results reported by the Filoceth^plus^ manufacturer and other groups [13].

Unpredictably, there were only significant differences in the cell viability after 180 days of storage in Filoceth^plus^ ($p = 0.0086$). However, these results cannot be attributed to the use of this CDM as freezing medium because similar results were observed in cells cultivated and stored in SSM ($p = 0.0057$) (Table 1). Perhaps, it seems to be more dependent on storage conditions, in terms of temperature fluctuation due to nitrogen restitution.

Since several invisible physico-chemical changes could occur into the cell during cell freezing and storage at low temperatures without affecting cell viability, we also compared the effect of a prolonged storage on the growing properties of cells frozen in Filoceth^plus^. For this purpose, cells thawed after seven and 180 days of storage, respectively, were seeded at 3×10^5 cells/mL in 25 cm^2 T-flasks containing SSM (8 % FBS) and HyQ® SFM4CHO™. Results evidenced no statistical differences ($p > 0.05$ in the maximum viable cell density, cell viability and time to reach the maximum viable

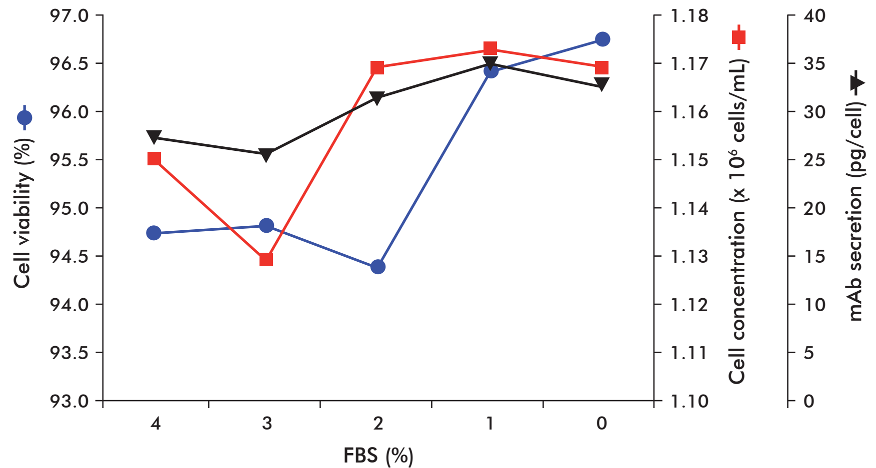

Figure 2. CB.Hep-1 hybridoma cell weaning in HyQ® SFM4CHO™. Parameters are represented as the mean value at the best adaptability of cells. The serum percentage corresponds to culture passages as follows: 4 % (passages 1 to 3), 3 % (4-6), 2 % (7-9), 1 % (10-12) and 0 % (13-17). Five passages in serum-free medium were required for full cell adaptation.

cell density (~1×10^6 cells/mL) between cells frozen in both conditions.

These results are also illustrated in figure 3. CB.Hep-1 hybridoma cells cultivated in PFM, frozen in CDM and stored in liquid nitrogen did not show substantial loss of the maximum viable cell density compared to cells cultivated and frozen in SSM. A significant decrease (up to 6 %; $p = 0.0057$ for CDM and $p = 0.0086$ for SSM) (Table 1) was only observed in the cell viability of cells stored for 180 days. These results were cell culture medium independent. Thus, this storage condition represents a useful alternative for an effective long-term storage of the CB.Hep-1 hybridoma cell avoiding the direct contact with FBS.

Once a hybridoma cell is generated, it can be used for producing antibodies by several methods. One of them comprises injecting hybridoma cells into the intraperitonial cavity of a mouse. The antibody-producing cells proliferate, releasing the target antibody at higher concentrations in the ascites. Then, the antibody is harvested by tapping the fluid from the intraperitonial cavity and further purified by different procedures [24, 25].

Prior to the inoculation of cells cultivated in the PFM and SSM into the animals, both cultures were compared in spinner flasks. As shown in table 2, there were no differences in the maximum viable cell density, cell viability, and doubling time of cell populations cultivated in both media. This was important because a higher growth rate and doubling time usually denote overfeeding, which may in turn lead to harmful lactate and ammonium accumulation in the culture supernatant. Thus, the use of this PFM minimizes the accumulation of waste products to a level that can be detrimental.

Furthermore, we were interested in comparing mAb production in ascites of cells weaned to growth in this PFM. In the ascites production study, cells cultivated in PFM were inoculated at 1×10^6 cells/mL per each Balb/c mouse approximately (Figure 1). Sixty animals were inoculated per each independent

20. Shinmoto H, Dosako S. Adaptation of mouse-human hybridomas to a protein free medium. Biotechnol Lett. 1993;15(4):327-30.

21. Darfler FJ. A protein-free medium for the growth of hybridomas and other cells of the immune system. *In Vitro* Cell Dev Biol. 1990;26(8):769-78.

22. Liguori MJ, Hoff-Velk JA, Ostrow DH. Recombinant human interleukin-6 enhances the immunoglobulin secretion of a rabbit-rabbit hybridoma. Hybridoma. 2001;20(3):189-98.

23. Schneider YJ. Optimisation of hybridoma cell growth and monoclonal antibody secretion in a chemically defined, serum- and protein-free culture medium. J Immunol Methods. 1989;116(1):65-77.

24. Patchornik G, Albeck A. A general approach for antibody purification utilizing [Protein A-catechol:Fe3+] macrocomplexes. Bioconjugate Chem. 2006;17(2):258-60.

25. Hahn R, Shimahara K, Steindl F, Jungbauer A. Comparison of protein A affinity sorbents III. Life time study. J Chromatogr A. 2006;1102(1-2):224-31.

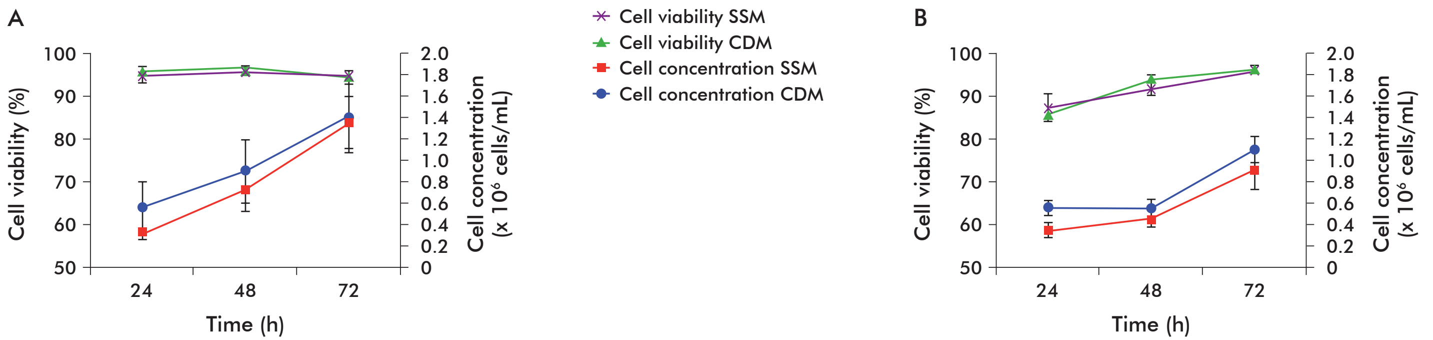

Figure 3. Analysis of cell concentration and viability of CB.Hep-1 hybridoma cells after storage frozen for 7 (A) and 180 (B) days in SSM and CDM media, after culture for 24 to 72 hours. There were only statistically significant differences for cell concentration in CDM between samples frozen for 7 and 180 days, and cultured for 24 hours after thawing ($p = 0.0247$).

Table 1. Results of the stability study of the CB.Hep-1 hybridoma cell line stored in liquid nitrogen using RPMI or Filoceth[plus] media for cryopreservation (n = 3) with or without fetal bovine serum (FBS)

Cell culture media for cultivation / freezing	Days 0		7		21		60		180		p values	
	CC	CV	CC	CV	CC	CV	CC	CV	CC	CV	CC	CV
SSM (8% FBS) / SSM (20% FBS)	3.3	96.0	3.4 ± 0.5	95.8 ± 1.5	3.2 ± 0.3	95.4 ± 0.4	3.0 ± 0.5	95.2 ± 1.6	3.0 ± 0.7	89.9 ± 3.3	0.7356	0.0086
PFM / CDM	3.3	96.0	3.2 ± 0.5	93.6 ± 1.7	3.3 ± 1.0	95.9 ± 1.6	3.0 ± 0.3	94.5 ± 1.9	3.0 ± 0.4	90.7 ± 0.9	0.9079	0.0057
p values	-	-	0.5097	0.1722	0.8402	0.6086	1.0000	0.6496	0.9456	0.7049	-	-

CC: Mean cell concentration values per 10^6 cells/mL.
CV: Mean cell viability values, expressed as percentage of viable cells.

experiment and twenty per replicate. In order to determine the effect of cells cultivated in PFM on ascites and mAb production, several critical factors were assessed. In the ascites production step, results were as follows: 96.6 ± 5.7 % of animals with ascitic tumor for cells cultivated in PFM vs. 95.0 ± 8.6 % for cells cultivated in SSM. The volume of ascites produced per animal was 3.8 ± 0.8 mL and 4.8 ± 1.3 for cell cultivated in PFM and SSM, respectively. The average amounts of IgG mAb were 39.8 ± 9.2 mg IgG per mouse in the ascites harvested from mice inoculated with cells cultivated in PFM, and 53.5 ± 34.9 mg IgG per mouse in the case of cells cultivated in SSM. The IgG mAb/total protein ratio, a parameter used for estimating the amount of IgG per total protein content in the ascites ('indirect measure of purity') was also statistically similar between these two experimental variants; 19.2 ± 3.1 % for PFM and 19.7 ± 6.0 % for SSM. In general, probability values for these parameters were not statistically different among values obtained in PFM and SSM (Table 2).

Particularly, costs associated to the culture system were not analyzed because they were irrelevant for mAb production in both media, CDM and PFM, as being part of the whole process.

Differences in the physico-chemical properties and yields of purified IgG mAb from ascites obtained from cells cultivated in CDM and PFM media should not be expected. Nevertheless, as the amount of the IgG in the ascites produced with cells cultivated with SFM was lower (Table 2), we assessed the purity, recovery and yield of the purified molecule, rejecting the hypothesis that this difference could be provoked by degradation of the IgG molecule in the ascites.

Consequently, the impact of using PFM for cell culture on mAb purification was also studied. To perform the CB.Hep-1 mAb purification, an affinity chromatography step based on PASFF was carried

26. Hober S, Nord K, Linhult M. Protein A chromatography for antibody purification. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;848(1):40-7.

27. Hahn R, Schlegel R, Jungbauer A. Comparison of protein A affinity sorbents. J Chromatogr B Analyt Technol Biomed Life Sci. 2003;790(1-2):35-51.

28. Tavares RM, Karmali A, Clemente A, Pais MS. Production and characterization of a specific rubisco monoclonal antibody, and its use in rubisco quantification during Zantedeschia aethiopica spathe development. Hybridoma. 1999;18(2):203-9.

Table 2. Results of spinner-flasks cell culture behavior and ascites production after the inoculation of the CB.Hep-1 hybridoma in BALB/c mice*

Medium	Spinner-flasks cell culture parameters				Ascites production parameters									
	CC	CV	EGR	DT	Animals with tumors (%)	Volume of ascites (mL)	Volume of ascites per mouse (mL)	IgG concentration in ascites (mg/mL)	IgG amount in ascites (mg)	IgG per mouse (mg)	Total protein in ascites (mg/mL)	Total protein (mg/L)	Total protein in ascites (mg)	IgG/total protein-ratio (%)
SSM (8 % FBS)	1.3[a]	95.3[a]	0.018[a]	38[a]	95 ± 8.6	139.3 ± 46.1	4.8 ± 1.3	7.2 ± 2.1	1073.5 ± 692.0	53.5 ± 34.9	36.7 ± 4.2	5135 ± 1954.0	256.7 ± 97.7	19.7 ± 6.0
PFM	1.4[a]	95.7[a]	0.017[a]	40[a]	96.6 ± 5.7	111.6 ± 31.5	3.8 ± 0.8	7.1 ± 1.6	777.3 ± 198.4	39.8 ± 9.2	36.9 ± 3.0	4092 ± 1142.1	205.4 ± 58.3	19.2 ± 3.1
p values	-	-	-	-	0.7952	-	0.8247	-	-	0.5190	-	0.4816	-	0.8964

* Values are shown as mean ± standard deviation, unless null standard deviation. Ascites production was evaluated in 20 animals.
[a] Only one determination per spinner flask.
CC: Maximum cell concentration, expressed as values per 10^6 cells/mL.
CV: Cell viability (%).
EGR: Exponential growth rate (h^{-1}).
DT: Doubling time (h).

out. Protein A is a cell wall component of the *Staphylococcus aureus*. This protein contains a linear series of five highly homologous antibody-binding domains [26, 27]. The application of this affinity chromatography in the purification of the CB.Hep-1 mAb evidenced a mAb recovery higher than 75 % (Table 3), in agreement with published reports [28], and no significant differences among mAb purification from ascites harvested from animal inoculated with cells cultivated in both media.

Finally, the characterization of purified molecules by agarose gel electrophoresis showed that SDS-PAGE profiles observed in both groups of samples matched with the typical IgG pattern (two bands at 50-55 kDa and 20-25 kDa, corresponding to the heavy and light chains, respectively) under reducing conditions. The purity of the eluted fraction was higher than 95 %, in all the assessed samples (Figure 4). However, PASFF could also co-concentrate non-specific antibodies. For instance, ascites averaged about 1 mg of host polyclonal IgG/mL (most of them IgG1 in mice). Thus, purity of the mAb was also corroborated by a different method, the CB.Hep-1 mAb/total protein ratio which was named 'specific activity' (Table 3). The 'specific activity' of the purified mAb was above 94 % in SSM samples and 100 % in PFM samples, corroborating the high purity and specificity of the purified molecules and with no significant differences between them (p = 0.1406). Moreover, the slight bands observed in the SDS-PAGE were the heavy chain bands and also heavy chain of the CB.Hep-1 mAb over it. This might be explained by differences in the heavy chain glycosylation patterns.

Conclusions

As shown above, the process using PFM to produce CB.Hep-1 mAb can meet purity specifications for pharmaceutical-grade mAb. CB.Hep-1 hybridoma cells weaned well and relatively quick to HyQ® SFM4-CHO™ medium. CB.Hep-1 hybridoma cells cultivated in HyQ® SFM4CHO™ and frozen in Filoceth^plus can be cryo-preserved in liquid nitrogen for at least 180 days showing non-significant differences in regards to the viability, maximum viable cell density and growth conditions with cells cultivated in RPMI + 8 % FBS and frozen in RPMI plus 20 % FBS. CB.Hep-1 hybridoma cells cultivated in HyQ® SFM4CHO™ exhibited similar performance relative to those cultivated in RPMI plus 8 % FBS in stationary suspension cultures and success in the mAb production by the ascites production method, which point out to the application of the PFM HyQ® SFM4/CHO™ for CB.Hep-1 mAb and hepatitis B vaccine manufacture.

Received in February, 2012.
Accepted in September 2012.

Table 3. Results of the CB.Hep-1 mAb chromatography purification for each culture medium used (n = 3)*

Hybridoma cell culture medium	Applied (mg)	Eluted (mg)	Recovery (%)	SDS-PAGE purity (%)	IgG/total protein ratio (%)
SSM (8 % FBS)	144.1 ± 45.3	108.3 ± 28.6	75.2 ± 3.6	97.9 ± 0.2	94 ± 3.6
PFM	138.2 ± 25.4	105.9 ± 19.6	76.7 ± 5.2	98.9 ± 0.4	100 ± 4.3
p values	0.8302	0.9105	0.7158	0.0531	0.1406

* Values are shown as mean ± standard deviation.

Acknowledgments

Authors are thankful to Gnotobiotic Animal Production Group at the National Center for Animal Breeding for supplying mice and also thank to Lic. Ernesto Galbán for his excellent edition work of the article. This work is supported by the Center for Genetic Engineering and Biotechnology of Havana, Cuba, as part of the monoclonal antibody production for vaccine production.

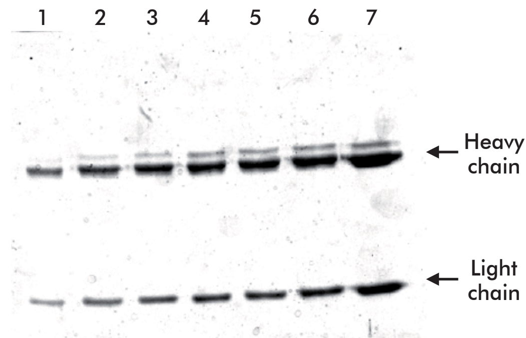

Figure 4. Analysis of CB.Hep-1 mAb by SDS-PAGE. Lanes 1-3: CB.Hep-1 in SSM, replicates 1 to 3; Lanes 4-6: CB.Hep-1 in PFM, replicates 1 to 3; Lane 7: Reference material (mouse IgG).