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A Summary of the Manufacture, Biochemical Characterization, and Virological Safety Demonstration of the Mouse mAb CB.Hep-1 Used to Produce the Hepatitis B Vaccine

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Introduction

Monoclonal antibodies (mAb) are highly selective molecules, and an unlimited amount of mAbs with equal quality can be produced using mammalian cell cultures and animals. These molecules have remarkable applications in biomedicine, diagnosis and therapy due to the ability to reproduce exactly the same binding properties. The mAbs have been generated against an ostensible set of compounds such as toxins, drugs, blood proteins, cancer cells, viruses, hormones, environmental pollutants, food products, metals and plant materials.

In general, mAbs can also be used for creating sensitive tests to detect small amounts of substances, and in therapies, abzymes, and for isolating specific compounds from complex mixtures by immunoaffinity chromatography (IAC).[1-3] The IAC is a very specific method which involves the recognition between an antibody and an antigen in a reversible manner.[4] Applying this technique in the purification of drug substances can increase costs. However, because a high purity and concentration factor can be achieved from the start of the purification process, it can enormously simplify the successive downstream processing.[5,6]

In vitro technologies are currently the most commonly used mAb manufacturing[7,8] methods. There are other methods for producing mAbs that are less expensive and do not require a large initial investment of capital for the facilities. Also, this goal of reducing production costs becomes difficult to obtain because several cell lines cannot be adapted to *in vitro* growth conditions, and production and stability rates of some hybridomas in cell culture can be very low (3–4% fail).

Among the main advantages of mAb production by ascites method are: 1) high ascites volumes and antibody concentration can be reached; 2) animal housing and staff are available in most of the labs; and 3) low risks for contamination with mAbs containing mouse DNA.[9] Nevertheless, because of its relatively high cost, extended virological safety validation programs, and ethical and market-associated problems, researchers are trying to replace mAb production by the ascites method.[10,11]

The main concern with the use of mAbs obtained by ascites as parental products or as immunoreagents used in drug substance manufacture is the introduction of adventitious agents (viruses, bacteria, fungi and mycoplasmas) and process-related impurities (protein and DNA

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Rosario Alemán, of the CGEB, and Enrique Noa, of the National Reference Center for AIDS Research, conducted most of the viral validation experiments.

Gisela Calás, Cristina García, Biunayki Reyes and Lorely Milá, of the mAb Production Department and Quality Assurance Direction at the CEGB, were involved in the establishment of the documentation system.

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contaminants, column leachables, media components) into the drug substance.[12] Thus, acceptability of products obtained by this methodology will depend on the demonstration of robust methods of production, validation, characterization of the molecule, virological safety, and compliance with regulatory standards (*e.g.*, the FDA's "Points to Consider") for mAb production for human use.

With that in mind, this work summarizes conclusive evidences on the methodology and results of the manufacture, biochemical characterization, virological safety demonstration, and viral validation of the mAb CB.Hep-1 used in the hepatitis B vaccine production.

Materials and Methods

mAb CB.Hep-1 Manufacture

Hybridoma CB.Hep-1 Cell Generation and Cultivation

The SP2/0-Ag14 derived hybridoma (CRL-1581; ATCC, Manassas, Virginia, USA) CB.Hep-1 was obtained using a Balb/c mouse subcutaneously immunized with the hepatitis B surface antigen (HBsAg) emulsified in complete and incomplete adjuvants of Freund (Sigma, St. Louis, Missouri, USA).[13] Cells were cultivated in 1L spinner-flasks, starting from 3×10^5 cells mL^{-1}, using RPMI-1640 (GIBCO-BRL, Gaithersburg, Maryland, USA) supplemented with 8% of foetal calf serum (FCS) (GIBCO-BRL), 2 mM L-glutamine, 1 mM sodium piruvate and 17 mM sodium bicarbonate. Cells were always maintained at 37°C in 5% CO_2 atmosphere and the medium was replaced every 48 h up to reaching the highest cell density. The cell viability was measured by the trypan blue exclusion method.[14]

Ascites Fluid Production

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

mAb CB.Hep-1 Purification

The ascites fluid was harvested, filtered, and then underwent two ammonium sulfate precipitations. In both precipitations, the material was centrifuged at 4800 x g for 20 min at 4°C. The dissolved pellet was desalted by size-exclusion chromatography using Sephadex G-25 coarse (Amersham Biosciences, Uppsala, Sweden) and a BP113/60 column (Amersham) at 130 cm h^{-1} and 150 mM phosphate buffered saline solution (PBS); pH 8.0, as mobile phase. The desalted material was purified by Protein A-Sepharose Fast Flow (PASFF) affinity chromatography[15] using 150 mM PBS; pH 8.0 as adsorption buffer and 100 mM citric acid; pH 3.0, as elution buffer. The column used was a BPG100/50 (Amersham) at a linear flow rate of 100 cm h^{-1}.

Extensive washings with PBS; pH 8.0, were done to remove endotoxins and other protein contaminants. Subsequently, an incubation of the purified mAb in 100 mM citric acid; pH 3.0 at 4°C, was performed as a virus inactivation step. Afterward, the buffer of samples was exchanged to 20 mM Tris/150 mM NaCl; pH 7.6, by size-exclusion chromatography using Sephadex G-25 coarse in a BP113/60 column (Amersham) at 130 cm h^{-1} and filtered under sterile conditions by 0.22 µm membranes.

Beaded Agarose Activation for mAb CB.Hep-1 Immobilization

The Sepharose CL-4B (Amersham) was moderately activated with CNBr (Merck, Darmstadt, Germany) according to the Wilcheck classification (6–12 µM of cyanate esters mL^{-1} of matrix).[16] The activation reaction was made as Axen, Porath and Ernback´s work and the concentration of cyanate esters was determined by the modified König reaction.[17, 18] The mAb CB.Hep-1 immobilization to the Sepharose CL-4B (Amersham) was performed according to a previously described procedure.[19]

Immunoaffinity Chromatography mAb CB.Hep-1 Immunosorbent Quality Evaluation

The IAC developed for evaluating the quality of the immunosorbents was performed in PD-10 columns (Amersham) packed with 12.1 mL of immunosorbents under hydrostatic pressure. Immunosorbents were previously equilibrated with 60 mL of 20 mM Tris/3 mM EDTA/1 M NaCl; pH7.0. Columns were directly loaded with the purified recombinant hepatitis B surface antigen (rec-HBsAg) in the same buffer. Columns were washed with the equilibrium buffer and the elution was carried out using 20 mM Tris/3 mM EDTA/1 M NaCl/3 M KSCN; pH 7.0. The flow rate was the same for all the steps.[20]

mAb CB.Hep-1 Biochemical Characterization

Colorimetric Assay for Protein Quantification

The quantification of proteins was performed following the procedure described by Lowry.[21] The range of the calibration curve was from 10–100 µg mL^{-1}. The absorbance was measured at 730 nm in an Ultrospec UV/Visible Spectrophotometer (GE Healthcare, Cambridge, England).

Enzyme-Linked Immunosorbent Assay (ELISA) for mAb CB.Hep-1 Quantification

MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 µg mL^{-1} of the rec-HBsAg in carbonate/bicarbonate buffer; pH 9.6. After incubation, plates were washed. Standard and control samples were diluted in 150 mM PBS/0.2% of bovine serum albumin/0.005% Tween 20 and incubated again for 20 min at 50°C. Subsequently, wells were washed five times and incubated with 100 µL $well^{-1}$ of a goat anti-mouse IgG horseradish peroxidase conjugate for 20 min at 50°C.

Plates were finally washed and the reaction was revealed using O-phenylenediamine (OPD) as substrate and 0.015% H_2O_2 in citrate buffer; pH 5.0. The reaction was stopped by adding 50 mL of 2 M H_2SO_4 and immediately measured at 492 nm using an ELISA Reader (Labsystems, Helsinki, Finland).

SDS-PAGE and HPLC-GF to Determine mAb CB.Hep-1 Purity

The identity pattern and the purity of the mAb CB.Hep-1 and rec-HBsAg were determined following the procedure described by Laemli.[22]

The molecular distribution and purity of the mAb were also estimated by using a HPLC-GF column TSK G3000 PW (600 mm/57.5 mm I.D., TosoHaas, Japan). The chromatographic mobile phase was 150 mM PBS; pH 7.0 and 100 µg of the samples dissolved in 150 mM PBS; pH 7.0 were directly applied into the system. The volumetric flow rate employed was 200 µL min^{-1} and the absorbance was measured at 226 nm.

Digestion of mAb CB.Hep-1 and Extraction of Peptides

The tryptic digestion of the mAb CB.Hep-1 followed a published protocol.[23] Gel digestions with the lysyl endopeptidase (LEP) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), endoproteinase Asp-N (Boehringer Ingelheim GmbH, Ingelheim, Germany), and quimotrypsin (Sigma-Aldrich, Inc., St. Louis, Missouri, USA) were performed with a standard method reported for tryptic digestions. Peptides were recovered and desalted with a ZipTip C18 column (Millipore Corp., Billerica, Mass., USA) followed by an elution step using 60% acetonitrile and 1% formic acid. Finally, solutions were mixed and loaded into the gold-coated capillaries.

Mass Spectrometry to Determine mAb CB.Hep-1 Amino Acid Sequence

The low-electrospray ionization (ESI)/mass spectrometry (MS) and MS/MS spectra were acquired using a hybrid quadrupole orthogonal acceleration tandem mass spectrometer QTof 2 (Waters Corp., Milford, Mass., USA) fitted with a Z-spray nanoflow ESI source. Other measuring conditions and data processing were done according to González *et al.*[24]

mAb CB.Hep-1 Specificity Confirmation

A cellulose membrane containing several peptides of the HBsAg was washed two times with dimethylformamide/ethanol/water and with 0.05% Tween/TBS (0.137 mM NaCl/0.0026 mM KCl/0.05 mM Tris); pH 7.0, three times for 10 min per wash. Subsequently, the membrane was blocked with 5% milk in TBS and incubated with the mAb solution for 3 h at room temperature.

The membrane was then washed in 0.05% Tween/TBS four times and incubated with an anti-mouse alkaline phosphatase conjugate dissolved in 5% milk/0.5% Tween 20/TBS for 30 min. Then, after several washings with TBS, 0.05% Tween/TBS, 5-bromine-4 chlorine-3 indol phosphate was added to the membrane for 10–30 min.

The reaction was stopped by washing the membrane with TBS. The amino acid sequence of the peptides is shown in Table 1 (1–37).[25] In this assay, the mAb CB.IFN-2,4 was used as negative control.[26]

ELISA to Determine mAb CB.Hep-1 Affinity Constant

The affinity constant was determined by the method described by Betty *et al.*[27] Microtiter plates were coated with the rec-HBsAg and incubated with the mAb CB.Hep-1. Plates were sequentially incubated with a horseradish peroxidase-antibody conjugate and the reaction was revealed using OPD as substrate and 0.015% H_2O_2 in citrate buffer; pH 5.0. The reaction was stopped by adding 50 mL of 2 M H_2SO_4. The amount of antibodies adherent to the antigen on the plates was reflected by the enzyme product measured by optical density at 492 nm using a Multiskan ELISA reader.

mAb CB.Hep-1 Isoelectric Point Determinations

A high-resolution electrophoretic technique to split proteins and peptides based on their isoelectric points was applied. Conditions used were a lineal pH gradient, ranging from 5.0–7.0 (PhastGel IEF, Amersham), and a homogeneous polyacrylamide gel (5% T, 3% C).[28]

Isotyping mAb CB.Hep-1

The isotype of the mAb CB.Hep-1 was determined by means of a mouse mAb isotyping commercial kit following the manufacturer's recommendations (HyCult biotechnology b.v., Uden, The Netherlands).

ELISA to Determine mAb CB.Hep-1 Released from Agarose Beads

Briefly, a plate (Costar, Cambridge, Mass., USA) was coated with a sheep anti-mouse polyclonal immunoglobulin (Ig) overnight at 4°C. The plate was blocked for 30 min at 37°C. Wells were washed and the eluted samples from immunosorbents were added and incubated for 3 h at 37°C with 1% non-fat milk/150 mM PBS; pH 8.0. After washing the plate, it was incubated with 100 µL $well^{-1}$ of a goat anti-mouse polyclonal immunoglobulin-horseradish peroxidase conjugate (Sigma-Aldrich).

The reaction was then revealed using 100 µL $well^{-1}$ of 0.05% OPD and 0.015% H_2O_2 in citrate buffer; pH 5.0, and stopped with 50 µL $well^{-1}$ of 1.25 M H_2SO_4. The absorbance was measured in a Multiskan ELISA reader using a 492 nm filter.[19, 20]

Quantification of the Main Contaminants

ELISA to Quantify Staphylococcal Protein A

Polystyrene plates (Costar) coated with anti-Protein A polyclonal antibodies were used to quantify the staphylococcal Protein A. After the sample addition, polyclonal antibody fragments conjugated with horseradish peroxidase were applied, and the reaction was then revealed with peroxidase substrate solution. The absorbance was measured in a Multiskan ELISA reader using a 492 nm filter.[29]

Dot-Blot to Quantify Mouse DNA

The mouse DNA content in the purified mAb CB.Hep-1 preparation was determined according to the method reported by Brown.[30] The oligonucleotides used as probes are shown in Table 1.

Transmission Electron Microscopy (TEM) for Murine Virus Determinations

Detection of retrovirus-like particles (RVLP) in hybridoma cells was done by thin section TEM. Cells were fixed in 2.5% glutaraldehyde/100 mM cacodylate during 1 h at 4°C, and cells were fixed again in 1% osmium tetroxide for 1 h at 4°C. Subsequently, cells were washed with 100 mM cacodylate and dehydrated using growing concentrations of ethanol, 30%, 50%, 70% and 100%, for 10 min at 4°C each time. Then cells were included in the Spurr resin following the procedure described by Spurr.[31]

Ultrafine sections of 400–500 Å were made using the LKB NOVA Ultrotome (Leica Microsystems, Inc., Deerfield, Illinois, USA) and placed in grids made from copper and nickel (400 mesh) to be contrasted with saturated uranyl acetate in methanol and lead citrate. Finally, cells were examined at a 15,000 magnification (Figure 1) using a JEM-2000 EX model TEM (JEOL Ltd, Tokyo, Japan) and microphotographed. Chosen at random, 200 cells were analyzed by DIGIPAT software (EICISOFT, Havana, Cuba).

Retrovirus Characterization By S^+L^- Focus Assay

In the assay, 0.5 mL of the hybridoma CB.Hep-1 supernatant was diluted 1:1 (v/v) with the cell culture medium RPMI 1640 (Invitrogen, Carlsbad, Calif., USA)/10% FCS (HyClone, Logan, Utah, USA)/polybrene (at 10 µg mL^{-1}) and inoculated, in duplicate, onto a subconfluent monolayer of $MiCl_1$ cells (CCL-64.1, ATCC) in 5 cm^2 plates. The plates were incubated at 37°C for 2 h after which time, 5 mL of fresh medium was added to each plate. The cell cultures were maintained for 10 days, and during this time, a microscopic examination was made for focus development. Foci are recognized by the fact that they are rounded cells that appear raised above the level of the contact-inhibited monolayer. The virus titre that can induce countable foci was expressed as focus forming units (FFU) per unit of volume. The xenotropic feline leukemia virus (strain FeLV-C) was employed as positive control while the culture medium was assayed as negative control.

The assay was considered valid when the number of foci was within 0.5 Log_{10} of the specified reference virus titre. The theoretical assay sensitivity, based on the Poisson distribution at 98% confidence level, is 4 FFU mL^{-1}.[32, 33]

Table 1. DNA oligonucleotides used to quantify the mouse DNA and HBsAg peptides used in the mAb CB.Hep-1 specificity assay.

5′- GAAAAACGTG-3′ and ′-GATCCTCATTTTTCACGTTTTT3-′

1. MENITSGFLGPLB	14. RFIIFLFILLLCB	27. AFAKYLWEWASVB
2. GFLGPLLVLQAGB	15. FILLLCLIFLLVB	28. WEWASVRFSWLSB
3. LVLQAGFFLLTRB	16. LIFLLVLLDYQGB	29. RFSWLSLLVPFVB
4. FFLLTRILTIPQB	17. LLDYQGMLPVCPB	30. LLVPFVQWFVGLB
5. ILTIPQSLDSWWB	18. MLPVCPLIPGSTB	31. QWFVGLSPTVWLB
6. SLDSWWTSLNFL	19. LIPGSTTTSTGPB	32. SPTVWLSAIWMMB
7. TSLNFLGGSPVCB	20. TTSTGPCKTCTTB	33. SAIWMMWYWGPSB
8. GGSPVCLGQNSQB	21. CKTCTTPAQGNSB	34. WYWGPSLYSIVSB
9. LGQNSQSPTSNHB	22. PAQGNSMFPSCCB	35. LYSIVSPFIPLLB
10. SPTSNHSPTSCP	23. MFPSCCCTKPTDB	36. PFIPLLPIFFCLB
11. SPTSCPPICPGYB	24. CTKPTDGNCTCIB	37. LLPIFFCLWVYI
12. PICPGYRWMCLRB	25. GNCTCIPIPSSWB	
13. RWMCLRRFIIFLB	26. PIPSSWAFAKYLB	

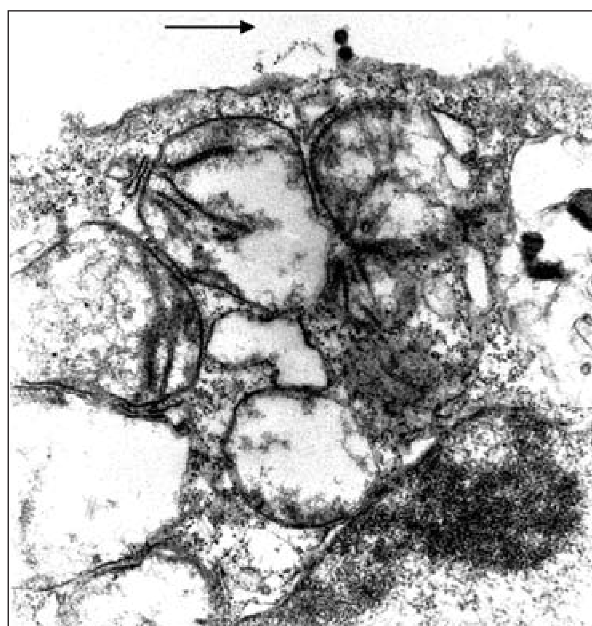


FIGURE 1. Electromicrograph of the hybridoma CB.Hep-I obtained by transmission electron microscopy. The arrow indicates type C RVLP (100–200 nm) in the external space of the cells. A magnification of 15,000 x was employed.

Retrovirus Characterization By XC Plaque Assay

In the assay, 0.5 mL of the hybridoma CB.Hep-1 supernatant was diluted 1:1 (v/v) with 0.5 mL of the cell culture medium MEM (Invitrogen)/1.5 g L^{-1} sodium bicarbonate/mixture of non-essential amino acids/0.1 mM sodium pyruvate/10% FCS (HyClone)/polybrene (at 10 µg mL^{-1}) and inoculated in duplicate onto SC-1 cells (CRL-1404, ATCC) in 5 cm^2 dishes. After the culture incubation at 37°C for 2 h, 5 mL of the culture medium was added to each dish and the cultures were incubated at 37°C.

The inoculated SC-1 cells were maintained for 5–7 days under this temperature and then cells were irradiated under UV light for 60 seconds. XC cells (CRL-165, ATCC) in fresh medium were added to the irradiated monolayer. These cultures were incubated at 37°C until the plaque formations occurred in the positive dishes. The cultures were stained with a crystal violet solution for 30 min, washed, dried and observed (appearing as holes in the XC cell sheet surrounded by multinucleated giant cells). The ecoptropic-MLV (strain mov-3) and cell culture medium were used as positive and negative controls respectively.

The assay was considered valid when the number of plaques was within 0.5 Log_{10} of the specified reference virus titre. The theoretical assay sensitivity based on the Poisson distribution at 98% confidence level is 4 PFU mL^{-1}.[32, 33]

Mouse Antibody Production (MAP) Tests Directed Against Murine Viruses

NMRI mice were inoculated with hybridoma CB.Hep-1 supernatants by different routes to assure the maximum opportunity for the adventitious viruses to infect animals. The serological techniques used to detect the presence of mouse virus-specific antibodies in the inoculated mice were ELISA, indirect fluorescent antibody (IFA) and hemoagglutination inhibition (HAI). Hybridoma supernatants were diluted 1:10 (v/v) in the cell culture medium RPMI-1640 (GIBCO-BRL) and injected into different animal groups using the following volumes: 50 µL (intranasal), 100 µL (intracraneal), and 500 µL (intraperitoneal, subcutaneous and intravenous). The cell culture medium was used as negative control.

The test was rejected when at least one animal from the first group died within 14 days after inoculation, or if one animal of the second group was eliminated by any unknown cause. Three sera of the non-inoculated mice and three sera of the animals obtained before the inoculation of the supernatants were also used as negative control. Hyperimmune sera, specific for each murine virus, were used as positive control. Samples were taken between 3 and 14 days after the supernatant inoculation for determining the lactate dehydrogenase (LDH) activity. The rest of the sera were analyzed after the 28th day post-inoculation.

Samples were considered contaminated with viruses when virus-specific antibodies were detected in the sera of the mice inoculated with cell supernatants. A similar procedure was employed for indirectly detecting viruses in the ascites and mAb CB.Hep-1 preparation. (See Table 2.)

TABLE 2.
Murine virus-specific antibody testing.

POTENTIAL VIRAL CONTAMINATION	ASSAY
Sendai virus (SeV)	ELISA
Pneumonia virus of mice (PVM)	ELISA
Mouse hepatitis virus	ELISA
Minute virus of mice (MVM)	ELISA
Mouse encephalomyelitis VIRUS (TMEV)	ELISA
Reovirus type 3 (Reo-3)	ELISA
Virus of epizootic diarrea of infant mice	ELISA
Mouse ectromelia virus (MEV)	ELISA
Polyomavirus	HAI
Mouse adenovirus	ELISA
Lymphocytic choriomeningitis virus (LCMV)	ELISA
Mouse cytomegalovirus (MCV)	ELISA
Mouse thymic virus	IFA
Hantaan virus	IFA
Lactate dehydrogenase-elevating virus (LDHV)	Enzyme

In Vitro Assay for Viral Contamination Detection in the Hybridoma CB.Hep-1 Cells

Hybridoma cells were cultivated and lysated prior to inoculation. Cells and supernatant were harvested from the cell culture and centrifuged at low speed. In parallel, duplicate cultures of detector cell lines were prepared in 75–80 cm^2 flasks and inoculated, with 3 mL per monolayer, the negative control (culture medium), test article and the positive control.

Cultures were maintained at 36°C for 14 days to be regularly observed microscopically for evidence of the cytopathic effect (CPE). At the end of the two-week culture period, the negative control and culture inoculated with the test article were tested for the ability to haemoadsorb (HA) guinea pig red cells at 4°C. A blind passage was made and the observation continued for another 14 days for cytopathogenic changes and HA. Cell lines used are shown in Table 3. Negative samples (culture media) were assessed alone as positive and negative controls. The test was considered valid if the virus induced cytopathogenic

TABLE 3.
Cell lines used for *in vitro* assays.

LOW PASSAGE
Human embryonal lung cells (HEL)
Tertiary cultures of African green monkey kidney cells (TAGMK)
Buffalo green monkey cells (BGM)
Human rhabdomyosarcoma cells (RD)
Mink lung cells (MVLu)
Subline of human cervix tumor cells
HeLa (rhinovirus-sensitive strain [Ohio])
Cloned mouse liver cells (NCTC 1469)
Cloned mouse connective tissue cells (L929)
Primary mouse embryo cells (ME)
KNOWN POSITIVE
Human poliovirus type 1
Human cytomegalovirus (CMV)
Herpes simplex virus type 1 (HSV-1)
Influenza A virus

changes and/or HA in positive controls and not in negative controls. The test samples were considered positive when cytopathogenic changes or HA were found.[36]

In Vivo Test in Chicken Embryonated Eggs for Viral Contamination Detection in the Hybridoma CB.Hep-1 Cells

This determination was made inoculating 200 µL of the hybridoma CB.Hep-1 supernatant in the allantoidal fluid and yolk sacs of ten embryonated eggs. The viability of embryos was checked during the first three days post-inoculation. Ten days later, the allantoidal fluid was harvested for the virus detection by means of HA of human, chicken and guinea pig erythrocytes.

The assay was considered valid when 70% of embryos did not present virus infection symptoms in 10 days and 80% of embryos inoculated with positive controls showed virus infection measured by HA and lesions in the chorioallantoidal membrane. All negative samples were additionally inoculated in embryos to corroborate the absence of viruses. The influenza type A (strain PR8) and RPMI 1640 were employed as positive and negative controls respectively.

In Vivo Test in Guinea Pigs; and Adult and Suckling Mice for Viral Contamination Detection in Hybridoma CB.Hep-1 Cells

Adult mice and guinea pigs were intracerebrally, intramuscularly and intraperitoneally inoculated with the hybridoma CB.Hep-1 supernatant. The clinical evolution of the animals was observed for 40 days. After this period of time, the animals were sacrificed by means of cervical dislocation to perform the pathological examination. In addition, three groups of suckling mice were inoculated with the hybridoma CB.Hep-1 supernatant in order to monitor animal growth and virus-related pathologies during an additional 45 days.

Serology Test to Detect Murine Viruses in Mice

Animal sera were incubated for 1 h at 37°C and subsequently for 1 h at 4°C. Then samples were centrifuged at 2000 x g for 5 min and diluted in 150 mM PBS; pH 7.2. After this procedure, sera were inactivated at 56°C for 30 min in order to proceed to the detection of antibodies directed against mouse viruses by ELISA or IFA. The number of the evaluated sera was determined according to Nicklas *et al.*[37] Where:

$$S = \frac{\log 0.05}{\log P} x\, N$$

S: size of the healthy animal group,
P: percentage of disease prevalence,
Log 0.05 at 95% of confidence.

Determination of Adventitious Viruses in the Ascites Fluid Using Cell Lines Susceptible to Virus Replication

Cell lines were maintained in 5 mL of cell culture medium in 25 cm² T-flasks for 24 h at 37°C. Then the cell culture medium was completely replaced by 5 mL of ascites fluid (in triplicate). The inoculated cultures were maintained at 37°C and monitored for 14 days using microscopy to observe the CPE. Finally, a HA assay was performed using guinea pig, Rhesus monkey and chicken erythrocytes for detecting viruses. Cell lines, cell culture medium and strains used are shown in Table 4.

Validation of the Viral Removal and Inactivation Capacity of the mAb CB.Hep-1 Purification Process

Virus Origins and Cell Lines Used to Measure the Cytopathic Effects of the Viruses

The model viruses used in this study cover a wide range of physico-chemical and structural characteristics of the viruses.[38] (See Table 5.)

Protein A Virus Removal with Beaded Agarose Affinity Chromatography

The validation and revalidation studies were done following the same principles of the viral validation studies.[38-41] These studies were performed in a laboratory separate from the mAb CB.Hep-1 production

TABLE 4.
Hemoagglutination inhibition assay components.

CELL LINES	CELL CULTURE MEDIA	STRAIN
Vero (ATCC 81)	MEM supplemented with 2% FCS	Bovine parainfluenza type 3 (SF-4, ATCC VR-281)
A9 (ATCC 1.4)	DMEM supplemented with 2% FCS	Measles virus (Edmonton, ATCC VR-24)
MRC-5 (ATCC CCL-171)	MEM supplemented with 2% FCS	MMV (ATCC VR-1346)

TABLE 5.
Model virus types, donors, and means of titration.

VIRUSES	DONATED BY	TITERED IN
Sendai virus (SeV)	National Center for Animal Breeding of Cuba (Cenpalab)	chicken embryonated eggs
Human herpes simplex virus type 1 (HSV-1)	National Veterinary Institute of Hungary	African green monkey kidney cells (Vero), guinea pig fibroblast cells (LFBC), and the HIV-negative human T cells (MT4)
Human immunodeficiency virus type 1 (HIV-1)	Dr. Mark Wainberg, McGill University, Canada	
Human poliovirus type 2 (HPV-2, Sabin strain)	National Institute for the Biological Standard and Control of England	
Canine parvovirus (CPV, origin 7164)	The Cuban National Center of Epidemiology and Diagnostic	

facility, and keeping constant the affinity chromatography residence time and the protein concentration in the column applied material. The scaled-down version of the processes represented 1% of the real purification scales. The starting material was individually loaded with each model virus and applied into the PASFF affinity column (in triplicate for each virus). The IgG adsorption buffer was 150 mM PBS; pH 8.0, while 100 mM citric acid; pH 3.0, was used as elution buffer.

All experiments were conducted at 4°C. The affinity columns used were the XK26/30 (Amersham Bioscences) loaded with 2.5 mL of matrix (validation) and 54.2 mL (revalidation). Both columns were operated at 100 cm h^{-1}. The protein absorbance was registered using a 280 nm filter and the applied IgG per run was 90% of the dynamic IgG binding capacity of the matrices.

Virus Inactivation By Acid pH

The viral inactivation at acid pH study was carried out by incubating samples of mAb CB.Hep-1 containing virus at 4°C for 7 h in 100 mM citric acid; pH 3.0, and taking samples every hour. Samples were neutralized by the addition of 2 M Tris to check the CPE for each virus. The initial titers were 8.0, 5.8, 9.0, 11.0, and 9.8 for SeV, HIV-1, HSV-1, HPV-2, and CPV, respectively. The mAb CB.Hep-1 antigen recognition capacity was also assessed without the presence of viruses at different temperatures.[38]

Virus Inactivation By Heat Treatment and a Chaotropic Agent (KSCN)

Each model virus (HSV-1, HIV-1, HPV-2, CPV) was inoculated in 1 mL of the eluted rec-HBsAg preparation and incubated at 60°C in the presence of 3 M KSCN; pH 7.0, for 2 h. After this, the temperature was reduced to 20°C and the buffer was exchanged to measure the CPE for each virus. The initial titers were 7.5, 5.5, 8.0, 10.0, and 8.5 for SeV, HIV-1, HSV-1, HPV-2, and CPV, respectively.[38]

Virus Inactivation During Protein A/Agarose Bead Matrix Sanitization With 70% Ethanol

Model viruses (HSV-1, HIV-1, HPV-2, and CPV) individually diluted 1:10 (v/v) were added to 9 mL of the PASFF matrix previously washed and equilibrated with a five-fold column volume of ethanol at 70%. The working temperature was 4°C and supernatants were collected after each exposure time (0–10 min, 12 h) to be dialyzed against 150 mM PBS; pH 7.2, for allowing the titration of viruses. Similar amounts of viruses added to the PASFF matrix previously equilibrated with 150 mM PBS; pH 7.2, and kept at 4°C during the whole experiment, served as control.[41]

Non-Enveloped Virus Inactivation During Protein A/Agarose Bead Matrix Sanitization with HCl 0.1 N

Non-enveloped model viruses (HPV-2 and CPV) were diluted 1:10 (v/v) and added to the PASFF matrix previously washed and equilibrated with five-fold column volume of 0.1 N HCl; pH 1.0. The working temperature was 4°C and samples were collected after the following exposure times: 0–10 min; 3, 4, 5, 8, and 10 h; to be dialyzed against 150 mM PBS; pH 7.2, to allow for virus titration. A similar amount of virus added to the affinity matrix previously equilibrated with 150 mM PBS; pH 7.2, and kept at 4°C during the whole experiment was used as control.[41]

Virus Inactivation During Protein A/Agarose Bead Matrix Storage With 20% Ethanol

Model viruses (HSV-1, HPV-2, HIV-1, CPV) individually diluted 1:10 (v/v) were applied to 9 mL of the PASFF matrix previously washed and equilibrated with five-fold column volume of ethanol at 20%. The working temperature was also 4°C, and samples (HSV-1 and HIV-1: 0-10 and 15 min, 1 and 2 h; HPV-2 and CPV: 0-10, 15, and 30 min, 1, 24, 48, and 72 h) were collected after each exposure time and the samples were dialyzed against 150 mM PBS; pH 7.2 to allow the virus titration. A similar amount of virus added to the affinity matrix previously equilibrated with 150 mM PBS; pH 7.2, at 4°C was used as control.

Calculation of the Removal and the Inactivation Capacity

The virus removal capacity (RC) was calculated individually as follows:[39]

$$10^{RC} = \frac{(\text{input virus titer x input volume})}{(\text{output virus titer x input volume})}$$

Titration of the Sendai Virus

The titers of the SeV were determined by the inoculation of test solutions into allantoic cavities of specific pathogen-free embryonated chicken eggs (supplied by the National Center for Animal Breeding of Cuba) and calculated using the Reed Müench method.[42] Endpoints were taken at the last dilution given virus infection effect, with the virus titer expressed as Log_{10}EID50 mL^{-1}. The titration of the virus was considered satisfactory when the difference between expected and true titer of the SeV used as control was less than 1 Log. Each sample was titrated by triplicate.

Titration of the Human Herpes Simplex Type 1 and Human Poliovirus Type 2

The titers of the HSV-1 and HPV-2 were determined by the inoculation of test solutions into Vero cell cultures and calculated using the Reed Müench method.[42] Briefly, the assay involved the inoculation of 50 µL of the viral sample in 150 µL of the medium MEM (GIBCO-BRL) supplemented with 2% FCS (HyClone) containing Vero-confluent cells. Ten serial dilutions were performed across the plates that were maintained at 37°C under 5–6% CO_2 atmosphere. On the fifth day, cultures were carefully observed. Endpoints were taken at the last dilution given CPE, with the virus titer expressed as Log_{10}TCID50 mL^{-1}.

The same virus preparation was used as control of the experiment and it was storage aliquoted at –70°C until thawing immediately prior to the assay of titration. The titration of viruses was considered satisfactory when the difference between expected and true

titers of the HVS-1 or HPV-2 used as control was less than 1 Log. Each sample was titrated by triplicate with eight determinations per each serial dilution.[38]

Titration of the Human Immunodeficiency Virus Type 1

The HIV-1 titers were determined by the inoculation of samples into MT4 cell culture and calculated using the Reed Müench method.[42] Fifty microliters of viral samples were inoculated in 150 µL of RPMI 1640 (GIBCO-BRL) supplemented with 2% of FCS (HyClone) containing MT4-confluent cells. Ten serial dilutions were performed across the plates. Plates were also maintained at 37°C under 5–6% CO_2 atmosphere. On the seventh day, the cultures were carefully observed and the virus titer was expressed as Log_{10}TCID50 mL^{-1} of the last dilution with CPE. The same virus preparation was used as control of the assay.

The titration of the virus was considered satisfactory when the difference between expected and true titers of the HIV-1 used as control was less than 1 Log. Each sample was titrated by triplicate with eight determinations per each dilution.[38]

Titration of the Canine Parvovirus

The titers of CPV were determined according to the Reed Müench method[42] of inoculating test solutions into LFBC cell cultures. The viral samples were added to 150 µL of MEM (GIBCO-BRL) supplemented with 2% of FCS (HyClone) containing LFBC confluent cells and maintained at 37°C under 5–6% CO_2 atmosphere. On the fifth day, the cultures were carefully observed.

The last dilution with virus CPE was taken for estimating the virus titers. The same CPV preparation was used as control of the experiment and the assay of the titration was considered satisfactory when the difference between expected and true titers of the CPV used as control was up to 1 Log. Each sample was titrated in triplicate with eight determinations per each dilution.[38]

Results and Discussion

The CB.Hep-1 is a mAb directed against the HBsAg routinely used as the immunoreagent for immunopurifying the rec-HBsAg employed as the active pharmaceutical ingredient of the hepatitis B vaccine.[13,43] In such sense, regulatory authorities such as the United States Food and Drug Administration have issued documents containing points to consider for the manufacturing of mAbs used as immunoreagents.[12]

These guidelines have been developed thanks to the accumulated experience from the required standards for mAb, vaccine and recombinant DNA product production. In general, the demonstration of the purity and efficacy is required, as well as the reliability and safety of the production process. There has not been much experience in using mAbs as immunoreagents for vaccine manufacturing on a large scale. Thus, the work outlined here could be generalized for any mAb produced by ascites method and used for drug substance purification.

Summary of the Manufacture of the mAb CB.Hep-1

The mAb CB.Hep-1 was generated by the fusion of a splenocyte of a mouse immunized with the HBsAg, with a SP2/0-Ag14 cell (a non-Ig-secreting or synthesizing line derived from a cell line created by fusing a BALB/c mouse spleen cell and a mouse myeloma P3X63Ag8).[13] The characterization of a mAb-producing cell line should include the specificity, class and subclass of the secreted Ig, viability, doubling time, cell density and production stability (main criterion). The antibody secretion should be stable regarding the type of antibody, class switch, and level of the expression beyond the population doublings used for the routine production. In such sense, a good deal of information concerning the loss of antibody productivity is available for myelomas.[44] In some myelomas, the mechanism of antibody loss involves losing the heavy-chain expression while in other cell lines, the loss of antibody production is due to the concurrent loss of the production of both chains. Hybridomas can also show loss of antibody production due to the loss of chromosomes containing the gene loci for the antibody chains. As a consequence, this non-secretory cell could have advantages in further cell cultivation.

The mAb CB.Hep-1 cell secretion capacity was maintained over 4.6 pg cell^{-1} (average = 9.6 ± 3.1 pg cell^{-1}) in spinner- and T-flasks during 60 passages, which characterizes this hybridoma as a low mAb producer, although quite stable. Additional passages were not studied because they were not necessary in the ascites production. The class and subclass of the IgG determined at the beginning of cell cultivation and at 60th passage showed coincidences with an IgG-2b kappa light chain (Figure 2). Thus, all further inoculations of cells in mice were made with a number of cell passages inferior to 60 and with a mAb secretion

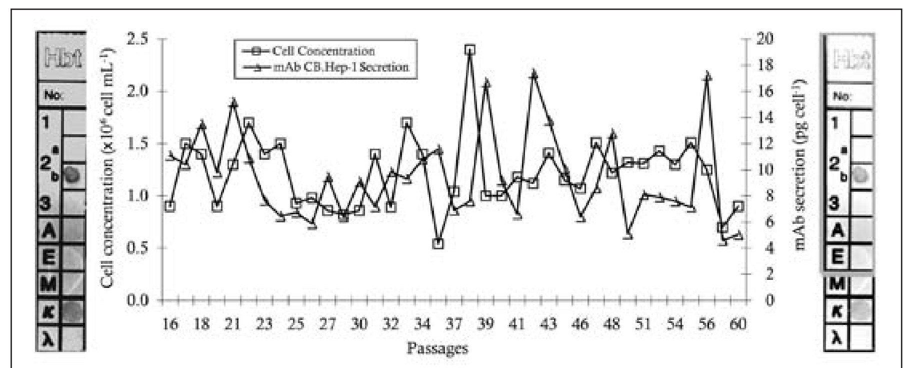


FIGURE 2. Specific secretion of the hybridoma CB.Hep-1 and isotype determination of the purified mAb CB.Hep-1 at the beginning (left) and at the end of the study (right).

consistently over 4.6 pg cell^{-1} (average = 8.48 ± 3.0 pg cell^{-1}).

Certainly we did not observe a clear correlation between mAb secretion in cell culture and ascites (R^2 = 0.014), but a secretion level over 4.6 pg cell^{-1} in the cell culture consistently guaranteed an average mAb CB.Hep-1 concentration of 3.13 ± 0.7 mg IgG mL^{-1} in the ascites (Table 6).

The mAb CB.Hep-1 purification process was designed combining saline precipitations to reduce the ascites volume and remove some contaminants (pre-purification treatment) with PASFF affinity chromatography (final purification). One of the advantages of the mAb purification from ascites fluid is the initial volume (a few liters) as opposed to the cell culture technology (several thousand liters). However, the complexity of the ascites is enormous. Therefore, the use of adsorption chromatographies, ion exchange, or affinity chromatographies is almost mandatory for obtaining a high recovery and purity of the final product with acceptable costs. In this method, the incubation of the mAb CB.Hep-1 at acid pH, ultrafiltration and sterile filtration steps were also introduced to increase the virus inactivation factor and achieve the required concentration and sterility of the preparation, respectively.[43]

The average recovery of the whole purification process was 68.02 ± 7.3% (Table 7). It processed up to 60 L of ascites in less than a week, a product concentration factor from 2.7 to 10-fold, and a stability of the affinity chromatography matrix, extended to 211 ± 46.6 purification cycles. These general results are optimum if the composition of the ascites and purification scale are taken into account.

In a particular analysis of the purification process, the pre-purification treatment consisted of two ammonium sulfate precipitations and a desalting by size-exclusion chromatography using Sephadex G-25. The recovery of this pre-purification treatment was 79.97% (Table 7) but the most important reasons for this treatment were the concentration of the products: 1) up to 30% of the initial volume; 2) the

TABLE 6. Outline of the mAb CB.Hep-1 controls employed in the characterization of the mAb and production process (n=22).

mAb CB.Hep-1 PRODUCTION STEPS	TESTS	LIMITS	TRUE VALUES
Cell Culture	Viability	90%	96.1 ± 4.1
	mAb secretion	4.6 pg cell^{-1}	8.4 ± 3.0
	In vitro test	Negative	Negative
	Specific *in vivo* test for murine viruses	Negative (viruses, group I)	Negative
	In vivo test for adventitious viruses	Negative	Negative
	TEM	RVLP	RVLP-Type C
	S^+L^- focus assay	Negative	Negative
	XC plaque assay	Negative	Negative
	Stability	60 passages	60
	Isotype	IgG-2b *Kappa* light-chain	IgG-2b *Kappa* light-chain
	Mycoplasmas	Negative	Negative
	Sterility of cell supernatants	Passes the test USP-29	Passes the test
Ascites Production	Mouse weight	≥ 21 g	24 male and 22 female
	mAb concentration	> 1 g L^{-1}	3.1 ± 0.7
	Specific *in vivo* test for murine viruses	Negative (viruses, group I)	Negative
	In vivo test for adventitious viruses	Negative	Negative
	Serology (animals)	Negative (viruses, group I)	Negative
	Mycoplasmas	Negative	Negative
	Sterility of the ascites	Passes the test USP-29	Passes the test USP-29
Purification of the mAb	mAb concentration	8 mg mL^{-1}	11.0 ± 0.9
	SDS-PAGE (reducing conditions)	90%	98.0 ± 2.7
	SDS-PAGE (no reducing conditions)	90%	98.7 ± 2.8
	HPLC-GF	95%	96.4 ± 0.9
	Mouse DNA	100 pg–10 ng mg^{-1} mAb	<7
	Staphylococcal PA	10 ppm	0.4 ± 0.38
	Specific *in vivo* test for murine viruses	Negative	Negative
	Process recovery	35.3–60.4%	68.0 ± 7.3
	Amino acid sequence	DNA coincident	DNA coincident
	Peptide mapping	Coincident in three lots	Coincident in three lots
	Lysines numbers	88	88
	Affinity constant	n x 10^8 M^{-1}	7.6 ± 6.9x10^8
	Isotype	IgG-2b *Kappa* Light chain	IgG-2b *Kappa* Light chain
	Isoelectric point range	4.9–8.2	6.7–7.1
	Isoproteins	up to 8 isoproteins	8
	Antigenic determinant recognized	"a"	"a"
	Amino acid sequence recognized	Cys-Lys-Thr-Cys-Thr-Thr	Cys-Lys-Thr-Cys-Thr-Thr
	Sterility of mAb preparation	Passes the test	Passes the test
	Stability of PASFF matrix	> 100 runs	211.0 ± 46.6
	Virus clarification capacity*	> 12 Logs	13.1–21.6
Activation of the Matrix	Volume/weight factor	1–4 mL	1.4 ± 0.04
	Cyanate esters concentration	> 3.8 µmoL mL^{-1}	11.0 ± 1.0
	Coupling efficiency	> 80%	99.2 ± 0.5
Immobilization of the mAb	Coupling efficiency	> 80%	99.3 ± 0.8
	Ligand density	3.5–4.0 mg mL^{-1}	4.0 ± 0.2
	rec-HBsAg elution capacity	100 µg mg^{-1} mAb	247.0 ± 7.4
	SDS-PAGE (rec-HBsAg, reducing conditions)*	> 80%	88.2 ± 3.2
	HPLC-GF (rec-HBsAg)**	> 95%	99.3 ± 0.8
	Ligand leakage	< 3 ng mAb µg^{-1} rec-HBsAg	0.3 ± 0.1
	Microbiology control	Passes the test USP-29	< 23

LEGEND:
*SDS-PAGE (rec-HBsAg) corresponds with the antigen purity after the affinity chromatography (n=29).
**HPLC-GF antigen purity at the end of the purification process (n=40).

elimination of some low molecular weight contaminants; and 3) the preparation of the samples to start the chromatographic purification process.

Protein A is a cell wall component of the *Staphylococcus aureus*. This protein contains a linear series of five highly homologous antibody-binding domains.[45, 46] Even though there are several described efficient purification methods for antibodies,[47, 48] affinity chromatography using Protein A as ligand is currently the most used.[49, 50] In general, PASFF affinity chromatography is capable of providing, under appropriate conditions: 1) a total IgG purity as high as 95%; and 2) between 70 and 90% of recovery while removing up to 4.5 Logs of DNA, 6.0 Logs of viruses, and 4.0 Logs of endotoxins in a single step. The primary binding site for Protein A is on the Fc fragment of the IgG (at the juncture of the CH_2 and CH_3 domains), but the affinity of the Protein A differs among IgG of different species and subclasses, because it is closely related to the net hydrophobicity of the contact residues of both molecules. For instance, the affinity of Protein A by the mouse IgG-2b is considered from moderate to high.[4]

The evaluation of this affinity chromatography in the purification process of the mAb CB.Hep-1 evidenced a mass recovery of the mAb in about 89.95% (Table 7), which shows correlation with published reports.[51] The purity of the eluted fraction was higher than 95%, but PASFF also co-concentrated non-specific antibodies. For instance, ascites average about 1 mg of host polyclonal IgG mL^{-1} (most of them IgG^{-1} [mouse]). Thus, the purity of the mAb must be corroborated by other methods.

After this powerful purification step, the purification process of mAb CB.Hep-1 was designed by the sequential combination of incubation at acid pH, size-exclusion chromatography (buffer exchange), ultrafiltration (concentration) and sterile filtration. The recoveries of these steps were 98.50, 97.98%, 98.65% and 99.68% respectively (Table 7). All of these steps performed according to expected results and none of them increased the purity of the mAb CB.Hep-1 preparation. Against this design, a further work demonstrated that the pre-purification treatment was unnecessary to get a high recovery and purity.[15] However, the influence of this process modification on virus removal efficiency is still under study.

Similar to affinity chromatography, the basis of the IAC is the specificity with which an immobilized antibody or antigen is recognized and adsorbs the corresponding partner.[4] Antibodies are ligands of exceptional interest in affinity chromatography because of their high bio-specificity. In regard to this application, several matrix activation procedures were being developed[17, 53, 54] over four decades ago. Within them, the CNBr activation (non-oriented method) is the most frequently used technique for immobilizing ligands to agarose because it is relatively simple and can be performed entirely in aqueous solutions.[55, 56] A limiting factor of the agarose is its low mechanical stability, which is especially important for large-scale processing. Nevertheless, cross-linking of the polysaccharide backbone resulted in matrices with higher rigidity.

In spite of the advantages of the IAC, there are currently very few examples of the application of this technique on large scale-purification. In the 1980s, almost 20 different proteins (*e.g.*, interferons, urokinase, tissue plasminogen activator, tetanus toxin, rec-HBsAg, etc.) were considered as candidates for their purifications, based on the IAC.[4] However, for vaccine purposes, the rec-HBsAg is one of the very few examples where a successful application of IAC has been finally demonstrated. An immunosorbent coupling efficiency greater than 98% was observed in all immobilizations of the mAb CB.Hep-1 to Sepharose CL-4B, but the amount of the antibody immobilized is, of course, dependent of the amount of active groups (cyanate esters).

The ligand density obtained was always higher than 4.0 mg mL^{-1} of matrix. A higher ligand density was not used because a high level of immobilized antibody decreases the specific activity and the purification efficiency due to steric factors. The elution capacity was about 247 ± 7.4 µg of the rec-HBsAg

TABLE 7. Recovery of the mAb CB.Hep-1 purification steps. The expected recovery values were estimated from values reported by each purification step (*n*=20).[51]

mAb CB.Hep-1 PRODUCTION STEPS	EXPECTED RECOVERY (average)	TRUE VALUE (average ± SD)
mAb CB.Hep-1 concentration (mg mL^{-1})	—	3.13 ± 0.70
Pre-treatment purification (%)	60–80	79.97 ± 14.38
mAb CB.Hep-1 concentration (mg mL^{-1})	—	7.67 ± 0.47
PASFF affinity chromatography (%)	70–90	89.95 ± 11.76
mAb CB.Hep-1 incubation at acid pH (%)	≥ 98	98.50 ± 2.10
mAb CB.Hep-1 concentration (mg mL^{-1})	—	4.98 ± 1.07
Size-exclusion chromatography (buffer-exchange) (%)	≥ 95	97.98 ± 2.50
mAb CB.Hep-1 concentration (mg mL^{-1})	—	2.99 ± 0.90
Ultrafiltration (%)	≥ 95	98.65 ± 3.29
mAb CB.Hep-1 concentration (mg mL^{-1})	—	8.80 ± 1.04
Sterile filtration (%)	≥ 95	99.68 ± 5.06
mAb CB.Hep-1 concentration (mg mL^{-1})	—	8.60 ± 1.03
Total recovery (%)	35.3–60.4	68.02 ± 7.32
Productivity of purification process (g mAb 30 h^{-1})	30–60	≅ 40

(Figure 3) mg^{-1} mAb and the purity of the eluted rec-HBsAg was higher than 80%, demonstrating the high purification capacity and consistency of this chromatography (Table 6).

In such sense, due to the high binding of the antibody-antigen complex, the most critical aspect in this chromatography is usually the antigen dissociation and elution. But in this case, the most critical step has been the adsorption because of the multiple-chemical forms and degrees of aggregation of the rec-HBsAg. In such sense, it is well known that the rec-HBsAg is produced by the expression of a monomer (Mr) 24,000 polypeptide in yeasts, where about 100 units of monomers are intracellularly self-assembled in lipoprotein particles.[57] In addition, rec-HBsAg retention into the matrix after the dissociation and elution step has been observed.[58]

Another crucial aspect of this technique is the level of the mAb leakage from the matrix. Isourea linkages are labile, especially at alkaline conditions in CNBr activated media. The mAb CB.Hep-1 that could be present in the hepatitis B vaccine could have several potential negative effects such as the stimulation of undesired immune responses and unintentional reactivity, or cytotoxicity for human tissues.[4, 59] In this case, the ligand leakage never exceeds 0.3 ± 0.1 µg of mAb mg^{-1} rec-HBsAg, which represents a very low amount of IgG, especially if we consider that it is the first chromatographic step of the rec-HBsAg purification process.[60] So these results identify a low risk for the application of this mAb in hepatitis B vaccine production and a poor influence of ligand leakage on the loss of the column efficiency (Figure 3).

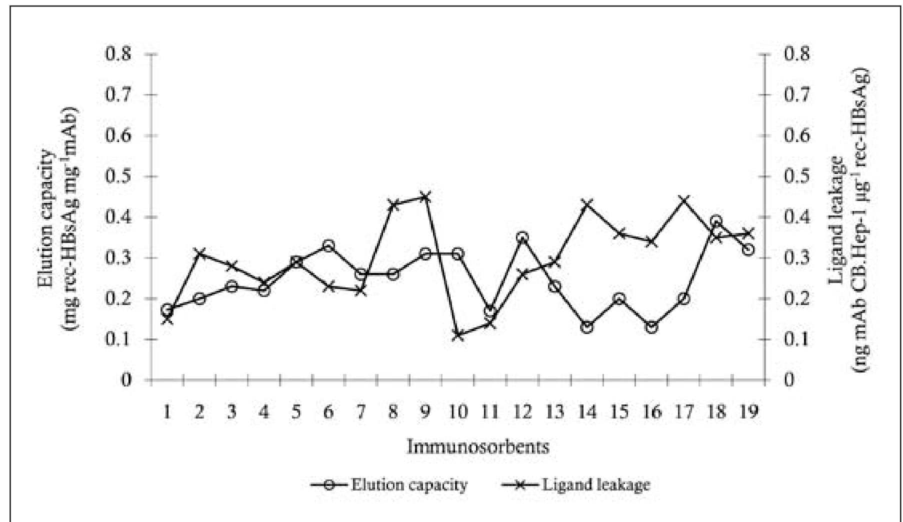


FIGURE 3. Antigen elution capacity and ligand leakage of the mAb CB.Hep-1 immunosorbents.

Summary of the Biochemical Characterization of the mAb CB.Hep-1

The mAbs are strongly variable molecules, especially in the isoelectric point and hydrophobicity. Thus, a rigorous characterization of the purified mAb molecule by chemical and biological methods is essential. At least the following parameters should be studied: class, subclass, light-chain composition, glycosylation patterns, integrity of the molecule by analysis of the heavy/light chain ratio, molecular weight, amino acid sequences, and secondary and tertiary structures.

Nevertheless, with the accumulated experience, and considering that this immobilization method does not involve carbohydrates and application of this mAb (IAC), glycosylation pattern, N and C terminal amino acid sequence, secondary and tertiary structures, and light-chain composition would be omitted. On the contrary, specificity assays are extremely important. Particular attention should be given to the use of a wide range of analytical techniques exploiting different physico-chemical properties of the molecule. Examples of suitable techniques for molecular characterization are: sodium docecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions, isoelectrofocusing, column

chromatography, and mapping of peptides.

Conditions used in the purification process of the mAb CB.Hep-1 provided an IgG absorption capacity of 15–20 mg mAb mL^{-1} of the PASFF and a purity of the purified mAb higher than 95% measured by SDS-PAGE and HPLC-GF methods (Table 6 and Figure 4). The homogeneity and identity of the purified mAb molecule were also checked by isoelectrofocusing, isotyping, MS and ELISA. These tests are valuables tools for testing the consistency of different production lots. The isoelectrofocusing pattern resulted in the presence of four majority isoproteins (eight isoproteins in total) which corresponds with an isoelectric point in a range from 6.75–7.04 (Figure 4C).[28] All isoproteins showed reaction against a serum specific for the mouse IgG-2b subclass (data not shown). These results are coincidental with those reported before, demonstrating values of isoelectric points ranging from 4.9 to 8.2 for mouse IgG-2b.[61]

Isotyping is a classification of mAb based on characteristics of the Ig in the samples. There are two formats of isotypes, the heavy and light chains. The heavy-chain includes all Ig types and subtypes, while the light-chain includes the κ and λ (L1C1) formats. Extrapolating the analysis to the human immune system, several isotypes such as IgG-2b have already been shown to be efficient in fixing the complement,[62] which suggests that among mAb, the IgG-2b subclass

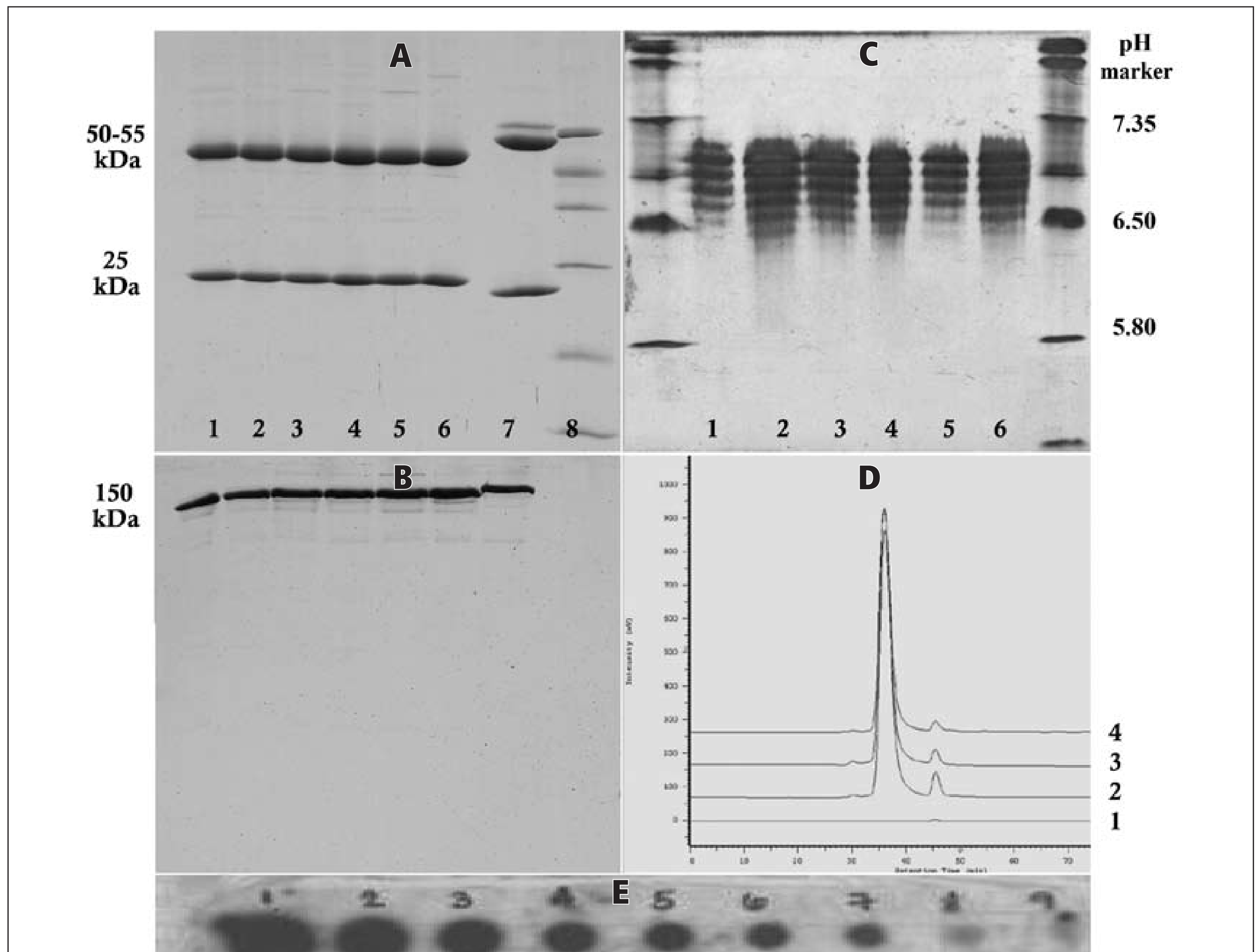


FIGURE 4. **A:** Purity of the mAb CB.Hep-1 estimated by SDS-PAGE under reducing conditions. Lines1-6, samples of the mAb CB.Hep-1. Line 7, inner reference material of a mouse mAb. Line 8, molecular weight marker, (bovine serum albumin 80 kDa, ovoalbumin 49 kDa, carbonic anhydrase 34.8 kDa, soybean trypsin inhibitor 28.9 kDa, lysozyme 20.6 kDa, aprotrinine 7.01 kDa). **B:** Purity of the mAb CB.Hep-1 estimated by SDS-PAGE under non-reducing conditions. Lines 1-6, samples of the mAb CB.Hep-1. Line 7, inner reference material of a mouse mAb. **C:** Isoelectrofocusing of the mAb CB.Hep-1. Lines 1-5, samples of the mAb CB.Hep-1. Line 6, inner reference material of the mAb CB.Hep-1. **D:** Purity of the mAb CB.Hep-1 estimated by size exclusion-HPLC. Line 1, 20 mM Tris/150 mM NaCl; pH 7.6. Line 2, inner reference material of the mAb CB.Hep-1. Lines 3-4, samples of the mAb CB.Hep-1. **E:** Mouse DNA content in samples of the mAb CB.Hep-1. Lines 1-9, mouse DNA standard curve (1 ng – 3.9 pg of ADN). Line 10, negative control (100 µg of bovine serum albumin). Line 11, inner reference material of the mAb CB.Hep-1. Line 12, sample of mAb CB.Hep-1.

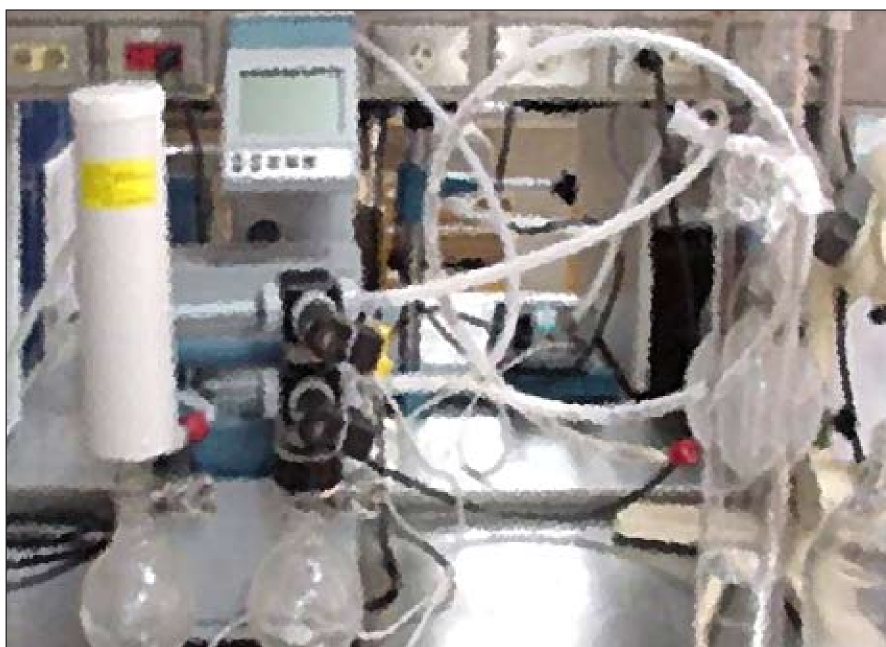
might be a good choice for serotherapy. Therefore, the isotype determination has remarkable importance for therapeutic uses. Nevertheless, for mAbs used as immunoreagents in IAC and obtained from ascites, it is just another important criterion of purity. This test is even more important if the mAb is purified by PASFF because an overlapping of eluted fractions can be observed, and there are also evidences of Protein A binding to the Fc regions of the IgAs and IgMs.[51] However, this purity criterion may also be relative because it does not permit discrimination among mAbs of the same subtype. The determination of the isotype of the mAb CB.Hep-1 demonstrated consistency for the IgG-2b κ light chain (Table 6 and Figure 1). The amount of mouse IgG-2b contaminant was indirectly estimated by the ELISA/Lowry ratio, where no contamination with mouse non-specific IgG-2b was detected.

The ESI/MS has been used to elucidate the structure of recombinant DNA-derived biotherapeutics. However, with the high molecular weight, complex structure, and large number of potential mass isoforms, there would be significant challenges to the analysis of mAbs. In this study, both heavy and light chains of the mAb CB.Hep-1 were previously isolated by SDS-PAGE for MS. The purified samples were submitted to a previous deglycosylation protocol with PNGaseF. It was due to sugar interferences in MS observed in previous experiments, where mAb CB.Hep-1 chain separations were performed by HPLC (data not shown). The mAb CB.Hep-1 light chains were completely verified. Meanwhile, only 94% of the heavy chains were sequenced because of peptide recovery difficulty from the SDS-PAGE. As a result, 100% of the sequenced amino acids coincided with the nucleotide sequences of the mAb CB.Hep-1 genes.[63, 64] On the other hand, since CNBr reacts with the hydroxyl groups on agarose to form cyanate esters and imidocarbonates, which react with amines (ε-amino group of the lysines [HO_2 CCH {NH_2}] [{CH_2}$_4$ NH_2]) in order to couple the protein onto the agarose matrix, the knowledge of the exact position of lysines into the amino acid sequence of the mAb was extremely important. The contribution of this analysis was to know that the total number of lysines was 88 (100%), 70 lysines (89.7%) are located at the Fc fragment and 18 (10.3%) at the Fab fragment (6, CDRs and 12, frameworks) and thus demonstrated that a higher probability to bind the mAb to the agarose is by the Fc fragments that do not compromise the binding properties of the immobilized mAb.

Detailed information on the antigenic determinant and amino acid sequence recognized by the mAb CB.Hep-1 was obtained from a synthetic peptide assay (criterion of specificity and identity). Several overlapping peptides of the rec-HBsAg were fixed to a cellulose membrane facing the mAb CB.Hep-1 (Table 1). Results indicate that the mAb CB.Hep-1 recognizes the following amino acid sequence Cys-Lys-Thr-Cys-Thr-Thr (Table 6), which corroborated results obtained by Fernández de Cossio *et al.*[65] This region has been reported to be quite immunogenic, corresponding with one of the most important antigenic determinants of the HBsAg.[66] Thus, the use of this mAb is a guarantee that the protein used as active pharmaceutical ingredient has this important epitope for vaccine use.

In the present study, the affinity constant (K_{aff}), based upon the law of mass action, and using serial dilutions of both antigen and antibody, was determined using the ELISA protocol described by Betty *et al.*[27] The amount of the mAb CB.Hep-1 adherent to rec-HBsAg on the plate was reflected by the enzyme product measured by optical density (OD). The use of serial dilutions of the mAb CB.Hep-1 resulted in a sigmoid curve of OD versus the logarithm of the total mAb added to the well. This method was valuable for measuring the true association constant and found to be simple, reproducible, and accurate. The K_{aff} values for the mAb CB.Hep-1 were $7.61 \pm 6.9 \times 10^8 M^{-1}$ (Table 6).

The variability in the K_{aff} values could be a consequence of using an aggregated antigen with a repeated epitope for this mAb. The determination of this parameter was critical because one of the reasons for the loss of column capacity is the strong binding of the antibody-antigen complex. A strong interaction usually requires hard antigen elution conditions, denaturing the involved proteins. Several authors report that mAb with lower affinity ($<10^8 M^{-1}$) would require moderate elution conditions such as pH changes.[4] Thus,



the mAb CB.Hep-1 won't be a proper candidate for the IAC. However, the application of a chaotropic agent for the antigen dissociation would be profitable for virus inactivation. Other researchers speculate that KSCN would contribute to the rec-HBsAg conformation.

Several toxic effects such as alteration of the tissue uptake of immunocomplex, complement activation, potentiation of natural killer activity in human lymphocytes, and toxic shock syndrome are associated with the presence of the staphylococcal Protein A.[67,68] In mAb CB.Hep-1 preparations, the Protein A leakage was determined by means of a specific ELISA which permitted quantification (with high sensitivity and without non-specific reactions) of the content of Protein A in the mAb CB.Hep-1 samples. Fab fragments conjugated with horseradish peroxidase were used to reduce the interference provoked by Fc fragments of the antibodies. The content of this contaminant was less than 0.4 ± 00.3 ppm for all CB.Hep-1 samples[29] (Table 6). It represents a very low amount of Protein A and, as a consequence, it is not a biological safety problem.

Other authors reported values of Protein A leakage up to 64 ppm using ELISA in ng range of sensitivity and as criterion of validation mAbs purified by ion-exchange chromatography.[69, 70] In addition, the Protein A content in the CB.Hep-1 preparations was drastically reduced during the immobilization of the mAb CB.Hep-1 and also during the rec-HBsAg downstream process, where this contaminant was undetectable (data not shown).

Due to the malignant transformation properties of the mammalian cellular DNA (fragments >500 nucleotides), the quantification of mouse DNA content in the mAb preparation is another imperative point to consider. In such sense, regulatory agencies are very strict. Nowadays, the acceptable level ranges between 100 pg–10 ng of residual cellular DNA per human dose.[71]

One of the advantages of the production of mAbs by the ascites fluid method is the relatively low DNA content in the downstream starting material. In addition, the high concentration of salts used in the pre-purification treatment helps to dissociate the complexes formed between DNA and proteins, improving the DNA clearance of the purification process. The dot-blot analysis performed to estimate the mouse DNA content in the mAb CB.Hep-1 preparations corroborated that the mouse DNA present in the purified mAb CB.Hep-1 did not exceed 7 pg mg^{-1} of IgG (Table 6 and Figure 4E).

Summarizing the Virological Safety Demonstration of the mAb CB.Hep-1 and Hepatitis B Vaccine

Saving all these key points, the problem resides now in the potential viral contamination of the hepatitis B vaccine. In such sense, there were some negative experiences in the biopharmaceutical industry in the last few years[72, 73] which is why several controls have been recommended by different regulatory agencies.[74, 75] The discovery of SV-40 in polio vaccines derived from primary Rhesus kidney cells made viral contamination a very real issue. Avian leukosis viruses have also been identified as endogenous agents that were carried out into inactivated influenza vaccine-derived chicken eggs.[76] More recently, several incidents of viral contaminations have been found such as mouse minute virus, bovine enterovirus/reovirus, and human rhinovirus.[77]

Murine hybridoma cell lines used for the production of mAbs are known to produce RVLP such as infectious murine leukemia viruses and/or intracisternal particles.[78,79] Perhaps this is one explanation for the tumorigenicity capacity of parental cells. Regarding the retrovirus contamination, there are two main types of retrovirus (type-A and intracisternal) that have been identified in mammalian cells. This type of particle lacks enveloped proteins which affects the outbreak of the particles.[78] For this reason, these particles are not associated to the pathogenicity of these viruses. Nevertheless, transposable genomic sequences that could activate oncogenes have been demonstrated in host cells. Type C retroviruses have the capacity to pass through the cytoplasmatic membrane and thus, infect other cells.

General testing for retrovirus uses biochemical tests such as viral reverse transcriptase enzymatic activity. However there are problems associated with the use of this test for determining retrovirus in cells. Cellular DNA polymerase can use the same RNA template as the reverse transcriptase assay which may give false positive results. Other assays used for determining retrovirus are the TEM and co-cultivation with detector cell lines assays such as S^+L^- focus assay and XC plaque assay. These assays would also allow the definition of the retrovirus as xenotropic (x-RVLP) or ecotropic (e-RVLP).

In 1982, Weiss reported a NS-1 cell containing a high level of type A-RVLP and also type C-RVLP.[80] In addition, some of these type C-RVLP infected human cells in cell culture. Further studies demonstrated that more than 20% of hybridoma cell lines expressed this type of retrovirus.[81] The TEM of the hybridoma CB.Hep-1 showed the presence of type C-RVLP.[33] Due to the fact that this type of particle was also observed in the myeloma cell lines, it is logical to suppose the origin of these RVLP in the hybridoma CB.Hep-1, although considering other possible origins cannot be overlooked. A strategy for eliminating RVLP would be the use of myelomas that do not contain type C-RVLP. However, as the retrovirus sequence is codified in all chromosomes, it is difficult to suppose that a myeloma without RVLP sequences could be isolated.[82]

A TEM positive result should be accompanied by S^+L^- and XC assays. The cells themselves may be co-cultivated with sensitive detector cell lines. The S^+L^- assay is performed for detecting the presence of xenotropic type C-RVLP.[33] Due to the *in vitro* infection with the murine-leukemia virus (MuLV) which does not induce morphological changes in infected cells,

the cell line ($MiCl_1$, ATCC CCL-64.1) that contains a defective genome of the murine sarcoma virus (MSV) was used. A super-infection of these cells with RVLP reconstitutes the MSV replication and as a consequence, the formation of focus. The XC plaque assay is a specific assay for detecting type C-RVLP with the capacity to infect only murine cells (ecotropics).[34] In this assay, the cell line SC-1 was used, which does not have Fv-1 restriction and is able to detect ecotropic MuLV. This cell line is highly sensitive to MuLV infection but it is not cytopathic in this cell line. Therefore, the SC-1 cells were co-cultivated with the XC virus-susceptible cells.

In the case of the hybridoma CB.Hep-1, the mink S^+L^- focus forming virus and the XC plaque assays demonstrated that these type C-RVLP did not show infection capacity.[83] But other works have also found xenotropic and ecotropic type C-RVLP in only 1% and 10% of the hybridoma cultures respectively. This apparent contradiction could be explained by the sensitivity of the assays, because other authors have also reported that only one in one million RVLP has the capacity to show FFU or plaque forming units (PFU).[84, 85] What has caused these viruses to lose their infection capacity is yet to be discovered. Even so, retroviruses are considered to be within the second group of viruses, according to the World Health Organization (WHO). This means that the production of mAbs for human applications can only be performed if the measurement of the removal and inactivation capacity of the purification process demonstrates that it is higher than the contamination level.[75]

Due to the fact that *in vitro* procedures are number and sensibility limited tests, *in vivo* tests constitute reliable complementary analysis for determining virus contamination. MAP testing is the most recommended assay for determining murine viruses. It is an indirect test because it detects antibodies directed against murine viruses. Thus, the main advantages of this test are its sensitivity and the specificity. In this work, no viruses were found in the monitoring of master cell bank, animals (by serology test), ascites fluid and purified mAb. In theory, problems associated with the test could be related to the infection of animals with viruses that affect host physiological functions such as antibody depression, lysis of T- and B-lymphocytes, and also some antigens of virus that show cross-reactivity with other viruses.[86]

In consequence, other *in vivo* tests using model animals like adult mice, guinea pigs, and chicken embryonated eggs were also employed to detect mouse and adventitious virus. In summary, no particular signs were noticed between the moment of the inoculation of the samples and the euthanasia of the animals. Besides, three groups of suckling mice were also inoculated with cell lysates to corroborate the negative results of the previous experiments in adult mice and guinea pigs. The assay was performed with cell lysates because problems have been observed with live cell inoculation that can grow and cause death in the test animals.

Several animals of these groups were born and injected while other animals were injected one day later. In this part of the test, researchers remarked that a high diversity in the growth was observed. Both extremes in large and small animals were found. Although these size differences were striking, no signs of illness or weakness were observed. Therefore, no obduction was performed to observe the animals for a longer period of time (six weeks). None of the suckling mice died within this period of observation.

Virus isolation from the hybridoma supernatant was also performed in chicken eggs, because it is another reliable and sensitive assay for detecting a large range of viruses. In general, several changes can be induced by viruses in eggs, depending on the nature of the infecting virus, route of inoculation, and age of the embryos. In this assay, embryos were inoculated by two different routes and kept for a period of three to ten days, depending on the route of inoculation.

The eggs were examined for embryo deaths, lesions into the chorioallantoic membrane and HA of the allantoic fluid. As results, no viral pocks were seen in the chorioallantoic membranes, and the number of dead embryos was similar in both the sample eggs and the control eggs. No HA was found in the embryonated eggs inoculated with the hybridoma supernatant and, as expected, the positive control eggs infected with the influenza A virus displayed HA in the allantoidal fluid. Similar results were obtained in one blind passage in a new set of eggs (only inoculation of yolk sac was performed in this case).

Summary of the Viral Validation Studies

Medicinal products should be sterile, which means to be free of adventitious viruses. However this goal is difficult to achieve because there are no simple physical or chemical methods that can assure that the product is free of viruses. Therefore, regulatory agencies usually do not ask for the absolute sterility of products—validation studies are required instead. Unprocessed bulks (ascites fluid or cell culture supernatant) showing evidence of virus infection may require additional testing to determine whether retrovirus or adventitious agents can be removed or inactivated during the processing for obtaining final products (in this case, hepatitis B vaccine). Similarly, in the absence of any negative findings, it may be useful to lend assurance by using model viruses in the validation studies.[74, 75] In this study, the starting material is spiked with deliberate amounts of viruses and the amounts quantified after each, or in determined steps of the processing. In general, these validation studies provide a high level of assurance that the final product will be free of these kinds of contaminants.

To demonstrate the removal and inactivation capacity of the mAb CB.Hep-1 and hepatitis B vaccine production processes, two steps were performed: PASFF affinity chromatography and incubation of the mAb CB.Hep-1 at acid pH. They were spiked with high-infectivity viruses during the mAb CB.Hep-1 purification process together with the treatment of

the rec-HBsAg using a combination of high temperature and KSCN. This demonstration was done with viruses representing a wide range of physico-chemical properties of murine viruses (Table 8). In this outline, the model viruses employed were enveloped and non-enveloped, varying in size (large: 50–200 nm; intermediate: 30–50 nm; and small: 20–30 nm) of both genome types (RNA and DNA) and with different resistances to physico-chemical agents.[38, 87]

To design the virus removal validation study, a scaling down on the mAb CB.Hep-1 purification process was done. For our experiments, the scale-down was brought to within 1% of the full production scale and the level of product purification mimicked the production process, and products were similar in terms of purity, specific activity, mouse DNA quantity and yield.[38] Results demonstrated that the PASFF affinity chromatography showed a maximum reduction factor of 7.2 Logs in the case of viruses larger than 120 nm size (SeV, HIV-1, HSV-1), while for small viruses, 18–30 nm (HPV-2, CPV), maximum reduction factor observed was 4.8 Logs.[38, 87] These small viruses

TABLE 8. Removal and inactivation capacity of model viruses in the Hepatitis B vaccine production. (ND: non-determined.)

VIRUS PROPERTIES AND STUDIED STEPS	SeV	HIV-1	HSV-1	HPV-2	CPV
Family	*Paramyxoviridae*	*Retroviridae*	*Herpesviridae*	*Picornaviridae*	*Parvoviridae*
Species	Murine	Human	Human	Human	Canine
Genome	RNA	RNA	DNA	RNA	DNA
Envelope	Envelope	Envelope	Envelope	Non-envelope	Non-envelope
Size (nm)	200	100-200	120	25-30	18-24
Resistance to physico-chemical agents	Low	Low	Low	Middle	Very high
Murine viruses represented by the model virus (They were grouped according to envelope and genome types)	Hantaan virus LCMV SV LDH VHR Rat coronavirus Retrovirus SDAV TMEV		MCV Thymic virus	Reo-3 PVM Rat rotavirus Polyoma virus Mouse rotavirus	MMV MAV MEV K Kilham virus Toolan virus
Cell line used for virus titration	Chicken eggs	MT4	Vero	Vero	LFBC
Virus titers used to spike the step (Logs)	8.4	5.7	7.5	10.2	9.7
Removal capacity of PASFF (Logs), validation	7.2	4.3	6.0	4.8	2.8
Virus titers used to spike the step (Logs)	ND	5.8	8.4	6.5	7.5
Removal capacity of PASFF (Logs), revalidation	ND	4.5	5.0	3.5	2.8
Virus titers used to spike the step (Logs)	ND	6.1	5.2	7.8	7.4
Inactivation capacity of ethanol 70% (Logs)	ND	4.6/15 min	3.0/15 min	1.8/12 h	3.9/12 h
Virus titers used to spike the step (Logs)	ND	5.0	5.26	7.7	6.48
Inactivation capacity of ethanol 20% (Logs)	ND	3.7/15 min	3.7/15 min	2.3/72 h	1.3/72 h
Virus titers used to spike the step (Logs)	ND	ND	ND	6.5	7.5
Inactivation capacity of sodium hydroxide 0.1 N (Logs)	ND	ND	ND	6.1/7 h	6.6/1 h
Virus titers used to spike the step (Logs)	8.0	5.8	9.0	11	9.8
Inactivation capacity of citric acid 100 mM; pH 3 (Logs)	7.4/1 h	4.8/10 min	8.3/1 h	0.1/7 h	5.2/1 h
Virus titers used to spike the step (Logs)	7.5	5.5	8.0	10	8.5
Inactivation capacity of heat treatment of rec-HBsAg (Logs)	7.1/10 min	5.8/1 h	6.9/10 min	8.2/10 min	6.4/10 min
Viral clarification capacity (Logs), validation*	21.7	14.5	20.8	13.1	14.4
Viral clarification capacity (Logs), revalidation*	ND	15.1	19.9	11.8	14.4

LEGEND:
*The viral clarification capacity was calculated as the sum of the removal capacity, viral inactivation capacity at acid pH and viral inactivation capacity of the heat treatment of the rec-HBsAg. Sanitization steps were not included.

can penetrate into the matrix, retarding their elution from the column.

It is important to remark that no viral inactivation was observed during the mAb CB.Hep-1 elution step. This is important because a robust purification process should include at least two robust orthogonal viral clearance steps, based on different mechanisms, to remove and inactivate viruses. To date, none of the viral clearance methods individually employed can be expected to remove all viruses present in a given process. Thus, the inactivation capacity of acid pH can be analyzed independently of the viral removal experiment results. Another important consideration is that removal and inactivation methods are limited by the sensitivity of the detection system utilized.

The frequency of revalidation studies is also another aspect of considerable discussion and attention because it has both regulatory and economic implications, and there are no standards regarding the preparation of viral spike material which may affect the material sensitivity of the physico-chemical conditions used. The consensus is that revalidation studies must be repeated after any critical changes of the manufacturing process. In this work, a revalidation of the viral removal capacity of the PASFF affinity chromatography was performed after the scale-up (factor=6) of the mAb CB.Hep-1 purification process. This was done to investigate whether this chromatography showed the same viral removal capacity of the validation study using the same model viruses. Experiments were also assessed using a scaled-down version equivalent to 1% of the new mAb CB.Hep-1 manufacturing purification scale which showed no significant differences in mAb yield, purity and specific activity. The recovery and purity showed significant differences with the validation study but the parameters were higher than the pre-determined specification limits.[41] Results demonstrated no significant differences in the removal capacity of these model viruses, corroborating the fact that the scale-up of the mAb CB.Hep-1 purification process does not modify the capacity of PASFF to remove the HIV-1, HSV-1, HPV-2 and CPV. As expected, PASFF affinity chromatography showed a higher removal capacity for enveloped and large viruses (Table 8).

Regarding the viral inactivation studies, the acid pH treatment of the viruses showed a maximum inactivation capacity of 8.3 Logs/1 h for enveloped viruses. Conversely, no inactivation capacity (0.1 Log/7 h) was observed for HPV-2. This result is in good correlation with the extensive number of publications for virus inactivation at low pH.[39, 40]

One remarkable change was observed in the inactivation of the CPV under this chemical condition. The "survival" capacity of this non-enveloped virus was drastically affected (5.2 Logs/1 h), as shown in Table 8. This result was somewhat surprising because usually non-enveloped viruses resist this inactivation condition. The combination of high temperature with a chaotropic agent showed a high inactivation capacity for all studied model viruses. We hypothesized that this inactivation capacity was mainly obtained due to a high concentration of the chaotropic agent. The agent causes molecular structures to be disrupted, particularly in those formed by non-covalent forces such as hydrogen bonding, Van der Waals interactions, and the hydrophobic effect. But we did not discard the effect of the temperature on the stability of the proteins used by the viruses to infect host cells. The total measured clearance factor of the hepatitis B vaccine was 21.7, 14.5, 20.8, 13.1 and 14.4 Logs for SeV, HIV-1, HSV-1 HPV-2, and CPV, respectively.[38, 87] Thus, it corroborated that the hepatitis B production process is very robust, in terms of virological safety.

Subsequent work was done to investigate virus inactivation capacities of the column sanitization protocol using ethanol at 70%, and matrix storage conditions in ethanol at 20%. In addition, a column sanitization protocol using HCl 0.1 N was also studied with non-enveloped viruses (HPV-2 and CPV) to increase the inactivation capacity for high-resistance viruses. To summarize, the results of the PASFF sanitization and storage studies confirmed that the HSV-1, HIV-1 and CPV were amenable to the incubation with ethanol at 70% (from 3.0 to 4.6 Logs/15 min), and the ethanol at 20% inactivated up to 3.9 Logs/12 h of enveloped viruses (HSV-1 and HIV-1) but it was not effective for inactivating non-enveloped viruses (HPV-1 and CPV, 1.3–2.3 Logs/72 h).[41] The isoelectric pH of the purified HPV-2 and CPV ranged from 5.5–7.0.[88] This attribute will be drastically changed under the presence of the HCl 0.1 N, in theory, allowing a high virus inactivation capacity. Results verified this suggestion. Both viruses were highly inactivated in 6.1 Logs/7 h and 6.6 Logs in just 1 h respectively. Therefore, a robust sanitization protocol of the PASFF matrix during the mAb CB.Hep-1 purification should involve the combination of ethanol at 70% and HCl 0.1 N in the studied exposure time.[41]

Currently we are involved in the revalidation studies of the PASFF affinity chromatography applying the ascites, loaded directly with model viruses, without the pre-treatment of the ascites fluid (saline precipitations and desalting by size exclusion chromatography). We expect a reduction in the removal capacity of viruses of this chromatography. Nevertheless, the high inactivation capacity of the processes and the other non-studied steps (immobilization, HPLC-GF, ion exchange chromatography) became the method for isolating the active pharmaceutical ingredient of the very robust hepatitis B vaccine, in terms of virological safety.

Conclusion

The manufacture, biochemical characterization and virological safety summarized here have been widely demonstrated. The mAb CB.Hep-1 can be obtained with high quality and virological safety for the immuno-purification of the rec-HBsAg used in the hepatitis B vaccine production. These results can be generalized as methodology for any mAb being purified for pharmacological use in humans.

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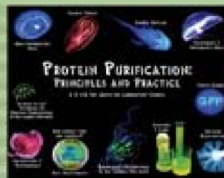

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