ARTICULOS ORIGINALES COMPLETOS

A MOUSE HYBRIDOMA CELL LINE SECRETING IgG AND IgM ANTIBODIES WITH SPECIFICITY FOR THE HEPATITIS B VIRUS SURFACE ANTIGEN

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SUMMARY

A mouse hybridoma cloned cell line secreting monoclonal antibodies (MAbs) which specifically recognizes the Hepatitis B Virus surface antigen (HBsAg) was originated by standard cell fusion procedures. The antibodies are directed against a sequential epitope located into the so-called "a" region, which is common to all HBsAg subtypes.

Ascitic fluid from this clone was produced, and two different molecules with the expected molecular weights of IgG and IgM were fractionated from a Protein A-purified sample using gel filtration HPLC. Both fractions retained anti-HBsAg activity in ELISA. The original hybridoma cell line was recloned by limiting dilution, and then by micromanipulation. The resulting clones continued to secret the two different specific immunoglobulin classes. Further demonstration of the presence of mouse heavy γ and μ chains in a single cloned hybridoma cell line was made at the RNA level by polymerase chain reaction selective amplification of either heavy chain variable regions, using specific primers. This co-expression of heavy chains could be explained either by a differential splicing of an unique active gene or by simultaneous activation of more than one allele.

RESUMEN

Un hibridoma de ratón que secreta anticuerpos monoclonales (AcMs) específicos para el antígeno de superficie del Virus de la Hepatitis tipo B (HBsAg), fue originado empleando procedimientos comunes de fusión celular. Estos anticuerpos reconocen un epitope secuencial situado en la región "a", común a todos los subtipos del HBsAg.

Se obtuvo fluido ascítico de este hibridoma y los anticuerpos fueron purificados mediante cromatografía de afinidad en Proteína A Sefarosa. Estos anticuerpos se fraccionaron mediante cromatografía de tamizaje molecular en HPLC para obtener dos moléculas, cuyos tiempos de retención en la columna y movilidad electroforética corresponden a los de la IgG e IgM. Ambas moléculas retuvieron la actividad anti HBsAg en ELISA. La línea de hibridoma original fue clonada y reclonada por dilución limitante y micromanipulación y los clones resultantes continuaron secretando anticuerpos de dos clases diferentes. Como demostración adicional a nivel de ARN, se logró amplificar ambas regiones variables por medio de la reacción en cadena de la polimerasa (RCP), utilizando cebadores específicos.

Este fenómeno de coexpresión de diferentes cadenas pesadas puede ser explicado a través de un mecanismo de "splicing" diferencial de un único gen activo o por la activación simultánea de más de un alelo.

INTRODUCTION

Infection by Hepatitis B Virus is one of the most important epidemiological problems, with more than 300 millions of carriers throughout the World (Lau *et al.*, 1989). For this reason, the development of serological methods for the screening of virus infected blood represents a first health priority in many countries.

Several authors have reported the obtention of murine monoclonal antibodies (MAbs; Kohler and Milstein, 1975) directed to the HBsAg and their use in

Table 1
Structures of Synthetic Oligonucleotides Employed for the Specific Amplification of Mouse Gamma and Mu Heavy Chain Variable Regions.

(A) γ CHAINS.

5' END:
MH-FR1 (amino acids 1 to 8):
5'...(CG)AGGTG(CA)AGCTC(CG)(AT)(AG)
(CG)A(AG)(CT)C(CG)GGG...3'

3' END:
MH-γ-Const. (amino acids 121 to 130):
5'...A(TC)CTCCACACACAGG(AG)(AG)CC
AGTGGATAGAC...3'

(B) μ CHAINS.

5' END:
MH-FR1 (amino acids 1 to 8):
5'...(CG)AGGTG(CA)AGCTC(CG)(AT)(AG)
(CG)A(AG)(CT)C(CG)GGG...3'

3' END:
MH-μ-Const.(amino acids 114 to 20):
5'...GAGCAGAGGACGCTCTCGAAAGAC...3'

(C) 3' END J2 PRIMER FOR NESTED PCR.
MH-J2 (amino acids 106 to 113):
5'...TGAGGAGACTGTGAGAGTGGTGCC...3'.

Note: Bases in parentheses are substitutions at a given position; i.e. (AT) indicates that A and T were present in equimolar amounts during the synthesis. Amino acid positions were classified after Kabat *et al.,* (1987).

immunoassays (Wands *et al.,* 1981, 1981a and 1982, Shafritz *et al.,* 1982, Liu *et al.,* 1985, Neurath *et al.,* 1986, Vieira *et al.,* 1988). The availability of these immunoreagents allowed the instrumentation of sensitive, specific and rapid diagnostic kits which are already in clinical use. They also allowed a more accurate classification and study of the different viral serotypes and their epidemiological distribution in different geographic areas (Wands et al., 1982a, Gaspar *et al.,* 1984).

In the present paper we describe the generation by standard cell fusion procedures of one cloned hybridoma cell line that secretes both IgG and IgM MAbs directed against the HBsAg.

MATERIALS AND METHODS

Hepatitis B Surface Antigen

Natural HBsAg was purified from plasma of infected individuals at the Immunology Department of the Faculty of Medical Sciences "Victoria de Girón" of Havana. This antigen was kindly provided by Dr. Antonio González Griego with more than 80% purity. This protein will be referred hereafter as nHBsAg.

Recombinant HBsAg, cloned and expressed in yeast, was provided as crude extracts by the Vaccine Division of the Center for Genetic Engineering and Biotechnology of Havana. This protein will be referred hereafter as rHBsAg.

Generation of Hybridoma

BALB/c mice were immunized subcutaneously with a first dose of 50 μg of nHBsAg, in Freund's Complete adjuvant, followed 15 and 21 days later by similar doses in Freund's incomplete adjuvant. Three days before the fusion, the animal with the higher anti-nHBsAg antibody titer (see below) received an intraperitoneal injection of 50 μg of antigen in phosphate buffered saline (PBS) and the spleen cells were fused with the myeloma cell line Sp2/0-Ag14. The fusion and culture procedures were similar to those described elsewhere (Duarte et al., 1987); briefly, spleen cells and myelomas were hybridized in the presence of 45% polyethylene glycol 1450 (Sigma), at a 10:1 ratio, and hybridoma selected in HAT medium (Sigma).

Cell Cloning Procedures

Hybridoma cells were cloned by limiting dilution, at 0.5 cells/wells, and at one cell per well by micromanipulation with elongated Pasteur pipettes, under microscopic surveillance.

Screening for Specific Antibodies

MicroELISA polyvinyl chloride plates (Dynatech) were coated overnight at 4°C with 2 μg/ml of nHBsAg in 0.1 M NaHCO₃ buffer (ph 9.6). The plates were washed three times with PBS-0.05% Tween 20, and 100 μl of diluted immune mouse serum or hybridoma culture supernatant were added to each well. After 2 hours at 37°C, the plates were washed again and incubated for one hour at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibodies. The reaction was developed with 100 μl/well of substrate solution (5 μl of H_2O_2, 5 mg of orthophenylenediamine, and 10 ml of 0.05 M citrate buffer, Ph 5.5), and arrested with 50 μl/well of 2.5 M H_2SO_4. Absorbance was read at 492 nm in a Titertek Multiskan MC-340. Non-related antigens and non-immune mouse serum were used for the determination of specificity. Samples with absorbance values three times higher than the negative control were considered as positive.

Production of Ascitic Fluid, Purification of MAbs and Coupling to Sepharose CL4B

Ascitic fluid was produced from each hybridoma as reported elsewhere (Duarte *et al.,* 1987). MAbs were purified using affinity chromatography on Protein A Sepharose as described by the manufacturers (Anonymous, 1987). Protein concentration in the samples was determined according to Lowry *et al.* (1951).

Purified MAbs were coupled to CnBr activated Sepharose CL4B (Pharmacia) at 5 mg of MAbs per ml of gel, according to the manufacturers recommendations (Anonymous, 1987).

Isotyping of MAbs

Isotyping was made by radial double immunodiffusion (Ouchterlony and Nilsson, 1973) using commercial antisera specific for different mouse immunoglobulin subclasses (ICN Immunobiologicals), and 20-fold concentrated culture supernatants.

Determination of the HBsAg Subtype Specificity of MAbs

Polyvinyl chloride plates (Dynatech) were coated with $10\,\mu g/ml$ of MAb for 15 min at 50°C. The plates were incubated with different concentrations of HBsAg "ad" and HBsAg "ay" standards (Paul Ehrlich Institute, Germany) for 30 min at 50°C. After two washes, $100\,\mu l$ of a proper dilution of peroxidase conjugated goat polyclonal anti-HBsAg antibodies was added and incubated for 30 min at 50°C. The reaction was developed as described before above.

Polyacrylamide Gel Electrophoresis

Electrophoresis were performed as suggested by Laemli (1970), using 12.5% gels for MAbs and 15% gels for the HBsAg samples.

Western blot

Different samples containing rHBsAg were run in a 15% acrylamide gel under reducing conditions and transferred to a $0.45\,\mu m$ nitrocellulose membrane during 1 hour at room temperature in a semi-dry transfer system. The membrane was blocked with Tris Buffer Saline (TBS) + 5% powdered milk for 1 hour at 37°C. Then it was incubated for an hour at room temperature with a $5\,\mu g/ml$ solution of MAb. The membrane was washed three times with TBS + 0.01% NP40 and the reaction developed with a Protein A-colloidal gold conjugate.

HPLC-Gel Filtration Chromatography

Protein A purified samples were loaded into a G 5000 PW column equilibrated in 50 Mm Na_2HPO_4, 0.1 M NaCl, Ph 7.0, at 0.15 ml/min. Two ml fractions were collected. Fractions on the same peak were pooled and assayed for anti rHBsAg specific activity in ELISA and for immunoglobulin subclass by double immunodiffusion.

Affinity constant determination

The affinity constant for the IgG and IgM components was measure by solid phase ELISA following the method of Beatty *et al.* (1988).

Oligonucleotide Primer Design

The rationale for the design of synthetic oligonucleotides for the specific polymerase chain reaction (PCR) amplification of the mouse chain variable region, using 5' end framework one and 3' end near γ constant domains as priming sites, has been described elsewhere (MH-FR1 and MH-γ-Const., respectively, in Table 1; Gavilondo *et al.*, 1990, Coloma *et al.*, 1991). For the amplification of the mouse μ chain variable region, we used the 5' end framework one primer described, together with a 3' end μ constant region specific oligonucleotide designed on the basis of the database of Kabat *et al.*, 1987 and GenBank (MH-FR1 and MH-μ-Const., respectively, in Table 1). Finally, a 3'end specific mouse heavy chain variable region J2 primer was employed to verify the amplifications by "nested" PCR (MH-J2 in Table 1).

Preparation of RNA

RNA was extracted either with the NP-40/SDS technique suggested by Gough (1988); 10^5 hybridoma cells were used as starting material.

First Strand Synthesis

First strand cDNA synthesis was performed using the Boehringer-Mannheim (Chicago, IL) cDNA kit; briefly, total RNA samples (approximately $0.5\,\mu g$; derived from 10^5 hybridoma cells) were heated at 65°C for 1-5 minutes, and incubated with a mixture of RNase inhibitor, deoxynucleotides, oligo $(dT)_{15}$ as primer, and AMV reverse transcriptase, for 60 minutes at 42°C.

Polymerase Chain Reaction

Eighty μl of PCR mix was added to the $10\,\mu l$ of first strand cDNA. The PCR mix was made following the instructions of the Perkin-Elmer Cetus (Norwalk, CT) PCR kit. Five μl of each primer was added to give a final primer concentration of $1\,\mu M$ and the mixture was subjected to PCR amplification using the Hybaid (UK) thermal cycler sets, for 25 cycles. The temperatures and times used for PCR were: melting at 94°C, minute; primer annealing at 55°C, 1 minute; primer extension at 72°C, 1 minute. Normally one minute ramp times were used between these temperatures. Ethidium bromide stained 2% agarose (NuSieve) gels were used to visualize PCR fragments.

Chromosome Number Determination

Air-dried chromosome preparations were made by the standard method of Rothfels and Siminovith (1958). Briefly, exponentially growing cells were incubated with $0.2\,\mu g/ml$ Colcemid for 4 hours. Cells treated with a hypotonic solution (0.075 M KCl) for 20 min at room temperature, and were then fixed by three changes of acetic acid-methanol (1:3) before spreading in slides and air drying. Chromosomes were stained with 10% Giemsa solution. Mean chromosome number of each clone was estimated from the analysis of at least 20 metaphases.

RESULTS

Fusion and Cell Cloning

Seventy-six percent of wells had growing cultures after fusion and 12% of these were found to secrete anti-nHBsAg antibodies, and several cultures were cloned by limiting dilution at 0.5 cells per well. Focus was centered in clone number 48/1, as it gave repeatedly high ELISA values, with no cross reaction with unrelated antigens, and remained stable after several passages. After a second cloning by limiting dilution, 100% of the resulting clones were shown to secrete antibodies that recognized nHBsAg. Culture supernatant from clone 48/1/5 was studied for antibody isotype by radial immunodiffusion. Immunoprecipitation bands in the agarose gel corresponded to the IgG2b subclass of IgG, and the IgM class. Careful microscopic micromanipulation was performed to isolate single cells from this clone into individual wells, the cultures grown, and clones
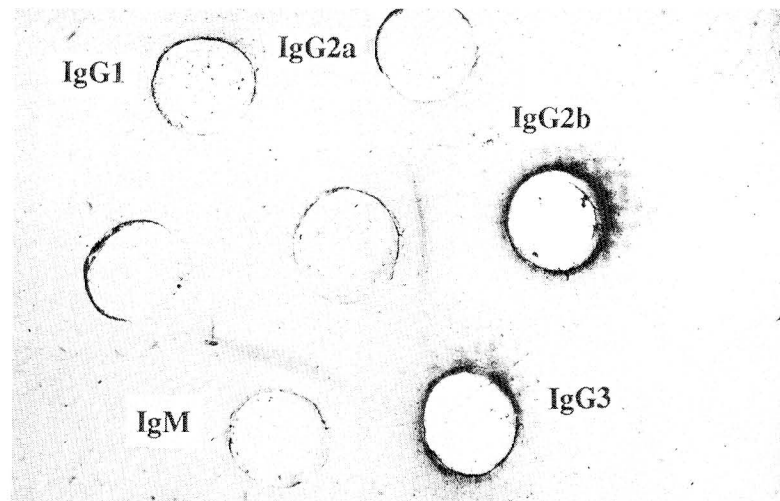
Fig. 1 Double radial immunodiffusion. Twenty-fold concentrated culture supernatnat from hybridomas was placed in the center and subclass-typing antisera at peripheral positions.

studied by immunodiffusion for immunoglobulin class and subclass. All isolated clones secreted the two antibodies (Figure 1).

Clone 48/1/5/4 (originated after two limiting dilutions and one isolation by micromanipulation, and denominated CB-Hep.1) was inoculated to BALB/c mice, and ascitic fluid was recovered with an anti-rHBsAg antibody titer of 1:540000 in ELISA (OD values 3 times higher than the negative controls). The antibodies were purified from ascites through Protein A Sepharose affinity chromatography, and the purified antibody samples run under denaturing conditions in SDS-PAGE. Three bands were observed, with molecular weights of 70, 55 and 24 kDa (Figure 2). When run under non-denatured conditions all three bands disappear to give place to the high molecular weight band characteristic of whole antibody molecules.

Purified antibody was applied to a gel filtration HPLC column and two peaks were resolved. Retention time of each peak corresponded to those expected for 150 and 900 kDa. SDS-PAGE (Figure 2) and radial immunodiffusion of these fractions indicated a correspondence with mouse IgG2b and IgM. The specificity of both fractions was confirmed to be anti-rHBsAg by ELISA, using anti-mouse IgM and anti-total mouse IgG conjugated antibodies.

Of the 2-2.5 mg of specific anti HBsAg MAb recovered from each ml of ascites, the relative proportion of the IgM has been found to vary between 5 and 60%. Prolonged culture in vitro and in vivo of hybridoma 48/1/5/4 causes a gradual loss of the ability

to secrete the IgM component (data not shown), and increases the growth rate in relation to the original culture.

The values obtained for the affinity constant of both antibodies were: $IgG = 4.6 \times 10^{10}$ M^{-1} and $IgM = 1.9 \times 10^{10}$ M^{-1}.

Immunochemical Characterization of CB-Hep.1

Western blot experiments with rHBsAg show that only two bands are recognized by CB-Hep.1: a major band of 24 kDa corresponding to the HBsAg monomer, and a band with the expected molecular weight of a dimer (Figure 3). This latter band is
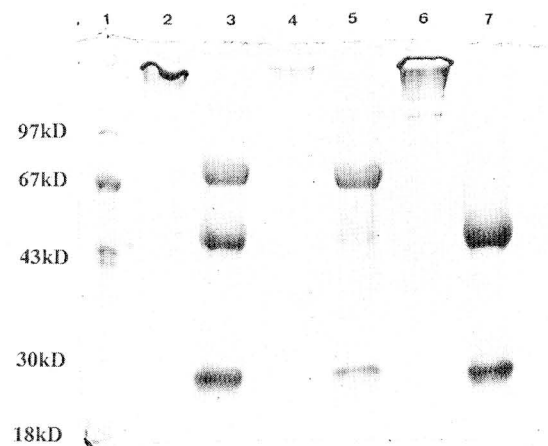

Fig.2 SDS-PAGE analysis of the purification of CB-Hep.1. Lane 1: Molecular weight standards: Lanes 2 and 3: Protein A chromatography fraction containing IgG and IgM heavy chain bands; Lanes 4 and 5: IgM fraction from gel filtration by the G 5000 PW column; Lane 6 and 7: IgG fraction from gel filtration by the G5000 PW column. Lanes 1, 3, 5 and 7: Reduced samples; lanes 2, 4 and 6: nonreduced samples.
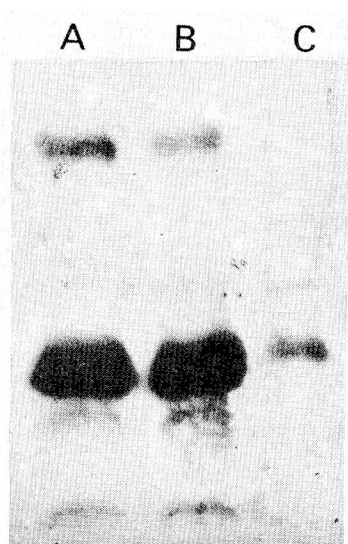
Fig.3 Western Blot assay with CB-Hep.1. Lanes A and B represent the rHBsAg eluted from a monoclonal antibody column prepared with CB- Hep.1. Lane C shows a crude extract from a recombinant yeast producing HBsAg. Samples on lanes A and C were heated for 5 min in Laemli buffer; the sample on lane B was heated for 20 min in the same buffer.
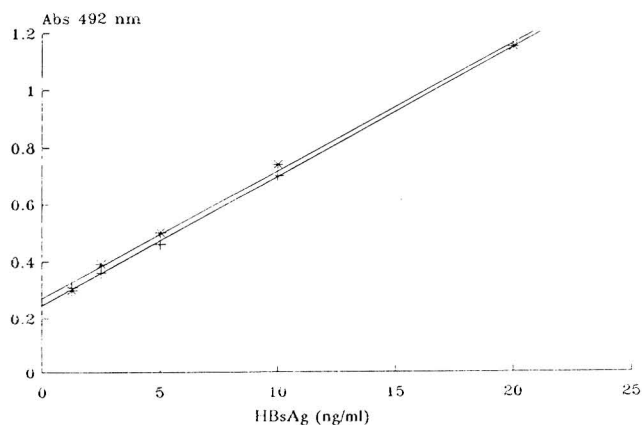

Fig. 4. ELISA with CB-Hep.1 as coating antibody, using HBsAg "ad" and "ay" subtype standards.

markedly reduced when the sample is incubated for an extended time in Laemli buffer. No unspecific bands corresponding to yeast proteins were detected.

In ELISA, it was shown that CB-Hep.1 detects the "ad" and "ay" HBsAg subtype standards with equivalent affinity (Figure 4).

PCR amplification of V regions.

Figure 5 shows the results of the PCR amplification of cDNA made from total RNA extracted from the CB-Hep.1 hybridoma, using the combinations of 5'end

framework one and 3' end γ or μ constant region primers detailed in Table 1. Both primer combinations produced fragments of expected size (400 bp). These bands were successfully reamplified using the combination of 5'end framework one and 3'end J2 region primers. As an additional control of primer specificity, the CDNA from a unrelated hybridoma that secretes IgG2a MAbs (IOR-T3) was amplified with the combination of framework one and γ region primers, but not with framework one and μ constant region primers (data not shown).

Chromosome Number Analysis

The myeloma fusion partner cell line (Sp2/0-Ag14) employed in this fusion, had a modal chromosomal number of 79, while the hybridoma 48/1/5/4 exhibited a modal number of 125. In the latter case, most chromosome preparations were difficult to count accurately, due to the extreme packing of the metaphases.

DISCUSSION

The results obtained suggest that hybridoma 48/1/5/4 secrets two antibody molecules of different class but with the same antigen specificity. One molecule was been identified as a pentameric IgM and the second one as monomeric IgG2b. We have not been able to detect bispecific antibody molecules composed by a mixture of both heavy chains. This is coherent with previous reports indicating that heavy chains from different immunoglobulin classes are not compatible enough to associate between them (Takahashi *et al.*, 1988).

The IgG2b and IgM MAbs secreted by clone 48/1/5/4 react specifically with the natural and recombinant HBsAg. The antigenic determinants recognized by these antibodies are preserved under denaturing conditions and, though, do not depend on antigen configuration. The antibody mixture present in the ascites showed strong avidity for the antigen, and high ELISA titters were obtained with very small amounts of MAbs.

These MAbs define a site on the protein which is located in the so called "a" domain, as demonstrated by the ability to react with standard preparations of both the HBsAg "ad" and HBsAg "ay" subtypes. These features make these antibodies promising candidates for immunoassays, due to their potential ability to detect all subtypes of HBV through its common domain (Vieira *et al.*, 1988, Corouce *et al.*, 1976).

Taking as true that we are in a case in which a single cell secretes two antibody classes, specific for the same antigen, we can advance three possible explanations for its origin.
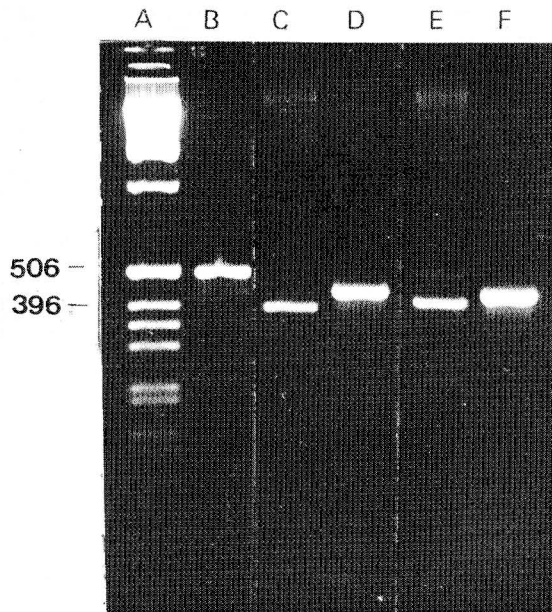
Fig. 5. PCR amplification of heavy chain variable regions of CB-Hep.1. Lane A: molecular weight markers (1kb DNA ladder, Life Technology Inc.), lane B: PCR positive control (500 bp), Oligonucleotides used: lane D: MH-FR1 and MH-γ-Const.; lane F: MH-FR1 and MH-μ-const, lanes C and E: reamplified material with MH-FR1 and MH-J2 primers.

The first one is that CB-Hep.1 was derived from a triple fusion event between one myeloma cell and two specific lymphocytes, a possibility that has been described previously (Arnold *et al.,* 1988). If the double fusion hypothesis is true in our case, it is logical to think that the modal chromosome number of CB-Hep.1 should be higher than the average for other hybridoma. To verify this point we determined the chromosome modal number of this cell line, and that of the myeloma Sp2/0- Ag14. We found that the modal chromosome number of the hybridoma is not higher than the expected from a single fusion event between a myeloma cell and a normal mouse lymphocyte.

Strelkaukas *et al.,* (1987) have also published the generation of a human hybridoma simultaneously secreting γ and μ heavy chain antibodies, and like the clone reported in this paper, both molecules display similar specificity. However, their cell line was essentially diploid and its origin could not be explained by a double lymphocyte fusion. If this is also our case, two alternative explanations could be advanced:

(2a) CB-Hep.1 cells posses different chromosomes with productively rearranged heavy chain genes, inherited from a single parental lymphocyte, a phenomenum that has been reported elsewhere (Lafrenz *et al., 1986*)

(2b) CB-Hep.1 cells bear only one productively rearranged heavy chain chromosome, and mRNA is alternative spliced producing two different heavy chain subclasses with an unique variable region. The latter has been also shown before for splenic B cells (Yaoita *et al.,* 1982, Pelmutter *et al.,* 1984) and B cell lines (Chen *et al.,* 1986 , Woloschack *et al.,* 1986, and Hellman *et al.,* 1988).

At this moment, it remains to be shown if the two MAbs secreted by CB-Hep.1 share the same light chain, and if the heavy chain variable regions genes have similar or different base sequences. As the DNA bands amplified by PCR with primer sets specific for either variableγ or μ regions (framework one primer is highly degenerate and common to all mouse antibodies; specificity is given by the 3' end primer) could be reamplified with a common 3'end J2 primer, it could be suggested that the IgG2b and IgM antibodies share the same framework four variable region sequence, but it is known that antibodies with different specificity share the same "J" region (Kabat *et al.,* 1987).

The fact that the constant affinity of both antibodies are very similar is also in favor of the mRNA splicing hypotheses.

In conclusion, until the complete sequence of both variable regions are elucidated, none of the alternative explanations for the origin and mechanism of secretion of CB-Hep.1 can be excluded.

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