ORIGINAL ARTICLES

PRODUCTION OF MONOCLONAL IgM AND IgG1 ANTIBODIES SPECIFIC FOR HEPATITIS B VIRUS SURFACE ANTIGEN

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SUMMARY :

Purpose: *Monoclonal antibody (mAb) allows isolation and identification of the antigen (Ag) with a unique and chosen specificity. Thus, monoclonal antibodies are powerful immunochemical tools. The aim of this study is to produce a hybridoma clone secreting mAb specific for hepatitis B virus (HBV) surface antigen (HBsAg).* ***Methods:*** *BALB/c mouse was immunized with HBsAg. Fusion between splenocytes of the immunized mouse and FO myeloma cells was performed by polyethyleneglycol procedure. Anti-HBs secreting hybridoma cells were screened with enzyme linked immunoassay. Hybridoma cells were cloned by a single cell pick-up technique.* ***Results:*** *Two hybridoma clones, named CAy-M and CAy-G, were isolated. CAy-M and CAy-G hybridoma clones were found to secrete anti-HBs IgM and anti-HBs IgG1 monoclonal antibodies respectively.* ***Conclusion:*** *These two hybridoma clones have been subcultured for ten months and are still secreting high levels of monoclonal antibodies.*

Key Words : *Hepatitis B Surface Antigens, Monoclonal Antibodies, Hybridomas.*

INTRODUCTION

In 1975, Köhler and Milstein described the hybridoma technology for generating monoclonal antibodies (1). Some of the advantages of this technique are: (a) Each hybrid produces only one antibody which recognizes a specific epitope on the antigen. (b) As the hybrid is immortal, it provides an unlimited supply of antibody. (c) Purifying of the specific antigen in large quantities could be performed with various methods by using mAb. (d) Diagnostic kits for antigen screening could be made by using mAb.

Microbial infections are frequently diagnosed by demonstrating specific antigen(s) or antibody(ies) in serum. Hepatitis B is a worldwide public health problem with a significant morbidity and mortality rate (2). Human hepatitis B virus is a member of the Hepadnaviridae family, which is characterized by a circular, partially double-stranded DNA genome approximately 3,000 bp long, an enveloped capsid, and the ability to infect liver cells (3). The viral envelope proteins (large, medium, and small hepatitis B surface antigens HBsAg) are considered to be crucial molecules in recognizing a possible receptor on plasma membranes of the human hepatocytes (4). As the HBsAg is an immunodominant antigen and is the predominant serum marker of HBV, the importance of detecting this antigen is well known. Detection of HBsAg is usually performed with anti-HBs mAb-based diagnostic kits. This study was

planned to develop a hybridoma clone producing monoclonal antibody specific for HBsAg. Two hybridoma clones (anti-HBs IgM producing CAy-M and anti-HBs IgG1 producing CAy-G lines) were produced. We believe that this is the first time in our country that two hybridoma cell lines producing mAb specific for HBsAg have ever been reported.

MATERIALS AND METHODS

Hepatitis B virus surface antigen (HBsAg) was partially purified as described before (5). Briefly, a serum sample from a HBV carrier, subtype ayw, was centrifuged through a layer of 20% sucrose-TNE (130 mM NaCl, 10 mM Tris-hydrochloride, 1mM disodium EDTA, pH7.4) and the antigen pellet, after resuspending in TNE, was passed through a column (60x2.5cm) of Sepharose-4B (Pharmacia, Uppsala, Sweden) equilibrated with TNE. HBsAg-positive fractions were pooled and then dialyzed (Spectra/Por3, m.w. cutoff approx 3.500, Spectrum Medical Indust. USA) against dH_2O. HBsAg concentration was determined with the use of a standard curve and then lyophilized. A 6 week old A 6-week-old female inbred BALB/c mouse (purchased from Harlan Inc, UK) was immunized by intraperitoneal injection of 30 µg of partially purified HBsAg in complete Freund's adjuvant (Sigma Chem. Co., MO, USA) and followed by second (in incomplete Freund's adjuvant) and third (in 0.9% NaCl) doses at 14 days intervals. After 3 days splenocytes were isolated and used for fusion. Polyethylene glycol (PEG, Wt 3000-3700, Sigma Chem. Co., MO, USA) fusion between $17x10^7$ splenocytes of the immunized mouse and $26x10^6$ FO myeloma cells (ATCC, CRL 1646) and hypoxanthine-aminopterine-thymidine (HAT, Sigma) selection were performed as described elsewhere (6) with some modifications. Briefly, hybridoma suspension was plated out in peritoneal macrophage seeded ($1x10^5$ cells per well) three 24-well plates (Greiner GMBH, Nürtingen, Germany) and then incubated for 24h at 37°C in a humidified atmosphere of 5% CO2. After incubation, HAT medium was added upon hybridoma cells. Exchange of supernatants with a fresh HAT medium was carried out three times at 4 days intervals. Because of the rapidly growing characteristics of the hybrids, replacing HAT with HT was not required. 19 days later after the fusion, supernatants were checked for the presence of specific antibodies. Five out of 71 wells were found to be positive. Cloning was performed by picking up a single cell with a thin-pointed pasteur pipette (hand-made) and transferred to wells of 96-well flat bottomed microtitre plates (Greiner GMBH, Nürtingen, Germany) under inspection with an inverted microscope in a biological safety cabinet. Single cells were cultured in a thymocyte-conditioned medium consisting of RPMI-1640 (Sigma) supplemented with 10% fetal calf serum (Seromed, Biochrom KG, Berlin, Germany) and 10% thymocyte supernatant.

Immunoassay. $2x10^5$ Hybridoma cells per ml were cultured in a 24-well plate for 24h. The production of IgM or IgG was assessed in the supernatants with an anti-HBs ELISA kit (Monolisa anti-HBs 3.0, Sanofi Diagnostics Pasteur, France. HBsAg phenotype ad and ay coated microtitre plate). Although this is a diagnostic ELISA kit for screening human anti-HBs in serum, it is also convenient to screen mouse anti-HBs because of its working principles (Fig. 1). In order to discriminate IgG and IgM isotypes, enzyme-labeled anti-mouse IgG (Fc specific, Sigma) conjugate was used instead of the original conjugate of the kit. Optical density produced by the reaction was read on an ELISA reader (LP400, Diagnostics Pasteur, France). The actual isotype of the monoclonal antibody produced by the hybridoma cells was determined using a commercially available isotyping kit (ISO-1,

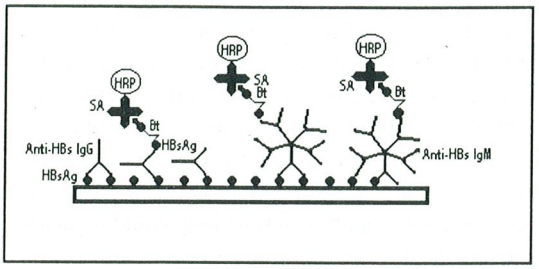

Fig. 1 : Working principles of anti-HBs ELISA kit. Because the tracer is Biotin-HBsAg/Streptavidin-Peroxidase, it is also convenient for searching mouse anti-HBs mAb regardless of isotypes (e.g. IgM and/or IgG). SA; Streptavidin, Bt; Biotin.

ImmunoType, mouse monoclonal antibody isotyping kit, Sigma).

Purification of anti-HBs IgG1 from ascites fluid by immunoaffinity chromatography. 0.5 ml of pristane (Sigma) was injected intraperitoneally to each BALB/c mouse. Seven days later it was followed by an injection of $10x10^6$ CAy-G hybridoma cells intraperitoneally. When ascites fluid (8-10 ml) was accumulated, it was withdrawn from each mouse and then pooled. In order to eliminate remnants of pristane, ascites fluid was centrifuged at 1500xg for 10 min and then the supernatant was passed through glass wool. Glass wool-passed ascites fluid was filtered (0.2 µm, Costar, Bodenheim, Germany), adjusted to pH 8.5 and then applied on staphylococcal protein A (SPA, Protein A-Sepharose CL-4B, Sigma) column for affinity purification. SPA column was attached to liquid chromatography system (HPLC, Waters 600E System controller, Millipore Corp. Milford, MA, USA) and equilibrated with PBS-Saline (pH 8.5) at a flow rate of 0.5 ml/min. 5 ml of ascites fluid was loaded on SPA column. SPA-bounded anti-HBs IgG1 was eluted with 0.1M citric acid (pH 4.5). Peaks were detected at 280 nm with the Waters 490E programmable multiwavelength detector. Chromatographic profile was evaluated with a computer program (Maksima 820 Chromatography workstation version 3.30). Fractions were collected and assessed for antibody content with Access Immunoassay system (Sanofi Diagnostics Pasteur, France).

RESULTS

Two anti-HBs high-positive wells were selected and the hybridoma cells were cloned. Isotypes of the monoclonal antibodies produced by the hybridoma clones were determined with a commercially available isotyping kit. It was found that the clones secreted different isotypes of mAb. One of them was positive for IgM and the other was positive for IgG1 (Fig. 2). Anti-HBs specific IgM- and IgG1-secreting hybridoma clones were named CAy-M and CAy-G respectively. Supernatants of 24h-cultured CAy-M and CAy-G hybridoma cell lines ($2x10^5$ cells/ml) demonstrated high levels of anti-HBs activities when assessed with ELISA (Table 1).

When the anti-HBs IgG1 containing ascites fluid was loaded on SPA-column, some of the anti-

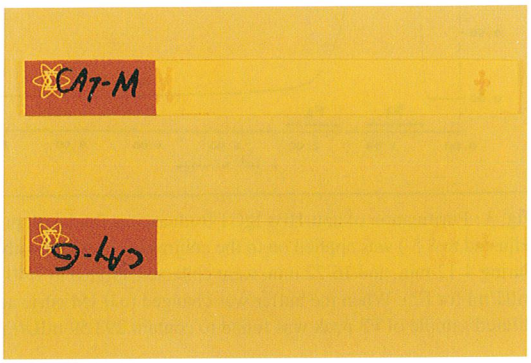

Fig. 2 : Self-descriptive strips of the monoclonal antibody isotyping kit. Culture supernatants of CAy-M and CAy-G assessed for their isotypes and found to be IgM (reaction resulted as the letter M on the left side) and IgG1 (reaction resulted as the letter G and the number 1 on the right side) isotypes respectively.

Table - 1 : Representative anti-HBs ELISA results of the culture supernatans of CAy-M and CAy-G hybridomas[a].

Conjugates	Samples					
	CAy-M	CAy-M/10[b]	CAy-G	CAy-G/10	(+) Control[c]	(-) Control
Biotin-HBsAg / Streptavidine-HRF[d]	2.900[e]	2.900	0.786	0.274	0.575	0.050
Anti-mouse IgG-HRP	0.096	ND[f]	2.305	1.879	ND	0.061

a. Hybridoma cells were cultured at a density of 2x105 cells per ml for 24 h in RPMI-1640 supplemented with 10% FCS.
b. Culture supernatants were diluted to 1:10 with 0.09 % NaCl before assessing.
c. Anti-HBs immunoglobulins of human origin containing 60mIU/ml.
d. HRP; Horse radish peroxidase
e. Optical density (OD) at 450 nm with reference to 630nm
f. Not done

HBs mAb did not bind to SPA. Samples (F1 and F2) were collected during the elution of the unbound fraction (Fig. 3A). It was assumed that some of the anti-HBs mAb had escaped (3.88 mIU/ml for F1 and 4.27 mIU/ml for F2) along with other non-specific proteins. However most of the anti-HBs mAb in the sample was obtained in a single peak (293.59 mIU/ml) when the buffer was changed to 0.1M citric acid pH 4.5 (Fig. 3B).

Hepatitis B virus surface antigen bears the B-cell epitopes which are important for the induction of protective antibody responses in humans. It has been clearly demonstrated that the region between residues 120 and 150 of HBsAg represents the determinant common to all HBV isolates and it is likely to be exposed on the surface of the HBV particle (7-9). Antibodies to these a determinants protect adults against the majority of infections

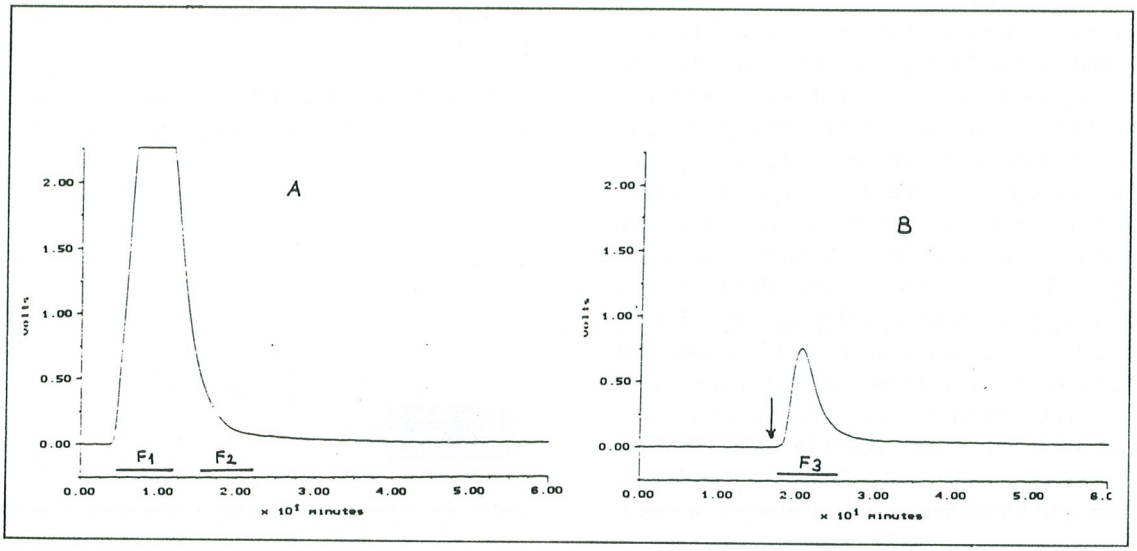

Fig. 3 : Purification of anti-HBs IgG1 from ascites fluid with protein A-Sepharose CL-4B affinity chromatography. 1 ml of sample (pH adjusted to 8.5) was applied on to the column and washed with PBS-saline (pH 8.5) at a constant flow rate (0.5 ml/min) (A). Fractions during 4-12 min. and 16-22 min. were collected, diluted to 1/40 and then assessed for anti-HBs IgG levels (3.88 mIU/ml for F1 and 4.27 mIU/ml for F2). When the buffer was changed to 0.1M citric acid (demonstrated by an arrow), SPA-bound IgG was released (B). 1/40 diluted sample of F3 peak was found to contain 293.59 mIU/ml of anti-HBs.

DISCUSSION

There are many different types of antibodies in serum and each one is specific for a different type of antigen. The use of this mixed antibody population causes a variety of different problems in immunochemical techniques. Thus, the preparation of homogenous antibodies with a defined specificity was a long-standing goal of immunochemical research. This goal was achieved with the hybridoma producing technology. Hybridization can be made between B lymphocyte and myeloma cells and it gives rise to a hybrid that possesses both the property of immortal growth in culture and the ability to produce an antibody of a pre-defined and single specificity. Thus, the importance of monoclonal antibodies originates from three characteristics; their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities.

irrespective of the subtype of the wild-type virus. In order to diagnose HBV infection or past infection, as for most of the other viral diseases, specific antigens or antibodies are screened in serum samples. This is usually performed with the mAb-based various techniques. But specific mAb should be produced first.

In this study we planned to produce a monoclonal antibody specific for HBsAg for multipurpose usage such as developing a diagnostic kit, isolating pure HBsAg from various samples and using for experimental researches. HBsAg was partially purified from serum as described before (5). Spleen cells of the HBsAg-immunized mouse was fused with FO myeloma cells. Anti-HBs mAb producing hybridoma cells were screened. Two out of five positive wells were cloned and it was found to be IgM and IgG isotype producers by using different conjugates (Table 1,

compare with Fig.1). The actual isotypes of the monoclonal antibodies were demonstrated to be IgM and IgG1 with an isotyping kit (Fig.2). CAy-M and CAy-G hybridoma lines were subcultered for nearly 10 months and during this time, re-cloning was needed only once for CAy-G to select the high producer again.

SPA binds to human IgG1, IgG2 and IgG4 but not IgG3. Mouse IgG1 binds to SPA to a lesser extent when compared to other mouse IgG subclasses (IgG2a, IgG2b and IgG3). Although leakage has occurred to some extent, anti-HBs IgG1 was isolated by SPA-affinity chromatography (Fig.3).

As a result, HBsAg-specific monoclonal antibodies could be purified and used as a valuable tool for various purposes such as purification of a specific antigen or development of diagnostic assays for detection of HBsAg or anti-HBs.

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