Production and Characterization of Monoclonal Antibodies Against Hepatitis B Viruses and Application of a Quick Sandwich ELISA

FATIMA YÜCEL, ALİ İHSAN MANAV, and AYNUR BAŞALP

ABSTRACT

Hepatitis B virus (HBV) infection is a major health problem worldwide. The diagnosis of acute and chronic hepatitis B infection is based on the detection of hepatitis B surface antigen (HBsAg). We report here the development of hybrid cell producing monoclonal antibodies (MAbs) specific for HBsAg using hybridoma technology. BALB/c mice were immunized with a mixture of HBsAg subtype “ad” and subtype “ay.” Spleen and lymph nodes were used as a source of high-titer antibody producing lymphocytes and removed and fused with myeloma cells of F0 origin separately. In the five fusion experiment, enzyme-linked immunosorbent assay (ELISA) tests showed that among 1594 hybridomas only 5 hybrids (9D12, 2B7, 4G5, 2G3, and 6E7) reacted with HBsAg. These MAbs were characterized for use in the development of diagnostic kits based on sandwich ELISA test system. The MAbs were conjugated with horseradish peroxidase (HRP) and used in the quick sandwich ELISA system. This system is a quite practical and time-saving test system when compared with common and commercial sandwich ELISA for diagnosis of hepatitis B surface antigen in human serum.

INTRODUCTION

Hepatitis B virus (HBV) is the major cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma occurring globally among humans. Acute and chronic viral hepatitis affects millions of individuals throughout the world and represents a major class of infectious disease in humans.\(^1,2\) It has been estimated by the World Health Organization (WHO) that 400 million people have been infected with HBV and that approximately 45% of the world population lives in regions of high HBV endemicity. In endemic areas like southeast Asia and equatorial Africa, vertical transmission perinatally by an HBV-infected mother to the newborn child is more common. In developed countries, the spread of HBV is usually horizontal, by blood products or through mucosal contact. More than 2 million people die annually from the sequel of HBV-associated liver disease: 100,000 die from fulminant, 500,000 from acute, and 400,000 through complications associated with chronic HBV. About 700,000 patients die annually from HBV-induced liver cirrhosis and approximately 600,000 patients die from hepatocellular carcinoma (HCC).\(^3\)

Transmission of HBV is done via percutaneous introduction. Those living in close contact with HBV-infected individuals, healthcare workers, and users of intravenous drugs who share their needles, form the high risk groups for infection. Uninfected individuals living with an HBV-carrier face an elevated risk of acquiring HBV infection. Newborns mostly develop a persistent infection and then develop chronic hepatitis later on in life. Infection during adulthood mostly results in an acute illness, followed by clinical recovery and viral clearance, although 5–10% of infected adults develop a persistent infection. Although many chronic carriers appear healthy, they can still transmit HBV to those with whom they have close contact, thereby starting a new cycle of the disease.

Presence of Hepatitis B surface Antigen (HBsAg) is a characteristic of acute or chronic HBV infections. One of the most sensitive and specific methods for diagnosing HBV infections is enzyme-linked immunosorbent assay (ELISA) and early detection of HBsAg may be helpful for simplifying the treatment of HBV-associated diseases.\(^4\) In the present study, anti-HBsAg monoclonal antibodies (MAbs) were generated by using lymphocytes obtained from spleen and lymph nodes. The
MABS were conjugated with horseradish peroxidase (HRP) and used in the quick sandwich ELISA system.

**MATERIALS AND METHODS**

**Reagents**

Hepatitis B surface antigen (HBsAg) ay and HBsAg ad types were purchased from Chemicon (AG-850, AG-852, Temecula, CA). HRP conjugated-commercial MAbs were used in Organon’s ELISA-based HBV diagnostic kit. Dulbecco’s Modified Eagles Medium (DMEM), gentamicin (antibiotics), hypoxanthine, aminopterine, thymidine (HAT) and hypoxanthine, thymidine (HT) were purchased from Gibco laboratories (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Biochrom (Seromed, Berlin, Germany). Peroxidase was obtained from Boehringer Mannheim (Mannheim, Germany).

**Immunization**

Eight-week-old female BALB/c mice were immunized with a mixture of two HBsAg proteins (subtypes ad and ay). A solution containing these antigens (containing 1 µg of each antigen) was prepared in phosphate-buffered saline (PBS: 10 mM K₂HPO₄, 10 mM KH₂PO₄, 0.15 M NaCl, pH 7.2) and mixed with equal volumes of Freund’s complete adjuvant (Sigma Steinheim, Germany). These complexes (0.2 mL) were given by intraperitoneal injections. Second and third injections were performed in 3 weekly intervals by using Freund’s incomplete adjuvant (Sigma). Four days before a fusion, a booster injection of 0.5 µg of each HBsAg antigen in 0.1 mL PBS without adjuvant was performed.

**Indirect ELISA**

The indirect ELISA was used for the screening of hybridoma supernatant and serum of the mice.(5,6) Enzyme immunoassay (Nunc, Roskilde, Denmark) plates were coated with 100 µL HBsAg per well at a concentration of 100 ng/mL in PBS buffer (pH 7.2) at 4°C overnight.

The unspecific bindings were blocked with nonfat milk powder for 1 h at 37°C and washed 3 times with 0.05% (v/v) PBS between-20 (PBST). The supernatant of hybridoma was incubated for 1 h at 37°C, with mouse serum as a positive control. Plates were then washed three times with PBST. The bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse polyvalent (IgG, IgM, IgA) antibodies diluted 1/1000 in PBS buffer for 1 h at 37°C.

Finally, Para-nitrophenyl phosphate (1 mg/mL solution in substrate buffer) was added and the reaction was measured by the absorbance at 405 nm on a Bio-Tech enzyme immunoassay (EIA) reader.

**Cell fusion and antibody production**

MAbs were produced by modification of the method of Köhler and Milstein.(7) We used lymphocytes from spleen cells and the lymph node for the antibody source. Initially, lymph nodes were collected from a different part of a mouse. Cells were resuspended in 10 mL of the PBS and washed three times in the same buffer. In the same fusion, the spleen was removed from the same mouse. Spleen cells also were suspended like lymphocytes from the lymph node. Then both lymphocytes from the lymph-node and spleen cells were fused with the F0 (ATTC CRL 1646) myeloma cell line in the presence of 50% polyethylene glycol (PEG) 4000 separately. (8) PEG was added slowly over a 1-min period while gently stirring and kept without any stirring for another 1 min. The fusion suspension was then diluted adding 4 mL of DMEM (Gibco, Paisley, Scotland) medium over a period of 2 min, followed by 40 mL at a rate of 10 mL per min. Fusion product, resuspended in DMEM containing 20% fetal calf serum (FCS; Biochrom, Seromed) and antibiotic were plated on 96-well plates and incubated overnight at 37°C, 5% CO₂, 95% humidity. At Day 15 the wells were screened for choice of the desired antibody by indirect ELISA. Positive wells were cloned by limiting dilution method using macrophages as feeder cells.(9)

<table>
<thead>
<tr>
<th>Table 1. Results of Fusion</th>
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<tr>
<td><strong>Fusion 1</strong></td>
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<tr>
<td>Spleen</td>
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<tr>
<td><strong>Fusion 2</strong></td>
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<tr>
<td>Spleen</td>
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<td>Lymph Node</td>
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<tr>
<td><strong>Fusion 3</strong></td>
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<tr>
<td>Spleen</td>
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<td>Lymph Node</td>
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<tr>
<th>Table 2. Immunoglobulin Class of Monoclonal Antibodies</th>
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<tr>
<td><strong>Monoclonal antibodies</strong></td>
</tr>
<tr>
<td>9D12</td>
</tr>
<tr>
<td>2B7</td>
</tr>
<tr>
<td>4G5</td>
</tr>
<tr>
<td>2G3</td>
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<td>6E7</td>
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Determination of antibody class and subclass

The class and subclass of MAbs were determined using a mouse hybridoma subtyping kit purchased from Boehringer Mannheim.

Purification of MAbs

MAbs were purified from the hybridoma supernatant by (NH₄)₂SO₄ precipitation between 30 and 50% saturation. The precipitate dissolved and dialyzed against PBS and the antibodies were purified by immunoaffinity chromatography using solidphase bound protein A (Staphylococcus aureus) as the IgG-immunotrap (Pharmacia, Uppsala, Sweden).

Preparation of MAbs-HRP conjugate

The enzyme antibody conjugate was prepared as described by Nakane and Kawaoi. Two milligrams HRP in 0.5 mL redistilled water, was added to 0.1 mL freshly prepared NaIO₄ solution 0.2 mol/L. This mixture was stirred for 20 min at room temperature. POD-aldehyde solution was dialyzed against sodium acetate buffer overnight at 4°C. Then purified MAb (4 mg) was added to this mixture and stirred for 2 h at room temperature. NaBH₄ solution (4 mg/mL) was added and allowed to stand for 2 h at 4°C with occasional shaking. The reaction mixture was dialyzed against PBS buffer and stored at 4°C. For the stabilization of conjugate, one granule of thymol was added.

FIG. 1. Comparison of specific activities (A₄05) of the five MAbs (2G3, 6E7, 9D12, 2B7, 4G5) of HBsAg with normal human serum and negative control, ad, ay, ad/ay; subtypes of HBsAg.
Sandwich ELISA

The antibody-sandwich ELISA was used for detecting HBV in human serum. The principle of the assay, MAb against hepatitis B viruses was bound to the ELISA microplate. The other MAb against HBV was conjugated with HRP. The two antibodies forming a “sandwich” complex with the antigen (HBV) were detected when present in the serum, by means of a two-step analytical system. If HBV is present in serum, it will bind to both the captured antibody and conjugated antibody. If the serum does not contain HBV antigen, it will not bind to any antibodies. After the final washing to remove sample and unbound conjugate, the enzymatic activity was detected by incubation with the mixed specific 3,3′,5,5′-tetramethyl benzidine (TMB) and hydrogen peroxide. Wells that contained HBV and hence bound conjugate developed a purple color that was converted to orange when the enzymatic reaction is terminated with HCl. In this assay, briefly, 96-well polystyrene plates were coated with 100-ng MAb and incubated at 4°C overnight. The plates were washed three times with washing buffer (0.005% Tween 20 in PBS). Then 0.2% nonfat milk powder in PBS was added to the wells and the plates were incubated for 1 h at 37°C followed by washing as above. Serum was added to the wells and incubated for 1 h at 37°C. After washing, MAb-HRP conjugate was added and incubated for 1 h at 37°C. After repeating the washing step 5 times with washing buffer, 100 μL of the substrate buffer was added. Peroxidase substrate solution was prepared as follows: 30 mg TMB was dissolved in 5 mL dimethylbenzidine (DMSO) and then diluted to 1/100 (v/v) with 0.1 M sodium acetate. The stock solution was adjusted to pH 5.0 with citric acid and then was mixed with 3% hydrogen peroxide at a ratio of 1000:0.5 (v/v) before use. After 15–30 min, the reaction was stopped with 1 M HCL, the optical density (OD) of each well was determined at 450 nm by a microplate reader (Biorad ELIA Reader, Tokyo, Japan).

Application of Quick Sandwich ELISA for Detecting HBsAg

To apply a quick sandwich ELISA for detecting HBsAg, the ELISA procedure for screening coated MAb Ag and the blocking step were the same as described above. After this step, MAb-HRP conjugate was diluted 1:1000 in PBS, and human serum was added to the wells simultaneously and incubated for 1 h at 37°C. After repeating washing 5 times with washing buffer, 100 μL of the substrate buffer was added. After 15–30 min the reaction was stopped as described above.

RESULTS AND DISCUSSION

Production and Characterization of Anti-HBsAg MAbs

As shown in Table 1, five cell fusions were done during this work. 1594 hybridoma colonies of 3642 wells were obtained. An initial screening for antibody production using an indirect ELISA method which used the HBsAg antigen (ad/ay) as a coating antigen, identified monospecific antibody producing cells from 5 of the 36 colonies.

These MAbs were found to be of the IgG class, three of which were IgG2b, and two IgG2a type by using a hybridoma subisotyping kit. All of these antibodies had kappa light chains (Table 2).

FIG. 2. Comparison of the activities (A_{405}) of the two home made HRP conjugated MAbs (2G3-HRP and 2B7-HRP) with HRP-conjugated commercial MAbs by using different sandwich ELISA. (A) Common sandwich ELISA. (B) Quick sandwich ELISA.
significant difference between two sandwich ELISA systems and the HRP conjugated-MAbs worked as efficiently as HRP-conjugated commercial MABs.

When the sandwich ELISA tests and our quick sandwich ELISA test were compared, it was shown that our quick sandwich ELISA test system gave reliable results as well as common sandwich ELISA test system.

This system seems quite practical and a time-saving test system for diagnosis of hepatitis B surface antigen in human serum.

REFERENCES


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