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Monoclonal Antibodies Against 24 kDa Surface Antigen of Hepatitis B Viruses

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Hepatitis B virus (HBV) infection is one of the major public health problems worldwide. Effective control of HBV transmission in areas of high and intermediate endemicity would not be possible without vaccination of the vulnerable group. The diagnosis of acute and chronic hepatitis B infection is based on the detection of hepatitis B surface antigen (HBsAg). In this study, we prepared nine hybridomas that produced monoclonal antibodies specific for HBsAg. BALB/c mice were immunized with 24 kDa HBsAg and the immune spleen cells were fused with SP2/0 myeloma cell line. We obtained seven IgG1, one IgM, and one IgG2b positive clones. The stable hybrids were sub-cloned and ascitic fluid was prepared in the BALB/c mice. After antibody purification by protein G, the affinity column was prepared and the pure 24 kDa HBsAg from cell extract was eluted from the column. Western blot analysis showed that all monoclonal antibodies are specific for 24 kDa antigen. Since 24 kDa HBsAg is used for vaccination against hepatitis, these monoclonal antibodies are the best candidate for the isolation and purification of recombinant HBsAg from yeast expression vector by affinity column to be used for vaccination.

Introduction

Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV). The virus is transmitted through contact with the blood or other body fluids of an infected person. According to World Health Organization (WHO), about 2 billion people worldwide have been infected with the virus and about 350 million live with chronic infection. An estimated 600,000 persons die each year due to the acute or chronic consequences of hepatitis B.[1] Although HBV is 50 to 100 times more infectious than HIV, about 90% of healthy adults who are infected with HBV will recover and be completely rid of the virus within 6 months. Hepatitis B is endemic in China and other parts of Asia. In the Middle East and Indian subcontinent, an estimated 2–5% of the general population is chronically infected.[1]

In Iran over 35% of the population have been exposed to the HBV and about 3% were chronic carriers before 1993.[2] After a national neonatal vaccination program, vaccination of high risk groups and introduction of disposable syringes, the overall prevalence of chronic HBV infection in the general population was decreased to about 1.7%.[3,4] Vaccination against HBV has shown to confer long-term immunity against HBV infection.[5] Recombinant hepatitis B surface antigen (HBsAg) has shown to induce a protective antibody response in 90–99% of immunized individuals.[6] Fifty-one to 56% of Iranian cirrhotic patients are HBsAg positive[2,7,8] and overall prevalence in blood donors in 2007 was 0.41%.[9] At present, vaccination is the most effective and cost-saving means of prevention of HBV infection. In this study, we produced monoclonal antibodies against HBsAg to be used for the isolation and purification of recombinant antigen for vaccination purposes.

Materials and Methods

Immunization and fusion

Seven-week-old female BALB/c mice were immunized intraperitoneally with 10 μg of HBsAg (obtained from production unit of the Pasteur Institute of Iran, Tehran) prepared in phosphate buffer saline (PBS, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 0.15 M/NaCl, pH 7.2) and mixed with equal volumes of Freund's complete adjuvant (Sigma, Seelze, Germany). Second and third injections (all with 10 μg antigen in 0.2 mL) were performed at 3 weekly intervals using Freund's incomplete adjuvant (Sigma). Three days before fusion, an intravenous injection of 10 μg of HBsAg in 0.1 mL PBS without adjuvant was performed. Spleen cells from immunized mice (about 10^8 cells) were fused with mouse SP2/0 myeloma cells (2×10^7) at a ratio of 5:1, for 1 min using 50% polyethylene glycol (PEG 1450, HYBI Max, Sigma). The fusion mixture was suspended in HAT medium (Sigma) and an aliquot of 200 μL (2.5×10^5 cells) were suspended into 96-well plates. After 2

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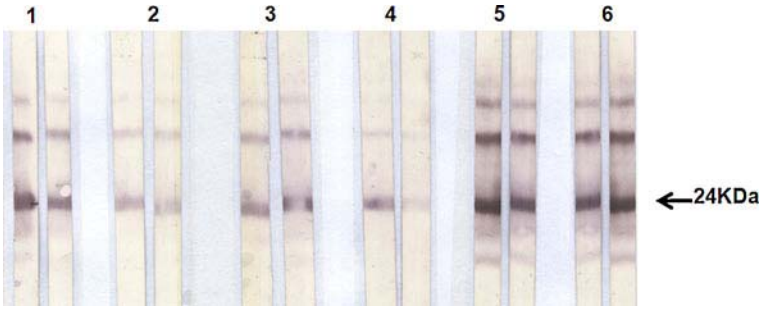

FIG. 1. Western blot analyses of six sera of BALB/c mice immunized with HBV 24 kDa antigen. After 10% SDS-PAGE, protein bands were blotted on the nitrocellulose sheet and analyzed with six immune BALB/c mice sera. All sera with 1:200 dilution (left lane) and 1:1000 dilution (right lane) could recognize the 24 kDa band and its apparent molecular weights of 42 and 66 kDa isotypes.

weeks, supernatants of growing hybridomas were screened for antibody production by ELISA. Positive wells were cloned by limiting dilution method using spleen cells and macrophages as feeder cells.

ELISA screening assay

Wells of 96-well microtiter plates were coated with 100 µL of HBsAg at a concentration of 10 µg/mL in PBS buffer (pH 7.2) for 1 h at room temperature (RT). Wells were washed with washing buffer (PBS, 0.1% BSA and 0.05% Tween-20, pH 7.2) and blocked with 250 µL of 1% BSA (Sigma). Then 100 µL of hybridoma supernatant were added to the wells and incubated for 1 h at RT. After washing, the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse polyvalent (IgG, IgM, IgA) antibodies. Finally, 100 µL per well 3,3,5,5′-tetramethylbenzidine (TMB) substrate was added and the positive wells were detected at 450 nm on a Bio-Tech ELISA reader. The positive clones were immediately sub-cloned by limiting dilution and were expanded and cryopreserved or grown as ascites into the pristane-primed mice.

Gel electrophoresis and Western immunoblotting

SDS-PAGE was performed according to the Laemmli method.[10] Gels were stained with Coomassie blue and later with silver staining. HBsAg separated by SDS-PAGE were transferred to nitrocellulose sheets (Schleicher & Schuell, 0.45 µm pore size) by semi-dry blotting technique.[11] Subsequently, the nitrocellulose sheet was incubated with 1% BSA in PBS buffer for 1 h and then incubated with hybridoma culture supernatant at 4°C overnight. The membrane was washed with PBS containing 0.1% BSA and 0.05% Tween-20, and secondary antibody (HRP-conjugated goat anti-mouse) was added for 1 h. After washing, the immune complex was detected by a color reaction using diaminobenzidine as enzyme substrate containing 0.03% $CoCl_2$ for color enhancement.

Determination of antibody class and subclass

Monoclonal antibody isotypes were determined by ELISA using goat anti-mouse isotype antibodies and HRP-conjugated mouse anti-goat antibody (Sigma isotype determination kit).

Purification of MAb by protein G column

The antibodies were purified from ascitic fluid and from hybridoma supernatant by precipitation with 45% saturated ammonium sulfate $(NH_4)_2SO_4$. The precipitate was dissolved and dialyzed against PBS and then antibody was purified with affinity chromatography using protein G Sepharose

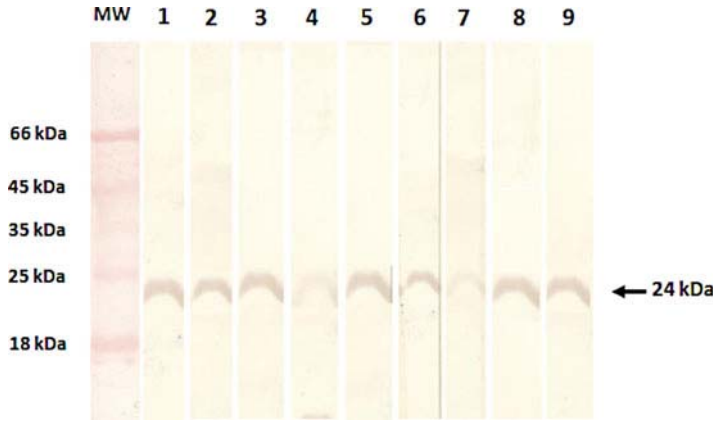

FIG. 2. Western blot analysis of nine MAbs secreted from nine hybridomas against 24 kDa antigen. After 10% SDS-PAGE, the antigen (5 µg) was blotted to the nitrocellulose sheet and processed with nine MAbs. Lanes 1–9, P1C7, P4E2, P7C1, P3D4, P5E9, P5E10, P4B1, P2F6, P3D2 MAbs, respectively.

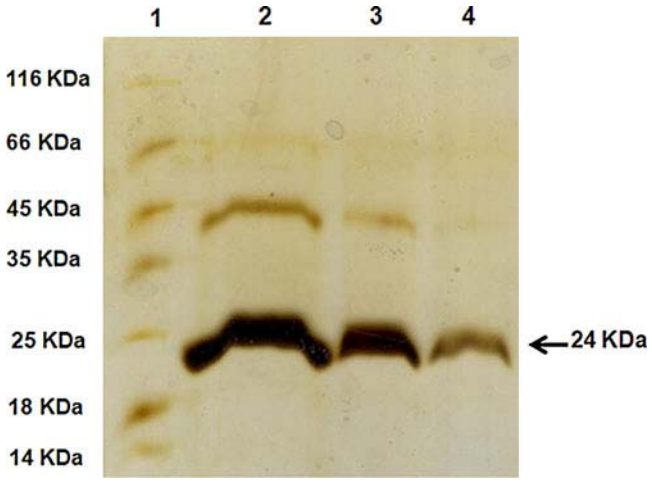

FIG. 3. Western blot analysis of P1C7-C2 MAb against different concentrations of 24 kDa antigen. After 10% SDS-PAGE, different concentrations of antigen were transferred to the nitrocellulose sheet and then incubated with P1C7-C2 antibody and silver stained. Lane 1, MW; lanes 2–3, different concentrations of 24 kDa antigen (1, 0.5, and 0.001 μg, respectively).

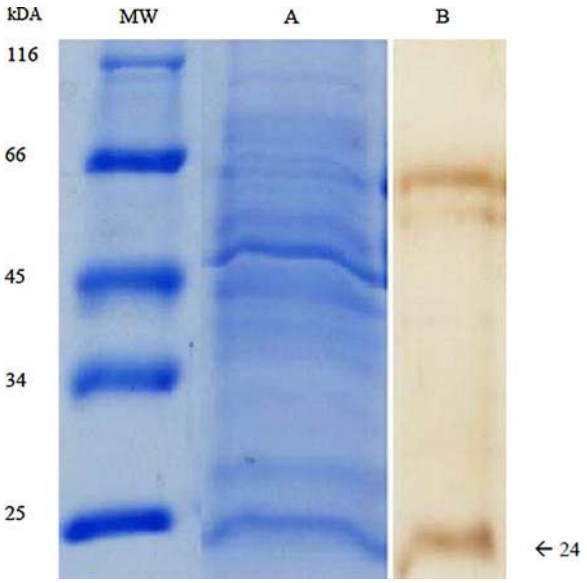

FIG. 4. Purification of recombinant 24 kDa HBV antigen from crude yeast extract by column chromatography. Affinity column was prepared by purified P1C7-C2 (IgG1) monoclonal antibody using CNBr-activated Sepharose 4B. Crude yeast extract containing expressed recombinant 24 kDa protein was applied to the column and the pure 24 kDa antigen was eluted from the column. (**A**) 10% SDS-PGE of crude yeast extract stained with Coomassie blue. (**B**) Purified 24 kDa antigen silver stained.

(Sigma) equilibrated in PBS buffer (pH 7.2). After applying the sample, the column was washed and the antibody was eluted with 0.1 M glycine-HCl buffer (pH 2.5). Eluted antibody was immediately neutralized with 1 M Tris-HCl (pH 9.0), and then concentrated and dialyzed against PBS buffer.

Results and Discussion

In order to get specific monoclonal antibodies (MAbs) against HBsAg in this experiment, we immunized BALB/c mice three times with recombinant 24 kDa HBsAg. Mice sera were analyzed 10 days after the last immunization with ELISA and Western blot for specific antibody titer against 24 kDa antigen. As seen in Figure 1, all six sera had specific antibodies against 24 kDa antigen in Western blot when 1:1000 dilution of sera was used. The immune spleen cells were fused with SP2/0 myeloma cell line and the hybrids were selected in HAT medium. About 70% of the wells had clones, of which 17% were secreting specific antibodies against HBsAg. We selected nine positive clones (P1C7, P4E2, P7C1, P3D4, P5E9, P5E10, P4B1, P2F6, and P4H1), and after sub-cloning twice by limiting dilution the isotypes were determined. Clone p2F6 produced IgM, clone P5E10 produced IgG2b, and the other seven clones produced IgG1 subclasses. Figure 2 shows that all nine hybrids produced specific monoclonal antibodies against 24 kDa antigen. These antibodies could detect the 24 kDa antigen in native or reduced form, indicating that these antibodies are against linear determinant but not against the conformational epitopes.

For further study, clone P1C7-C2 (IgG1) was selected and it was shown that it could recognize < 1 ng of pure HBsAg in the Western blot analysis (Fig. 3). In addition to the 24 kDa antigen, we identified two more protein bands of 66 and 42 kDa when a high concentration of pure antigen was used. It seems that these proteins are the isomers of HBsAg.

Clone P1C7-C2 was proliferated in pristane-primed mice, the ascitic fluid was collected, and the antibody was purified by ammonium sulfate precipitation and protein G column. Affinity column was prepared with CNBr-activated gel and the recombinant 24 kDa antigen was purified from the crude yeast extract. As shown in Figure 4, the 24 kDa protein was purified and could be used as a vaccine candidate. In fact, 24 kDa antigen together with alum as an accepted adjuvant is used as a vaccination program against hepatitis B in Iran.

It appears that the expression of the 24 kDa gene in yeast has advantages over other cells. Diminsky and colleagues[12] showed that the composition, structure, and immunogenicity of HBsAg particles derived from Chinese hamster ovary (CHO) cells and from cells of the yeast *Hansenula polymorpha* were almost the same. However, the CHO-HBsAg has three peptides (S, M, and L), each in two forms of glycosylation, while the *Hansenula*-HBsAg has only the non-glycosylated S peptide. Subcutaneous injection into mice of fluorescein-isothiocyanate-labeled HBsAg particles from both sources resulted in their accumulation in the marginal sinus of lymph nodes. The humoral responses to subcutaneous injection into mice of CHO- and *Hansenula*-HBsAg were similar; however, the cytotoxic T lymphocyte response to CHO-HBsAg was lower.

We intend to use these MAbs for determination of epitopes of 24 kDa antigen. Sa'adu and colleagues[13] used five human MAbs and found at least three different epitopes, two of which were within the *a* group determinant. Recently, a hepatitis B virus vaccine has been developed using a new adjuvant and HBV surface antigens produced from a CHO cell line.[14] The purified HBV surface antigens were composed of L, M, and S proteins in a mixture of 20 and 40 nm diameter particles and filamentous forms. The HBV surface

antigen, formulated with L-pampo, a proprietary adjuvant, induced 10 times more antibody than the same antigen with alum and was capable of inducing strong immune responses in three different HBV transgenic mice. Our aim is to make an industrial affinity column to purify enough pure HBsAg to be used as an HBV vaccine after the required specification.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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