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Human monoclonal antibody against Hepatitis B virus surface antigen (HBsAg)

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Abstract

Hepatitis B virus (HBV) is one of the main pathogens responsible for hepatitis and hepatocellular carcinoma. Human plasma-derived Hepatitis B immune globulin (HBIG) is being used for prophylactic and liver transplantation currently. However, it may be necessary to replace a HBIG with a recombinant one because of limited availability of human plasma with high anti-HBsAg antibody titer and possible contamination of human pathogens. A Chinese hamster ovary (CHO) cell line, HB-C7A, was established which produces a fully human IgG1 that binds HBsAg. The HB-C7A exhibits ~2600 units/mg of antibody. The affinity (K_a) of HB-C7A is $1.1 \times 10^8\ M^{-1}$ by Biacore analysis and estimated 6.7-fold higher than that of Hepabig® (a plasma-derived HBIG from Green Cross Corp., Yongin, Korea) by competition ELISA. The HB-C7A recognizes the conformational "a" determinant of HBsAg and binds HBV particle more efficiently than the Hepabig®. The HB-C7A binds to HBV-infected human liver tissue but does not bind to normal human tissues. This HB-C7A has several advantages compared to plasma-derived Hepabig® such as activity, safety and availability.

Keywords: Human antibody; Hepatitis B virus (HBV); Hepatitis B virus surface antigen (HBsAg); Hepatitis B immune globulin (HBIG); Chinese hamster ovary (CHO) cell; Tissue cross-reactivity

1. Introduction

Hepatitis B virus (HBV) is one of the principle pathogens responsible for hepatitis and hepatocellular carcinoma, and causes a serious public health problem worldwide (Gitlin, 1997). There are currently 300 million HBV carriers worldwide (Chisari and Ferrari, 1995; Lok, 2000). Chronically infected patients with active liver disease carry a high risk of developing cirrhosis and hepatocellular carcinoma (Chisari and Ferrari, 1995).

Antibodies have been shown to neutralize micro-organisms, destroy cancer cells and modulate the immune system, thus antibody therapy has a great potential for treatment of cancer, autoimmune disorders and viral or bacterial infections (Eren et al., 1998).

Hepatitis B immune globulin (HBIG), prepared from plasma of donors with high anti-HBsAg antibody titer, is a highly effective prophylactic agent (Heijtink et al., 1999; Keller and Stiehm, 2000). An increasingly important use of HBIG is to prevent hepatitis B recurrence in hepatitis B-seropositive liver transplantation recipients (Keller and Stiehm, 2000). However, the currently available HBIG is not an ideal source of therapeutic antibody due to its limited availability, low specific activity and possible contamination of infectious agents (Ehrlich et al., 1992; Witherell, 2002).

Murine monoclonal antibodies (mAbs) are being used rarely in human therapeutics because of their human anti-mouse antibody (HAMA) responses in patients. Humanization of mouse monoclonal antibodies is one approach to reduce the HAMA response, although humanized antibody contains 5–10% of rodent origin sequences (Mountain and Adair, 1992; Baca et al., 1997; Vaswani and Hamilton, 1998; Nakamura et al., 2001). Human antibodies might be more appropriate in a long-term application such as treatment of chronic hepatitis, and technologies for human antibody production are available such as

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phage-display (Hoogenboom and Chames, 2000 and references therein), transgenic mice (Green, 1999), trimera mice (Reisner and Dagan, 1998) and SCID mice (Nguyen et al., 1997), etc.

The safety and efficacy of a human monoclonal antibody (OST-577) directed against HBsAg was evaluated and this antibody demonstrated a reduction in serum HBV DNA and transaminases in patients with chronic HBV infection (Paar et al., 1994). In addition, combination of two human high-affinity monoclonal antibodies (XTL-001) with affinity to different regions of the HBV surface antigen (HBsAg) is under development as a potential passive immunotherapy for HBV infection (Witherell, 2002).

In the previous report, human Fabs against HBsAg were isolated from phage-displayed antibody library which was constructed using peripheral blood lymphocytes from vaccinated volunteers (Kim and Park, 2002).

The Fab was converted to a human immunoglobulin IgG1 and a Chinese hamster ovary (CHO) cell line that produces human monoclonal anti-HBsAg antibody was established (designated as HB-C7A). The HB-C7A antibody was produced in a serum-free medium, purified by affinity and ion-exchange chromatography and the purified antibody was characterized in terms of affinity to HBsAg, binding to HBV and reactivity to human tissues.

2. Materials and methods

2.1. Construction of vectors for CHO cell expression

The Fab clone HB 4-9, which was selected from phage library (Kim and Park, 2002), was inserted to mammalian expression vector. The vectors for expression of antibody were derived from the pCMV-HKR127HC for heavy chain and pKCdhfr-HKR127 (Hong et al., 2004).

The heavy chain expression vector pCMV-HKR127HC (Hong et al., 2004) was modified by replacing *Apa*I site at the end of CH3 with *Nhe*I site using primer P021 (CAG GTC GAC TAG AGC TAG CTA TTC TAT AGT G) and the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) by following the method of manufacturer and designated as pRC-12 (Fig. 1).

The leader DNA sequence was amplified by PCR using primers P001 (GAC TCA CTA TAG GGA GAC CC) and P026 (CAC CAG CTG CAC CTC ACT CTG GAC ACC ATT TAA GAG) on the pCMV-HKR127HC (Hong et al., 2004) and V_H was amplified by PCR using primers P024 (GAG GTG CAG CTG GTG GAG TCG GGT GGA GGC CTG GTC AAG) and P025 (GGG AAG ACC GAT GGG CCC TTG GTG GAG GCT G) on the HB4-9 (Kim and Park, 2002). Then the two fragments were fused by PCR using primers P001 and P025 after mixing same amount of two fragments and this PCR fragment was inserted to pRC-12 by digesting with *Not*I and *Apa*I and designated as pRC12-HB-H4.

The light chain expression vector pKCdhfr-HKR127 (Hong et al., 2004) was modified by replacing the *Hin*dIII site with *Nhe*I using primer P013 (GGG AGA CCC AAG CTA GCT CAG ACA GGC AG) and the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and designated as pKC-11. This pKC-11 was modified further by introducing *Bst*EII and *Hin*dIII sites using primers P022 (GGG GAC ATT GTG GTG ACC CAA TCT CCA GC for *Bst*EII) and P023 (GGA GGG GGG ACC AAG CTT GAA ATA AAA CGG for *Hin*dIII) and the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and designated as pKC-12 (Fig. 1). The V_L was amplified by PCR using primers P016 (GAG CTC GTG GTG ACC CAG TCT CCA) and P018 (TCG TTT GAT TTC AAG CTT GGT CCC TTG) on the HB 4-9 (Kim and Park, 2002) and inserted to pKC-12 by digestion with *Bst*EII and *Hin*dIII and designated as pKC12-HB-L9.

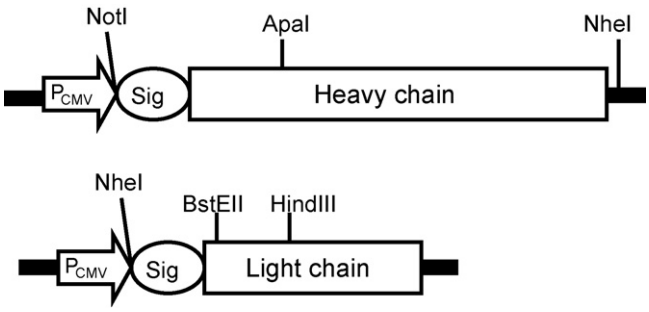

Fig. 1. The arrangement of genes in expression vectors of heavy (pRC12-HB-H4) and light (pKC12-HB-L9) chains. P_{CMV} denotes the cytomegalovirus promoter. *Sig* denotes signal peptide of human immunoglobulin. Restriction enzyme sites used for cloning are shown. The light chain expression vector contains dihydrofolate reductase (*dhfr*) gene (not shown in this figure) which is used for amplification of integrated genes by increasing concentrations of methotrexate (MTX).

2.2. Establishment of a cell line producing human anti-HBsAg antibody

The heavy and light chain expression vectors were co-transfected to CHO DG44 cells (originated from Urlaub et al., 1986), which were maintained in the MEM α Medium containing L-glutamine, ribonucleosides, and deoxyribonucleosides (GIBCO Invitrogen Cat No 12571-063, Grand Island, NY) and 10% fetal bovine serum (FBS) (GIBCO Invitrogen), using Effectene (Qiagen, Valencia, CA) following the manufacturer's instruction. Two days after transfection, cells were resuspended in MEM α Medium containing L-glutamine, but no ribonucleosides or deoxyribonucleosides (GIBCO Invitrogen Cat. no. 12561-056) containing 10% dialyzed FBS (GIBCO Invitrogen) and 550 μg/ml of Geneticin (GIBCO Invitrogen) and transferred to 96 well culture plate (NUNC, Roskilde, Denmark). The cells were screened for anti-HBsAg antibody expression by ELISA in which HBsAg was immobilized to 96 well ELISA plate (NUNC, Maxisorp) and the rest of the assay procedures are same as described (Kim and Park, 2002). Finally a cell line was established, which secretes a human anti-HBsAg IgG1 antibody and was adapted to grow in 1000 nM MTX by increasing the MTX concentration stepwise from 20 nM, and designated as HB-C7A.

The established cell line, HB-C7A, was adapted to serum-free media P1 (Green Cross Corp.) and antibody was produced in a bioreactor (New Brunswick Scientific, Edison, NJ). Antibody was purified using STREAMLINE rProtein A (GE Healthcare

Bio-Sciences, Uppsala, Sweden) from the culture media, then ion-exchange chromatography, and purification of antibody was analyzed by SDS-PAGE.

2.3. Determination of activity of HB-C7A

Activity of HB-C7A was measured by ELISA. For this, HBsAg (Berna Biotech Korea, Yongin, Korea) was diluted to 10 μg/ml PBS, then 100 μl of diluted HBsAg were dispensed into 96-well plates (Nunc Immuno Module Maxisorp, Roskilde, Denmark) and incubated overnight at 4 °C. Plates were washed once with PBS-T (phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20), then 200 μl of 1% BSA in PBS were added and incubated for 1 h at room temperature and washed once with PBS-T. The WHO International Unit of anti-hepatitis B immunoglobulin (International Laboratory for Biological Standards, Amsterdam, The Netherlands) and HB-C7A were diluted serially in 1% BSA-PBS and 100 μl of diluted antibodies were added and incubated for 2 h at room temperature. Plates were washed with PBS-T, then 100 μl of diluted goat anti-human IgG (Fab specific)-peroxidase conjugate (Sigma–Aldrich) in 1% BSA-PBS were added and incubated for 1 h at room temperature. After washing again with PBS-T, 100 μl of TMB solution (KPL) were added and the O.D. was measured at 405 nm. Activity of plasma-derived HBIG, Hepabig® (Green Cross Corp.) was measured as same way.

2.4. Determination of affinity of HB-C7A

Surface Plasmon resonance analysis was used to measure the association and dissociation rate constant for binding of HB-C7A mAb to immobilized HBsAg using BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden) and all Biacore experiments were performed at 25 °C. The carboxylated dextran matrix of a CM5 sensor chip (Biacore, Cat. BR-1000-14) was activated with a 7-min pulse (35 μl at a flow rate of 5 μl/min) of 0.2 M EDC/0.05 M NHS (Amine coupling kit, Biacore, Cat. BR-1000-50). Purified, recombinant HBsAg (Berna Biotech Korea) was diluted 5-fold in 10 mM sodium acetate, pH 4.0 (Biacore, Cat. BR-1003-49) and injected at a flow of 5 μl/min until maximum density (typically ∼1155 RU) was reached. This was followed by a 7-min pulse (35 μl at a flow rate of 5 μl/min) of 1 M ethanolamine hydrochloride, pH 8.5, to quench excess of active ester groups. The HB-C7A antibody was diluted to 125, 62.5, 31.3, 15.6, 7.8 and 0 nM in HBS-EP buffer (Biacore, Cat. BR-1001-88) and the samples were injected on the HBsAg surface at 30 μl/min (100 μl injection) for 200 s and dissociation of bound analyte was allowed to proceed for 200 s. The data were analyzed by the BIA evaluation software 3.2 assuming 1:1 model. Regeneration of the surface was achieved with a 30 μl injection (30 μl/min) of 50 mM NaOH and 1 M NaCl. Affinity of plasma-derived HBIG, Hepabig® (Green Cross Corp.), was measured as same way but with much higher concentrations of antibody starting from 50 μM.

Affinities of HB-C7A and Hepabig® (Green Cross Corp.) were compared by competition ELISA using HBsAg-coated plates and free HBsAg as described (Kim and Park, 2002). The HBsAg concentration that gives 50% inhibition of maximum binding (the ELISA reading performed without competitive HBsAg) was determined as affinity.

2.5. Determination of HB-C7A binding to HBsAg subtypes

Binding of antibody to different subtypes of HBsAg was determined by ELISA. For this, HBsAg *ad* (Fitzgerald, Concord, MA) and *ay* (Fitzgerald) were diluted to 10 μg/ml PBS, then 100 μl of diluted HBsAg *ad* and *ay* were dispensed into 96-well plates (Nunc Immuno Module Maxisorp) respectively and incubated overnight at 4 °C. Plates were washed once with PBS-T, then 200 μl of 1% BSA in PBS were added, incubated for 1 h at room temperature and washed once with PBS-T, and the rest of assay procedures were same as described in 2.3. Mouse monoclonal antibodies, clone H31 which is specific to "a" determinant of HBsAg and clone HY66 which is specific to "y" determinant of HBsAg (unpublished data), were included as controls.

2.6. Epitope mapping of HB-C7A

The epitope of HB-C7A antibody was investigated. Synthesis and screening of all overlapping linear 15-mers covering 226 amino acids of HBsAg *adr* were performed by Pepscan Systems (Lelystad, Netherlands). Fine-tuning of epitope was performed further by synthesis and screening of various length of peptides based on the alanine scan, length variants and looped versions running from 6-mer to 21-mers.

2.7. Immunoprecipitation of HBV by HB-C7A

Binding of antibody to HBV was determined by immunoprecipitation (Eren et al., 2000). The inherent IgGs in the patient serum were removed prior to immunoprecipitation reaction by incubating 1000 μl of HBV-infected serum (obtained from Ajou University, School of Medicine, Korea), which was diluted 10-fold with 0.2% BSA in PBS, with agarose conjugated goat anti-human IgG (Fc specific) (Research Diagnostics Inc., Flanders, NJ).

The HB-C7A antibody was bound to the agarose conjugated goat anti-human IgG (Research Diagnostics) prior to binding reaction with HBV in the patient serum by incubating 10 μl of HB-C7A antibody in different amount (0.1, 0.5, 1 and 5 μg and PBS as a control) with 50 μl of agarose conjugated anti-human IgG (Research Diagnostics) for 1 h at RT with gentle shaking, then 10 mg of human IgG (I.V.-Globulin-S, Green Cross Corp.) were added for 1 h at RT to mask the unoccupied anti-human IgG in agarose by HB-C7A antibody.

Two hundred (200) μl of IgGs-depleted HBV infected human serum were added to the HB-C7A-bound anti-human IgG-agarose (Research Diagnostics) and incubated for 1 h at RT with gentle shaking. The supernatant was harvested by centrifugation and analyzed with Cobas Amplicor HBV Monitor Test, v2.0 (Roche Diagnostics, Basel, Switzerland). The remaining agarose was washed 10 times with 0.2% BSA in PBS and resuspended in 100 μl of 0.2% BSA in PBS, then 5 μl of 10% SDS (final 0.05%), 2 μl of 50 mM EDTA (final 1 mM) and 200 μg of proteinase

K (Sigma–Aldrich) were added and incubated 30 min at 55 °C by mixing every 5 min. Finally the supernatant was harvested and DNA was prepared from the supernatant with QIAquick PCR purification Kit (Qiagen, Hilden, Germany) and the HBV specific DNA was amplified using LiquiMix GM PCR premix (Neurotics, Daejeon, Korea) with primers M3 (CTGGGAGGAGTTGGGGGAGGAGATT; Carman et al., 1989) and POL8 (AGGATAGAACCTAGCAGGC; Alexopoulou et al., 1997). The PCR reaction was performed 35 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C with pre-incubation for 5 min at 55 °C and final extension reaction for 10 min at 72 °C. And the amplified DNAs were analyzed in 1.0% agarose gel.

A humanized anti-HBsAg antibody HuS10 (Ryu et al., 1996) and an anti-tetanus human mAb TT-F9 produced in this lab (unpublished data) were included as controls.

2.8. Immunohistochemical staining of human tissues by HB-C7A

Binding of HB-C7A antibody to HBV-infected and normal tissues was determined by immunohistochemistry in the Covance Laboratories Ltd. (Harrogate, U.K). Both the HB-C7A antibody and human IgG1 (Sigma–Aldrich) were biotinylated using a biotinylation kit, Immunoprobe (Sigma–Aldrich). Prepared sections of human liver tissue known to be infected with the HBV (Spring Bioscience, Fremont, CA) were used as positive control tissue for the demonstration of specific positive immunohistochemical staining with the biotinylated HB-C7A antibody.

2.8.1. Human tissue panel

The supply and pertinent data regarding age, sex and any relevant clinical history are detailed in the study records and cryo-sections were prepared in accordance with Covance Laboratories Ltd. Standard Operating Procedures.

2.8.2. Immunohistochemical staining methods

Cryo-sections were fixed in acetone and incubated in hydrogen peroxide in methanol. Sections were then incubated with normal rabbit serum followed by sequential incubation with avidin and biotin. Incubation with biotinylated antibody was followed by treatment with StreptABComplex/HRP. DAB (3,3′-diaminobenzidine tetrahydrochloride) was employed as chromogen with haematoxylin used as a counterstain.

For the positive immunohistochemical staining control, the biotinylated anti-HB antibody was evaluated on human liver tissue known to be infected with the Hepatitis B Virus.

For the negative immunohistochemical staining, the biotinylated HB-C7A antibody was eliminated from the immunohistochemical staining and replaced with biotinylated IgG1 isotype negative control.

3. Results

3.1. Binding characteristics of HB-C7A

Mammalian expression vectors for heavy and light were constructed from the Fab genes selected from phage library and transfected to CHO cells. The cell line (HB-C7A) was finally established which produces human monoclonal anti-HBsAg antibody, and the antibody was prepared by protein A and ion-exchange chromatography purification from serum-free culture and purification was analyzed by SDS-PAGE (Fig. 2).

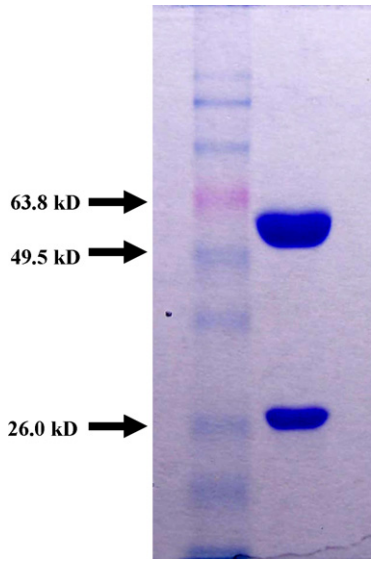

Fig. 2. Purification of antibody confirmed by SDS-PAGE. Antibody was purified by protein A and ion-exchange chromatographies from the culture supernatant of HB-C7A cells. Ten micrograms of purified antibody was analyzed. Prestained Protein Ladder (BenchMark, Invitrogen) was included.

The activity of HB-C7A antibody is ∼2600 units/mg and that of Hepabig® is ∼1.3 unit/mg based on the WHO International Unit of anti-hepatitis B immunoglobulin.

The affinity of HB-C7A by Biacore is $1.1 \times 10^8\ M^{-1}$ (Fig. 3), but that of Hepabig® could not be measured by Biacore. The affinities of HB-C7A and Hepabig® by competition ELISA are $8.0 \times 10^9\ M^{-1}$ and $1.2 \times 10^9\ M^{-1}$ respectively (Fig. 4).

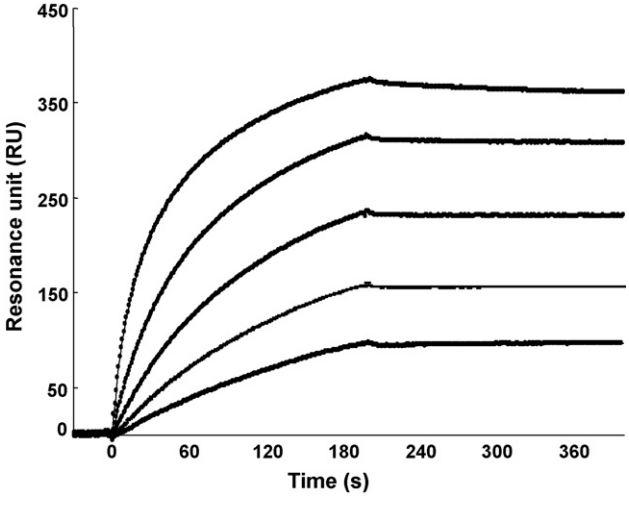

Fig. 3. Binding of HB-C7A to the immobilized HBsAg in Biacore analysis. The HB-C7A antibody was diluted to 125, 62.5, 31.3, 15.6, 7.8 nM in HBS-EP buffer and injected on the HBsAg surface at 30 μl/min for 200 s (100 μl injection) and dissociation of bound analyte was allowed to proceed for 200 s.

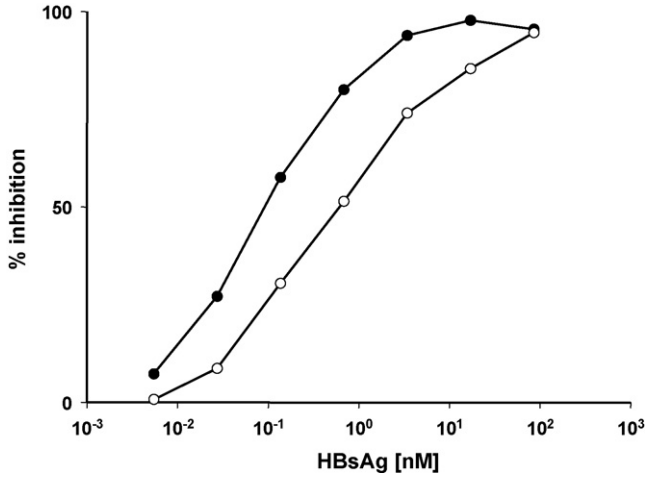

Fig. 4. Competition ELISA for measuring relative affinities of HB-C7A and Hepabig®. The inhibition of antibody binding to free HBsAg was analyzed on HBsAg-coated ELISA plates according to different concentrations of HBsAg. And the HBsAg concentration that gives 50% inhibition of maximum binding (the ELISA reading performed without competitive HBsAg) was determined as affinity. Each symbol denotes HB-C7A (●) and Hepabig® (◯).

The binding of HB-C7A antibody to a different HBV subtype is important for HBV neutralization irrespective of its subtype. The HB-C7A antibody binds the "ad" and "ay" subtype of HBsAg equally. The H31 mouse mAb which is specific to "a" determinant of HBsAg (unpublished data) exhibits the same binding characteristics with HB-C7A. In contrast the HY66 mouse mAb which is specific to "y" determinant of HBsAg (unpublished data) binds the "'ay" but does not bind the "ad" subtype of HBsAg (Fig. 5).

3.2. Epitope mapping of HB-C7A

The antibody did not exhibit any clear peaks on a serially synthesized 15-mer peptide and has a strong tendency to recognize all peptides with a carboxy terminal proline (data not shown) and four different epitope candidates were identified from all these peptides (SQSPTSNH**SPT**SCP**P**, YWGPSLYNI**LSP**FL**P**, VPFVQWFVG**LSPT**VW, EWASVRFSW**LS**LLV**P**).

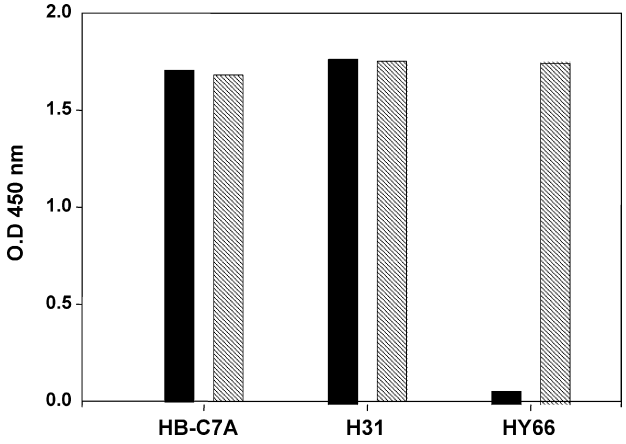

Fig. 5. Binding of HB-C7A to the immobilized HBsAg subtypes "ad" (black bar; ■) and "ay" (hatched bar; ▨) by ELISA. Mouse monoclonal antibodies H31 which was proven to be specific to HBsAg "a" and HY66 which was proven to be specific to HBsAg "y" were included as references.

A follow-up order was performed to identify which of the four regions is the actual epitope. To this end approximately 500 new peptides were synthesized. These include various types of looped peptides in which the native cysteines in between the cysteines used for loop configuration were replaced by alanine. Throughout this analysis, most of the top binders contain **PTSNHSPTSAPP** (the A is the replace C). The **SPTSAPP** is part of peptide candidate in the first analysis. The alanine scan shows that **SPXXXP** is the core of the epitope (data not shown). However any clear epitope could not be confirmed when the candidate epitopes were interpreted with the topology of HBsAg in the HBV.

3.3. Immunoprecipitation of HBV by HB-C7A

Binding of antibody to HBV was manifested by immunoprecipitation and the precipitated HBV particles were measured by PCR amplification and quantitation of HBV DNA. The precipitated HBV particles are proportional to the amount of antibody used for immunoprecipitation; in contrast the HBV particles remaining in the supernatant are inversely proportional to the amount of antibody used (Fig. 6A and B).

Binding ability of the HB-C7A antibody to HBV is much higher than that of Hepabig®. The HB-C7A antibody and the humanized anti-HBsAg antibody (HuS10) could precipitate the HBV particles, however both the Hepabig® and the anti-tetanus human mAb could not precipitate the HBV particles when the same amount of antibody (1 μg) was employed for immunoprecipitation (Fig. 6C).

3.4. Binding of HB-C7A to human tissues

The HB-C7A antibody was evaluated for the assessment of potential cross-reactivity with prepared cryo-sections from a selected panel of human tissues. The HB-C7A antibody binds to the HBV-infected human liver tissue (Fig. 7A) but the control antibody does not bind to the HBV-infected human liver tissue (Fig. 7B). The HB-C7A antibody does not demonstrate cross reactivity with the selected panel of human tissues examined: Adrenal, Bladder, Blood Cells, Blood Vessel, Bone Marrow, Breast, Brain (Cerebellum), Brain (Cerebrum), Cervix, Colon, Duodenum, Endometrium, Eye, Fallopian Tube, Gastric Antrum, Gastric Body, Heart, Ileum, Kidney, Liver, Lung, Lymph Node, Muscle, Oesophagus, Ovary, Pancreas, Parathyroid, Parotid, Peripheral Nerve, Pituitary, Placenta, Prostate, Skin, Spinal Cord, Spleen, Testis, Thymus, Thyroid, Tonsil, Ureter.

4. Discussion

The HB-C7A is a fully human IgG1 mAb that binds HBsAg and is produced from CHO cells.

The activity (units) of antibody was measured by ELISA in which HBsAg was immobilized and goat anti-human IgG-peroxidase conjugate was employed for the detection of

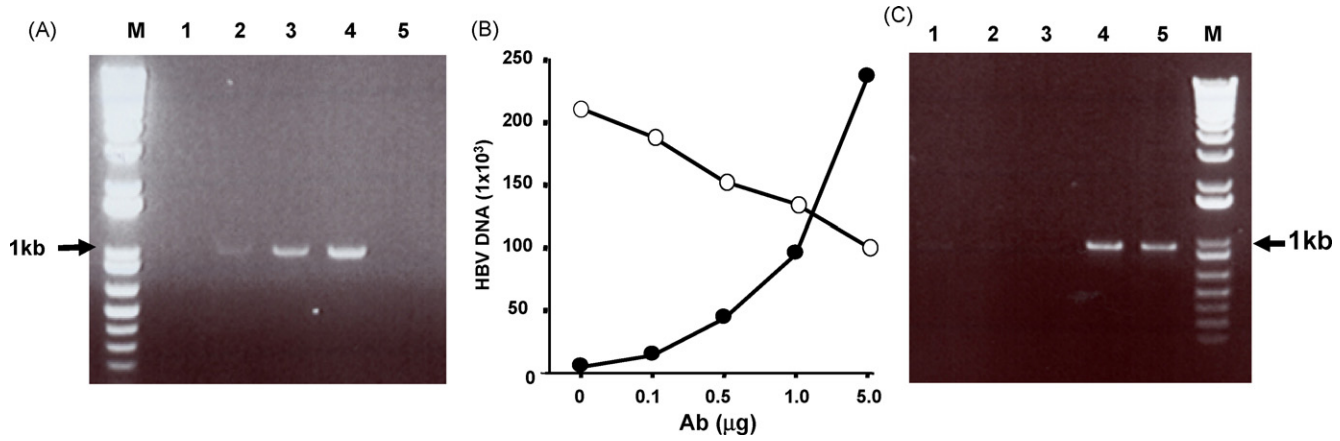

Fig. 6. Binding of HB-C7A antibody to HBV was proved by immunoprecipitation. The HB-C7A antibody was bound to the agarose conjugated goat anti-human IgG prior to binding reaction with HBV in the patient serum and the HBV infected human serum in which IgGs were depleted prior to immunoprecipitation was added to the HB-C7A-bound anti-human IgG-agarose. (A) The precipitated HBV particles were measured by PCR amplification of HBV DNA; M: DNA ladder, lane 1: 0.1 μg of HB-C7A, lane 2: 0.5 μg of HB-C7A, lane 3: 1 μg of HB-C7A, lane 4: 5 μg of HB-C7A, lane 5: PBS. (B) HBV DNAs were quantitated by Cobas Amplicor HBV Monitor Test, v2.0 (Roche Diagnostics) in the precipitates (●) and in the remaining supernatants (○). (C) When same amount of antibody (1 μg) was used for immunoprecipitation, the HB-C7A and the humanized anti-HbsAg antibody (HuS10) precipitated HBV; lane 1: PBS, lane 2: 1 μg of anti-tetanus human antibody (TT-F9), lane 3: 1 μg of plasma-derived HBIG, Hepabig®, lane 4: 1 μg of HuS10 antibody, lane 5: 1 μg of HB-C7A antibody, M: DNA ladder.

antibodies bound to immobilized HBsAg and the activity was calculated based on the WHO International Unit of anti-hepatitis B immunoglobulin.

The affinity of HB-C7A was measured by Biacore, however that of Hepabig® (Green Cross Corp.) could not be measured by the same method; the Hepabig® did not exhibit any association to the immobilized HBsAg in Biacore analysis even with much higher concentrations than that of HB-C7A used. The affinities of two antibodies, HB-C7A and Hepabig®, could be compared by competition ELISA and this method could reflect comparative affinities between antibodies.

The affinity of HB-C7A showed 7.3-fold differences between Biacore and competition ELISA ($1.1 \times 10^8\ M^{-1}$ by Biacore versus $8.0 \times 10^9\ M^{-1}$ by competition ELISA). Biosensor-based reaction constants may not match those obtained from solution-based methods due to a variety of potential artifacts (Day et al., 2002); the differences between surface affinities and solution affinities are so large that competition Biacore reflecting true affinities is recommended (Nieba et al., 1996).

HB-C7A antibody exhibits much higher activity and affinity than those of Hepabig® and this indicates that the recombinant HB-C7A mAb is highly potent than Hepabig® and might be more effective in HBV neutralization.

The binding ability of HB-C7A antibody to HBV was manifested by immunoprecipitation. The precipitation of HBV by antibody is proportional to the amount of antibody used, and when the same amount of antibody was employed (1 μg of each antibody) for immunoprecipitation the HB-C7A antibody precipitated HBV, however the Hepabig® could not precipitate the HBV. This strongly suggests that the in vitro activity is correlated with the binding activity of antibody to HBV and as a result the HBV neutralizing activity of antibody (Eren et al.,

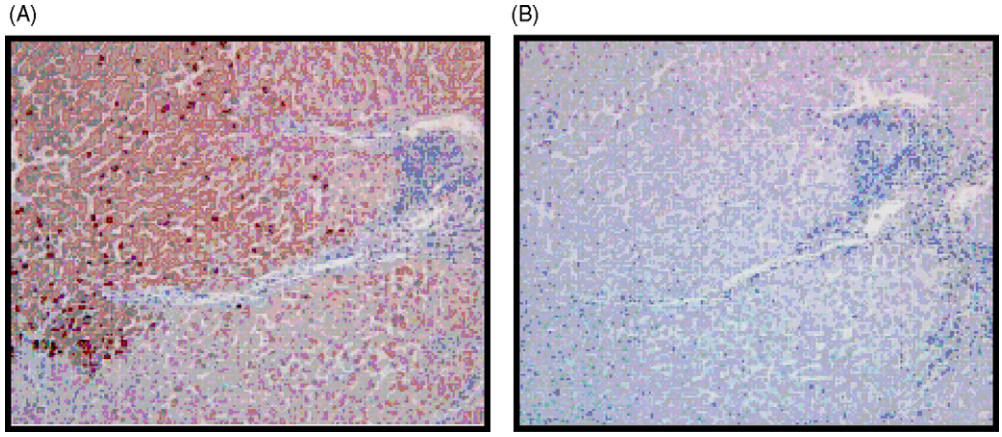

Fig. 7. Immunohistochemical staining of HBV-infected human tissue by HB-C7A and human IgG1 antibody. HB-C7A antibody and human IgG1 (Sigma–Aldrich) were biotinylated. For the positive immunohistochemical staining control, the biotinylated HB-C7A antibody was evaluated on human liver tissue known to be infected with the Hepatitis B virus (A). For the negative immunohistochemical staining, biotinylated IgG1 isotype control was used for the immunohistochemical staining on human liver tissue known to be infected with the Hepatitis B virus (B).

2000; Ryu et al., 1996). It is expected that the activity measured by ELISA or any other in vitro methods has correlation with in vivo neutralization activity although it is not demonstrated yet.

All HBsAg contains the group specific "a" determinant, although there are four major antigenic types of HBsAg such as adr, adw, ayr and ayw (Peterson et al., 1982) and this "a" determinant is considered to be the most important target for diagnosis and immune prophylaxis (Ijaz et al., 2003). The HB-C7A antibody exhibited same binding to the HBsAg "ad" and "ay" subtypes, indicating it recognizes common "a" determinant of HBsAg and as a result its binding to HBV is not dependent on the subtypes of HBV.

The epitope of HB-C7A antibody could not be determined by linear and looped pepscan analysis. Considering the HBsAg topology in the HBV, the region between amino acid 110 and 150 could be the epitope (Stirk et al., 1992). This region contains many P, T and S, and is exposed and is very conformational. Most of the identified better binding peptides in the pepscan analysis may be mimics of this epitope in which P, T and S seem to be important. This antibody did not bind HBsAg in Western blot of patient sera; in contrast mAb H128, which was proved to recognize linear epitope by pepscan analysis, exhibited binding to HBsAg in Western blot of patient sera (unpublished data). Considering the pepscan analysis and Western blot of patient sera, we assume that the epitope of HB-C7A antibody is conformational and this reflects the fact that neutralizing antibodies induced by HBV vaccine are largely targeted towards conformational epitope (Chiou et al., 1997).

Therapeutic antibody is recommended to evaluate antibody binding to the tissues from at least three unrelated human donors according to the FDA guideline because non-target tissue binding of antibody may have serious consequences. Immunohistochemical analysis of HB-C7A antibody to 32 normal human tissues did not demonstrate any tissue cross-reactivity to normal human tissues examined, indicating that this antibody might be safe when administered to the patient.

HBIG has been shown to be efficacious in the prevention of transmission of HBV post-exposure (Sawyer, 2000). In addition, it has been shown to be markedly effective in reducing the recurrence rate of HBV infection when used as prophylactic agent after orthotopic liver transplantation for HBV-related liver disease (Cirera et al., 2001).

Recently, lamivudine, an oral purine nucleoside analogue, is being used extensively for treating the chronic hepatitis B, but the emergence of lamivudine-resistant mutants limits the efficacy of the drug (Han et al., 2000 and references therein). However, combination of HBIG and lamivudine prevented HBV recurrence in patients with no evidence of the development of the lamivudine-resistant escape mutants during long-term follow-up (Han et al., 2000 and references therein; Saab et al., 2000). The conventional HBIG is required high amount of protein for neutralization of HBV because of its low activity and affinity, although it is less likely to be ineffective against escape mutants than a monoclonal antibody. However this HB-C7A could be applied much less amount considering its high activity. As a result this antibody may have several advantages compared to plasma-derived HBIG such as activity, affinity, safety and availability.

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