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Analysis of the epitope and neutralizing capacity of human monoclonal antibodies induced by hepatitis B vaccine

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ABSTRACT

Hepatitis B virus (HBV) is an infectious agent that is a significant worldwide public health issue. However, the mechanism by which vaccination-induced antibodies prevent HBV infection remains unclear. To investigate the mechanism by which antibodies induced by hepatitis B surface Ag (HBsAg)-vaccination prevent HBV infection in humans, we prepared human monoclonal antibodies (mAbs) against HBsAg using a novel cell-microarray system from peripheral blood B-lymphocytes from vaccinated individuals. We then characterized the IgG subclass, L-chain subtype, and V-gene repertoire of the H/L-chain, as well as affinities of each of these mAbs. We also determined the epitopes of the individual mAbs using synthesized peptides, and the HBV-neutralizing activities of mAbs using the hepatocyte cell line HepaRG. Consequently, IgG1 and kappa chain was mainly used as the mAbs for HBsAg. Seventy percent of the mAbs bound to the loop domain of the small-HBsAg and showed greater neutralizing activities. There were no relationships between their affinities and neutralization activities. A combination of mAbs recognizing the first loop domain showed a synergistic effect on HBV-neutralizing activity that surpassed conventional hepatitis B-Ig (HBIG) in the HepaRG cell line assay. These results may contribute to the development of effective mAb treatment against HBV infection replacing conventional HBIG administration.

1. Introduction

Hepatitis B virus (HBV) is a double-stranded DNA virus and causes chronic liver disease, leading to cirrhosis and hepatocellular carcinoma. About 350 million people are chronically infected with HBV around the world, and the infection is a worldwide public health problem (Ganem and Prince, 2004). In recent years, various therapeutic regimens, such as interferon or viral reverse transcriptase inhibitors, have been established (The EASL Jury, 2003; Beasley et al., 1983; Chayama et al., 1998). However, the effect of interferon is still unsatisfactory and long-term administration of reverse transcriptase inhibitors results in the emergence of resistant mutant virus strains (Chayama et al., 1998). Administration of specific hepatitis B immunoglobulin (HBIG) and vaccination with hepatitis B surface antigen (HBsAg), which is mainly composed from small-HBsAg (S-HBs), provide passive or active immunization against infection with HBV and useful ways to prevent infection with HBV (The EASL Jury, 2003; Alter, 2003; Beasley et al., 1983). Thus, antibodies directed HBsAg are useful to prevent the infection of HBV. However, the mechanism by which S-HBs contributes to HBV entry into hepatocytes and how antibodies against S-HBs neutralize HBV remain to be clarified. Furthermore, the currently available HBIG is not an ideal source of therapeutic antibody due to its limited availability, low specific activity and possible contamination with infectious agents. Therefore the attempt to prevent the infection of HBV with monoclonal antibodies (mAbs) has been performed, but the result is still unsatisfactory (Kim et al., 2008; Shin et al., 2007).

We established a cell-microarray system that allowed analysis of large numbers of lymphocytes on a single-cell basis with high sensitivity (Jin et al., 2009; Tajiri et al., 2007; Tokimitsu et al., 2007). Previously, we analyzed B-lymphocytes responding to HBV from S-HBs-vaccinated healthy volunteers and generated 31 different mAbs for HBV (Jin et al., 2009; Tajiri et al., 2007; Tokimitsu et al., 2007). In this study, we determined the epitopes of these mAbs on S-HBs and their neutralizing effects against HBV infection in vitro using the HepaRG cell line assay. Furthermore, we investigated the possibilities for the therapeutic antibody using these mAbs or their combination. The findings of the present study provide insight into the mechanism by which S-HBs vaccination prevents HBV infection and may contribute to the development of efficient recombinant anti-HBsAg antibodies for prophylaxis.

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2. Materials and methods

2.1. Generation of human monoclonal antibodies against HBsAg

All the experiments were performed with the approval of the Ethical Committee of the University of Toyama and all the volunteers gave their written informed consent prior to the study. HBsAg-specific mAbs were obtained using the cell-microarray system as described previously (Jin et al., 2009; Tajiri et al., 2007; Tokimitsu et al., 2007). Briefly, we detected single HBsAg-responding B-lymphocytes (Tajiri et al., 2007; Tokimitsu et al., 2007) or anti-HBsAg antibody-secreting cells (ASCs) (Jin et al., 2009) from peripheral B-cells of healthy volunteers who had been vaccinated with yeast-derived recombinant HBsAg (Bimmugen, *adr*, genotype C; Kaketsuken, Kumamoto, Japan) at 7 days before peripheral blood samples were drawn. We then retrieved the single cells from the microchip and amplified antibody cDNAs for heavy and light chain variable fragments (V_H and V_L, respectively) using a single-cell 5′-RACE method (Ozawa et al., 2006) with primers for gamma, kappa, and lambda chains, and then inserted into expression vectors containing the whole constant region cDNAs of heavy and light chains. We analyzed the V gene repertoire and IgG subclass of antibodies using the IMGT/V-Quest tool (http://imgt.cines.fr/) (Table 1). Concerning the isoform of antibodies, we screened only IgG-positive or IgG-secreting cells and amplified γ chain cDNA to produce IgG, because IgG is superior to other isoforms of Ig in physiological activity. We then cotransfected Chinese hamster ovary (CHO) cells with both the heavy and light chain expression vectors to obtain supernatants containing the whole antibody molecules, as described previously (Jin et al., 2009; Tokimitsu et al., 2007). We collected the supernatant of cultured cells and purified the antibody using a protein A column with ÄKTA Prime plus (GE Healthcare, Uppsala, Sweden), and then dialyzed against phosphate-buffered saline. The reactivities of all the obtained antibodies against HBsAg were confirmed by HBsAg-coated ELISA.

2.2. Determination of the affinity of each mAb

We measured the affinity of each mAb by surface plasmon resonance (SPR) using a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). We activated the carboxylated dextran matrix of a CM5 sensor chip (Biacore, Cat. BR-1000-14) 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 (Kaketsuken) was diluted in 10 mM sodium acetate, pH 4.0, and injected at a flow rate of 5 μL/min until an adequate density was reached. Excess active ester groups were blocked with 1 M ethanolamine hydrochloride. Each antibody was diluted to 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, and 0 nM in HBSS buffer containing 0.05% Tween 20. We then injected the diluted samples, and determined the value of dissociation constant (K_D) in accordance with the manufacturer's instructions (Biacore AB, Uppsala, Sweden).

2.3. Preparation of peptides and determination of antibody-binding site in small-HBsAg

We investigated the binding site of each mAb by peptide mapping of the extracellular domain of S-HBs. We determined the conformational structure of S-HBs with two disulfide bonded extracellular loop domains as reported previously by Stirk et al. (Carman, 1997; Stirk et al., 1992) (Fig. 2). We used 10-mer to 15-mer peptides covering the extracellular domain (aa 104–163) of S-HBs (genotype C, *adr*) synthesized by Operon Biotechnologies (Huntsville, AL) designed to conserve the disulfide bonded conformational structures. Briefly, the peptides were synthesized by standard solid-phase methods using 9-fluorenylmethoxy carbonyl chemistry and purified by reversed-phase high-performance liquid chromatography. The peptides were cyclized by oxidation between sulfhydryl groups of two cysteines in the peptide sequence. Cyclization of the peptides was confirmed by matrix-associated laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), as detection of $-2\,m/z$ to the molecular weight of the linear peptides. Biotin was added at the C-terminal of synthesized peptides. Their amino acid sequences are shown in Fig. 2(B).

We examined the binding activity of each mAb for recombinant peptides by ELISA with streptavidin-coated plates (Nunc, Roskilde, Denmark). Plates were coated with the peptides at 10 mg/mL and nonspecific binding was blocked with PBS containing 3% bovine serum albumin. Each mAb was added to the wells for 2 h, followed by washing and reaction with alkaline phosphatase-conjugated anti-human IgG (Sigma, Saint Louis, MO). The O.D. value at 405 nm was evaluated after addition of phosphate substrate (Sigma). Control human monoclonal IgG1 (cIgG, Athens Research & Technology, Athens, GA) was added at the same concentration as a control.

2.4. Analysis of HBV-neutralizing capacity

We investigated HBV-neutralizing capacity using the HepaRG cell line (supplied by Biopredic International, Rennes, France) that has been established as an in vitro HBV infection model (Gripon et al., 2002). The HepaRG cells were cultured as previously described (Gripon et al., 2002) in William's E medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 5×10^{-7} M hydrocortisone hemisuccinate for 2 weeks. For differentiation and infection, the HepaRG cells were cultured for 2 weeks in medium supplemented with 2% DMSO. About half of the HepaRG cells were differentiated to hepatocytes, confirmed by anti-albumin antibody staining (data not shown) as previously described (Cerec et al., 2007). We preincubated 2×10^4 copy of HBV (from serum of HBV-carrier, genotype C, *adr*) and 1 μg of each mAb for 1 h at room temperature, and

Table 1
Sequence in the 3′ region of the human immunoglobulin G CH1 domain.

	*	*	*	*	*	*	*
IgG1	CCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCG						
IgG2	CCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGC***G***CCCT***G***CTCCA***G***GAGCACCTCTG***A***G***A***GCACAGC***C***						
IgG3	C***T***TCCACCAAGGGCCCATCGGTCTTCCCCCTGGC***G***CCCT***G***CTCCA***G***GAGCACCTCTG***G***G***G***GCACAGC***G***						
IgG4	C***T***TCCACCAAGGGCCCATC***C***GTCTTCCCCCTGGC***G***CCCT***G***CTCCA***G***GAGCACCTCTG***A***G***A***GCACAGC***C***						
HB0116~	CCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCG						
HB0477~	CTTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCTGGGGGCACAGCG						
HB1089~	CCTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCGCCCTGCTCCAGGAGCACCTCTGAGAGCACAGCC						

The upper rows show the amino acid sequences of each human IgG subclass. Asterisk (*) indicates the key residues determining the subclasses. Lower rows show representative amino acid sequences of anti-HBsAg mAbs.

Table 2
Characteristics of human monoclonal antibodies for small-HBsAg.

mAb	Case[a]	Light chain	Subclass	V_H	Heavy chain		Light chain		$K_D(M^{-1})$
					D_H	J_H	V_L	J_L	
HB0116	a	Kappa	IgG1	3-48*02	3-10*01	2*01	3-15*01	4*01	3.63 n
HB0477	b	Kappa	IgG3	4-04*02	4-17*01	4*01	4-01*01	1*01	369 n
HB0478	b	Kappa	IgG1	3-30*04	3-09*01	6*03	1-12*01	4*01	2.91 n
HB0905	c	Lambda	IgG1	7-4-1*02	3-10*01	6*02	1-44*01	2*01	20.3 n
HB1089	c	Lambda	IgG2	3-23*04	2-02*01	4*02	4-69*01	3*02	27.7 n
HB1474	c	Lambda	IgG1	3-09*01	6-13*01	5*02	1-51*01	2*01	6.52 n
HBV2.09	c	Kappa	ND	3-33*01	ND	ND	ND	ND	7.48 n
HBV2.17	c	Kappa	ND	ND	ND	ND	4-01*01	3*01	67.4 n
HBV2.18	c	Kappa	ND	5-51*01	ND	ND	1-39*01	3*01	18.7 n
HBV2.22	c	Lambda	ND	ND	ND	ND	ND	ND	3.20 n
HBV2.23	c	Kappa	ND	ND	ND	ND	ND	ND	207 n
HB1363	d	Lambda	IgG2	3-13*02	ND	ND	ND	ND	1.22 n
HB1367	d	Kappa	IgG1	3*21*02	3-16*02	6*03	3-15*01	5*01	30.3 n
HB1368	d	Kappa	IgG3	3-21*02	6-13*01	6*02	2-28*01	2*01	2.93 μ
HB1370	d	Kappa	IgG1	3-30*02	2-02*01	5*02	ND	ND	194 p
HB1378	d	Lambda	IgG1	3-48*03	2-08*02	4*02	1-40*01	1*01	1.92 n
HB1381	d	Lambda	IgG1	3-48*03	2-15*02	4*02	1-40*01	1*01	3.59 μ
HB1421	d	Kappa	IgG1	3-21*01	3-16*02	6*03	3-15*01	5*01	239 n
HB1422	d	Kappa	IgG1	4-39*01	6-19*02	4*02	1-51*01	3*02	72.5 n
HB1425	d	Kappa	IgG1	3-23*04	6-13*01	6*02	2-28*01	4*01	103 n
HB1431	d	Kappa	IgG1	3-23*01	1-26*01	3*01	1-16*01	4*01	526 n
HB1434	d	Kappa	IgG1	6-01*01	3-03*01	6*03	1-05*03	2*01	107 n
HB1438	d	Kappa	IgG1	3-48*02	4-17*01	2*01	3-15*01	4*01	122 n
HB1440	d	Kappa	IgG1	3-33*01	2-21*02	4*02	1-39*01	4*01	107 n
HB1442	d	Lambda	IgG1	3-66*01	3-10*02	4*02	1-40*01	3*02	375 n
HB1444	d	Kappa	IgG1	3-33*01	6-19*01	4*02	1-39*01	4*01	311 n
HB1448	d	Lambda	IgG1	3-48*01	2-15*01	4*02	1-40*01	1*01	553 n
HB1453	d	Kappa	IgG2	3-33*01	5-05*01	4*02	1-39*01	3*01	803 n
HB1460	d	Kappa	IgG1	3-33*01	5-05*01	4*02	1-39*01	3*01	63.0 n
HB1462	d	Kappa	IgG1	3-23*04	6-13*01	6*02	2-28*01	4*01	6.20 n
HB1465	d	Kappa	IgG3	4-39*01	6-19*01	4*02	2-28*01	4*01	536 n

In K_D value, 'n' means nanomolar, 'μ' means micromolar, and 'p' means picomolar; ND, not determined.
[a] Each letter indicates each volunteer vaccinated with small-HBsAg.

then added them to HepaRG cells in medium with 4% PEG8000 (Sigma). After overnight incubation, the HepaRG cells were gently washed three times with medium and then cultured with fresh medium. On days 2, 4, 6, and 8 after infection, DNA was extracted from HepaRG cell lysate using a SMITEST kit in accordance with the manufacturer's protocol (Genome Science Laboratories, Tokyo, Japan). We amplified HBV-DNA by 50 cycles of PCR in a total volume of 20 μL and detected products using SYBR Green (Stratagene, Cedar Creek, TX), with quantification performed using a Stratagene Mx3000P qPCR machine (Stratagene, La Jolla, CA). The primer sequences were TTTGGGGCATGGACATTGAC (forward) and GGTGAACAATGTTCCGGAGAC (reverse) designed to amplify a 157 base pair product from HBV core antigen of HBV genome. We then harvested the culture supernatant, and quantified HBsAg in the supernatant using a commercial chemiluminescent immunoassay kit (Architect; Abbott Japan, Osaka, Japan) on the indicated days. We used the same concentration of control human monoclonal IgG1 (cIgG, Athens Research & Technologies) and HBIG (Japan Red Cross Center, Tokyo, Japan) as a control. We determined the concentration of HBIG in the binding to HBsAg as same as the HB0116 with HBsAg-coated ELISA (data not shown). We evaluated the neutralizing capacity compared to that of cIgG.

2.5. Immunohistological study

Eight days after infection, infected or uninfected (control) HepaRG cells were fixed in 4% paraformaldehyde for 30 min. Cell permeabilization was ensured by treatment for 30 min with a solution of 0.1% Triton-X. The first antibody was a specific rabbit polyclonal antibody directed against the HBV core protein (Dako, Hamburg, Germany). The second antibody consisted of a fluorescein isothiocyanate (FITC)-labeled antibody directed against rabbit immunoglobulin (DAKO). 4′,6-Diamino-2-phenylindole (DAPI; Sigma) was used for nuclear staining. We quantified the FITC-fluorescence signal with NIH image software.

2.6. Statistical analysis

Statistical analyses were performed using StatView 4.02J (Abacus Concepts, Berkeley, CA). Statistical evaluations between two groups were performed using the Mann–Whitney *U* test. $P < 0.05$ indicated significance.

3. Results

3.1. Characteristics of monoclonal antibodies against HBsAg

We obtained 31 mAbs, which bound specifically to HBsAg, from B-lymphocytes or antibody-secreting cells in the peripheral blood of 4 volunteers (cases a–d, male, 30–50 y.o.) who had been vaccinated with S-HBs vaccine 7 days before blood sample collection (Jin et al., 2009; Tajiri et al., 2007; Tokimitsu et al., 2007). Five mAbs (HB0116, HB0477, HB0478, HB0905 and HB1089) were obtained from memory B-lymphocytes (Tajiri et al., 2007; Tokimitsu et al., 2007) and other 26 mAbs were from ASCs (Jin et al., 2009), because amplification of mRNA from single B-lymphocyte is superior in ASCs. We first determined the IgG subclass of the mAbs by the sequence of amino acid at the C-terminal of the mAbs (Table 1). We next investigated the subtype of the light chain (kappa or lambda), V_H or V_L gene repertoire, as well as affinities (K_D value) of the mAbs as described in Section 2 (Table 2 and Fig. 1). The majority of mAbs had a kappa chain as the L-chain (22/31, 71.0%) and were of the IgG1 subclass (20/26, 76.9%). A minority of the mAbs had a lambda chain as the L-chain (9/31, 29.0%), and belonged to

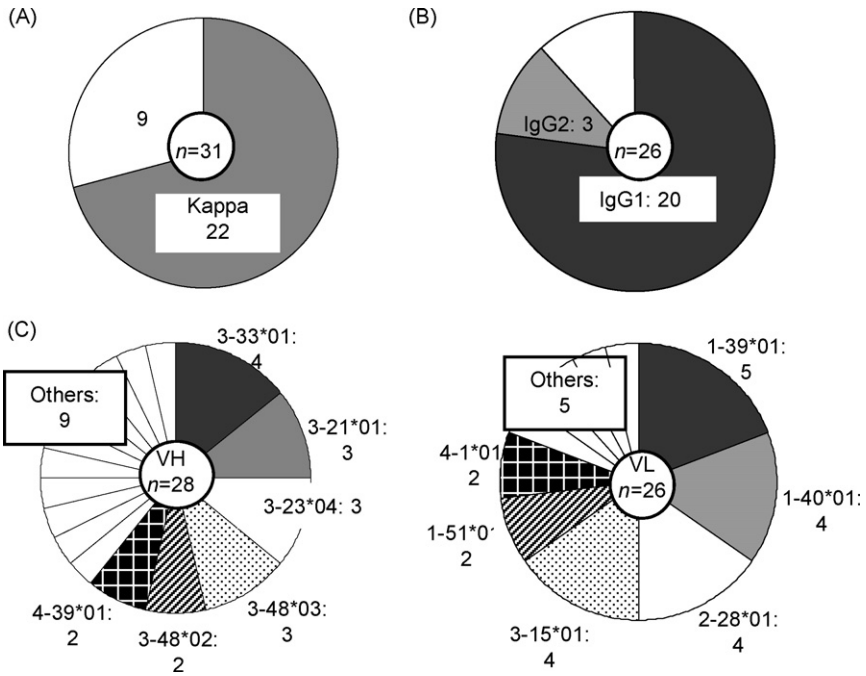


Fig. 1. Characteristics of mAbs obtained from S-HBs-vaccinated volunteers. (A) Proportion of light chain subtype of mAbs ($n=31$). (B) Proportion of IgG subclass in mAbs ($n=26$). (C) Repertoire of variable region in mAbs. The left panel shows the heavy chain variable region (V_H, $n=28$) and the right panel shows that of the light chain (V_L, $n=26$). IGMT database accession numbers are shown.

the IgG2 or IgG3 subclass (3/26, 11.5%). None of the mAbs belonged to the IgG4 subclass. The mAbs showed dissociation constant (K_D) values ranging from 10^{-6} to 10^{-10} (Table 2). With regard to the variable region repertoire, we could not find the significant repertoire (Table 2 and Fig. 1(C)). Collectively, the majority of the antibodies produced against HBsAg in vaccinated individuals were IgG1/kappa using various V genes with various affinities, playing a critical role in prophylaxis against HBV infection.

3.2. Binding sites of each HBsAg-specific mAb

To examine the binding site of each HBsAg-specific mAb using the peptide mapping method, we synthesized peptides covering the extracellular region of S-HBs (Fig. 2(A)). As the extracellular loop domain of S-HBs, known as the "a" determinant region, is an essential antigenic epitope for human B-lymphocytes (Carman, 1997; Stirk et al., 1992), we synthesized peptides with the appropriate conformational structures. The extracellular domain of S-HBs contains eight preserved cysteines and 2 disulfide bonds between 121 and 124 or 124 and 137 together with 139 and 148 establish the conformational structure (Carman, 1997; Qiu et al., 1996). Taking this conformational structure into consideration, we synthesized recombinant peptides corresponding to the extracellular domain of S-HBs, as shown in Fig. 2. We synthesized peptides L1–L4 with a linear structure and peptides C1–C3 with disulfide bonds to maintain the conformational structure.

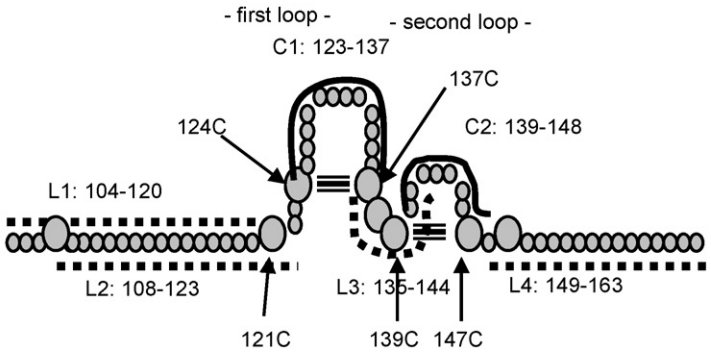
We then assessed the binding activity of each mAb to the peptides by ELISA. Representative binding activities to C1, C2, and L4 peptides with various concentrations of 6 different mAbs are shown in Fig. 3(A), and the binding sites of all the mAbs tested are shown in Fig. 3(B) and (C). As summarized in Fig. 3(C), 38.7% (12/31) of the mAbs bound to the first loop domain (C1: aa 123–137), and 29.0% (9/31) of the mAbs bound to the second loop domain (C2: aa 139–148). Thirteen percent (4/31) of the mAbs bound to the non-loop domain, and we could not determine binding of the remaining mAbs. These data suggest that the majority of mAbs against HBsAg bind to the extracellular domain of S-HBs, which would be responsible for their HBV-neutralizing activities.

3.3. HBV-neutralizing activities of each of the mAbs

First, we evaluated the HepaRG neutralization system using human mAbs; HB0116, HB0478, and HBW6 that had been reported to show neutralization activities for HBV in vivo (Sawada et al., 1995; Tokimitsu et al., 2007), and cIgG and HB0477 that have been shown to have no neutralization activities (Table 3) (Tokimitsu et al., 2007). We preincubated HBV with each of the mAbs or control IgG prior to infection of HepaRG cells, and quantified HBV-DNA in the cell lysates as well as HBsAg in the supernatant after 2–7 days in culture. As shown in Fig. 4(A) and (B), the levels of HBV-DNA in cell lysates and HBsAg in the culture supernatants increased gradually from day 2 after infection with HBV that had been pretreated with cIgG or HB0477. However, pretreatment of HBV with HB0116, HB0478, and HBW6 significantly inhibited the increases in intracellular HBV-DNA or HBsAg in the supernatant. We could not find an increase in HBV-DNA or HBsAg in both groups treated with effective mAbs and cIgG or non-effective mAb, until day 14 after infection. HBV attachment or entry to target cells can be well reflected in HepaRG cells assay whereas HBV replication may be less reflected in HepaRG as previously reported (Gripon et al., 2002; Lucifora et al., 2010). We also confirmed the neutralization activity of HB0116 using immunohistochemical methods. HBV pretreated with cIgG, HB0477 or HB0116 was used to infect HepaRG cells, and the cells were then stained with anti-HB core protein Ab after 7 days in culture. As shown in Fig. 4(C), treatment of HBV with HB0116 mAb inhibited infection of HepaRG cells. These results indicated that the HepaRG system is useful to analyze HBV-neutralizing capacity in vitro.

After evaluation of the HepaRG system as an in vitro HBV-neutralization assay, we determined the HBV-neutralizing capacities of each of 31 mAbs against HBsAg using HepaRG cells. The results indicated that 76.2% (16/21) of mAbs that bound to the

(A)



(B)

	a.a	sequence	structure
L1	104-120	NH2-LPVCPLLPGTSTTSTGP-K-biotin-CONH2	linear
L2	108-123	NH2-PLLPGTSTTSTGPCKT-K-biotin-CONH2	linear
L3	135-144	NH2-PSCCCTKPSD-K-biotin-CONH2	linear
L4	149-163	NH2-CIPIPSSWAFARFLW-K-biotin-CONH2	linear
C1	123-137	NH2-TCTIPAQGTSMFPSC-K-biotin-CONH2	cyclized
C2	139-148	NH2-CTKPSDGNCT-K-biotin-CONH2	cyclized
C3	120-136	NH2-PCKTCTIPAQGTSMFPS-K-biotin-CONH2	cyclized
C1m	120-137	NH2-P**A**KTCTIPAQGTSMFPSC-K-biotin-CONH2	cyclized

Cyclized peptide with disulfide linkage is underlined. In C1m, cystein at 121 were mutated to alanine (bold) to evaluate the effect of CKTC motifs.

Fig. 2. Synthesized peptide covering extracellular domain of small-HBsAg. (A) Conformational structure of the extracellular domain in S-HBs. The extracellular domain of S-HBs contains eight preserved cysteines (large circles). Small circles show amino acids other than cysteine. The triple lines indicate the proposed disulfide bonds. Dotted lines represent linear synthesized peptides (L1–L4), whereas bold lines indicate cyclized synthesized peptides (C1–C3). (B) Amino acid sequences of each of the synthesized peptides. C1m is the synthesized cyclized peptide with amino acid mutation 121C to A.

first and second loops of HBsAg showed HBV-neutralizing capacities, while only 20.0% (2/10) of mAbs that bound to non-loop regions showed such activities (Fig. 4(D) and Table 3). We next compared the levels of neutralization capacities of mAbs binding to the first loop, the second loop, and the non-loop regions. The mAbs that bound to the first and second loops showed significantly higher neutralization capacities than those binding to the non-loop regions (Fig. 4(E)). We also examined the relationship between the neutralization capacities and affinities of each mAb to HBsAg. As shown in Fig. 4(F), no such relationships were found ($R^2 = 0.167$). As for the subclass or repertoire of IgG, we could not find the difference in the neutralizing capacity due to the small number. These observations implied that the neutralizing capacitiy of mAbs toward HBsAg depends mainly on the epitopes to which each of the mAbs binds, but not their affinities. The results also demonstrated that both the first and second loops of S-HBs may play key roles in infection of hepatocytes by HBV.

3.4. Synergistic effects of combinations of two mAbs directed toward the 1st loop domain on HBV-neutralizing activities

We then examined the effects of combination of mAbs on their HBV-neutralizing activities using the HepaRG system. We pretreated HBV with combinations of each of the mAbs and determined their neutralization activities. In several cases, the mAb combinations showed increased neutralizing effects (data not shown), but a marked increase in HBV-neutralizing capacity was observed when HB0116 and HB0478, both of which bind to the first loop domain, were combined (Fig. 5(A) and (B)). Importantly, the neutralizing capacity was much greater than that of HBIG at the same concentration to bind to HBsAg. We confirmed the synergistic effect of the neutralization capacity using immunohistochemical methods. As shown in Fig. 5(C) and (D), we observed a marked decrease in HB core protein with the combination of HB0116 and HB0478 compared to HB0116 alone. These observations indicated that two mAbs directed to the epitopes within the first loop of S-HBs show a synergistic neutralizing effect on HBV infection. This was unexpected as we initially presumed that a combination of one mAb that binds to the first loop and another that binds to the second loop would show a synergistic effect on HBV-neutralizing capacity.

To further analyze the mechanism underlying the synergistic effect on neutralization capacity, we examined the precise locations of the epitopes bound by the mAbs. As the amino acid motif between 121C and 124C is important as an antigenic epitope for HBV (Alves Vianna et al., 2006; Chen et al., 1996; Qiu et al., 1996), we synthesized peptides with an amino acid change from 121C to A in the first loop (120–137, C1m). We also prepared a peptide without the disulfide bond between cysteines 124 and 137 (C3, 120–136), and then determined the binding activities of HB0116 and HB0478 to C1, C1m, and C3 peptides. As shown in Fig. 5(E), both HB0116 and HB0478 bound to C1 and C1m peptide, but not to C3 peptide. We found that the binding of HB0116 to C1m was not significantly less than that to C1, while HB0478 showed significantly less binding to C1m than to C1. These results imply that both HB0116 and HB0478 bind to the first loop containing a disulfide bond between cysteines124 and 137, and the binding of HB0478 is more dependent on the KTC motif of the first loop (aa 122–124) than that of HB0116. Thus, we concluded that the extracellular domain of S-HBs, especially the first loop domain (aa 122–137), plays a key role in infection of hepatocytes by HBV, and that a combination of different mAbs that recognize adjacent epitopes of the KTC motif of the first loop domain somehow exhibits a synergistic HBV neutralization effect, probably due to the structure of the KTC motif of the first loop (aa 122–124).

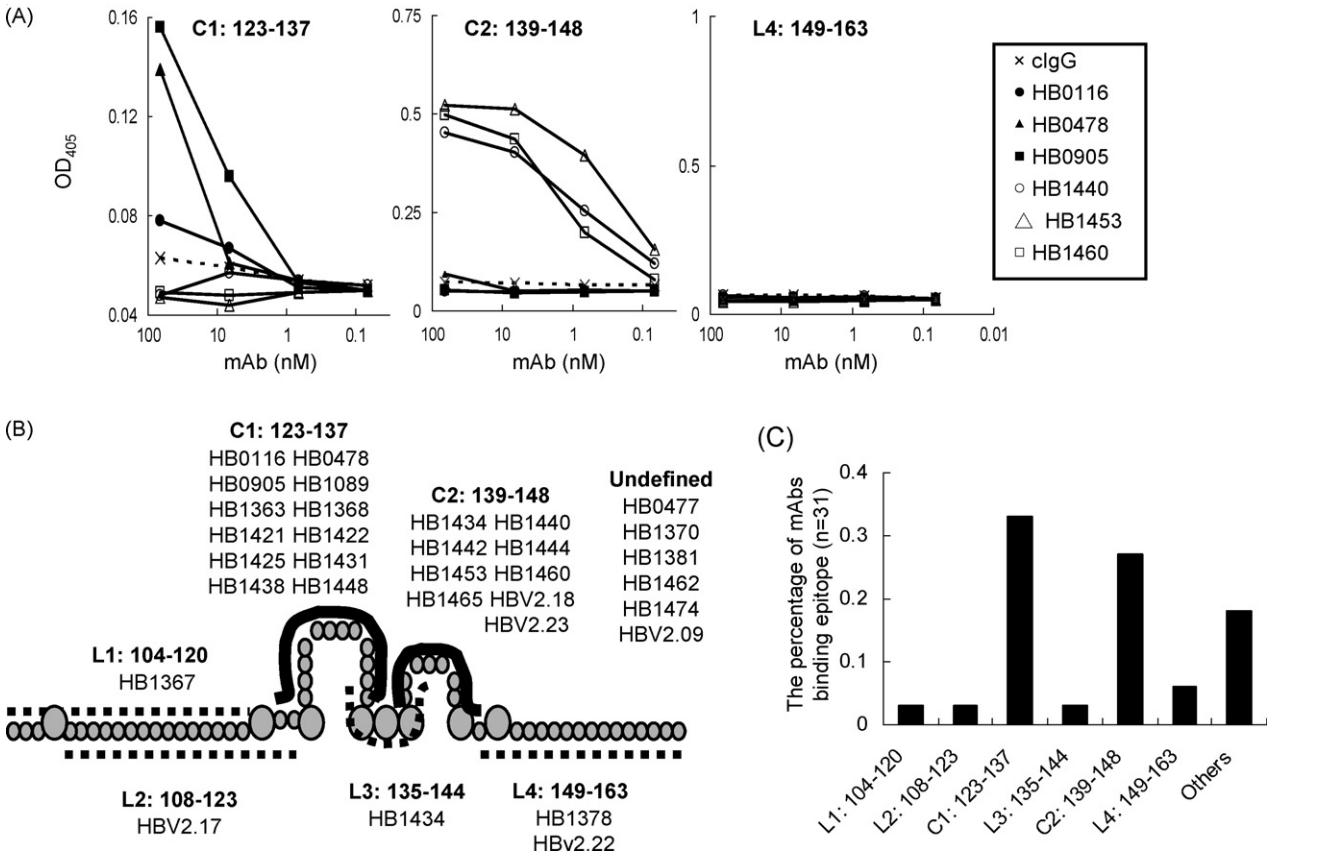


Fig. 3. The binding of each mAb to the synthesized peptides of S-HBs. (A) Representative data of binding of mAbs to synthesized peptide, C1 (123–137), C2 (139–148), and L4 (149–163). The data shown are averages of three independent experiments. The *Y*-axis shows the OD at 405 nm. The *X*-axis shows the concentrations of the applied mAbs. (B) Summary of mAb binding to each of the synthesized peptides of S-HBs. (C) Proportion of mAbs binding to each peptide. *Y*-axis shows the proportion of each mAb binding epitope.

4. Discussion

This study was performed to determine the characteristics of anti-HBV mAbs obtained from peripheral blood memory B-lymphocytes or ASCs in peripheral blood of HBsAg-vaccinated volunteers. The results showed that the majority of the anti-HBV mAbs were IgG1-kappa and bound to the extracellular loop domain of S-HBs. They exhibited higher HBV-neutralizing capacities than those that bound to the extracellular non-loop domain, and there were no correlations between their affinity and neutralization capacity. Importantly, two different anti-HBV mAbs directed against the first loop of S-HBs showed a synergistic effect on HBV neutralization that surpassed the neutralizing effect of HBIG in the HepaRG cell system. These observations regarding the efficient production of mAbs from human B-lymphocytes in peripheral blood of vaccinated volunteers or patients suffering from infectious diseases may not only contribute to our understanding of the mechanism by which antibody-mediated immunity prevents infection of the host, but may also facilitate rapid and efficient drug-discovery of mAbs to prevent and treat various infectious diseases, including AIDS, pandemic influenza, bioterrorism, *etc*.

HBV has envelope proteins consisting of the S-HBs, middle-HBsAg (M-HBs), and large-HBsAg (L-HBs). The S-HBs are the major components of the HBV envelope, while L-HBs and M-HBs are present in very small amounts in the HBV envelope (Glebe and Urban, 2007; Seeger and Mason, 2000). Although there is no doubt that the envelope protein of HBV plays an essential role in entry of HBV into hepatocytes, the precise mechanism of HBV entry into hepatocytes, especially the role of S-HBs in HBV infection, remains unresolved. Sequence 2–48 of the preS1 region (L-HBs) was reported to play a certain role in HBV entry into hepatocytes (Gripon et al., 2002; Le Seyec et al., 1998; Neurath et al., 1986). Furthermore, recent analysis using hepatitis delta virus suggested that amino acid sequence around Cys-121 is essential for the antigenic loop formation and hepadnavirus entry (Abou-Jaoudé and Sureau, 2005, 2007). In the present study, we demonstrated that human mAbs for S-HBs, especially those directed toward the first loop domains, efficiently inhibit HBV infection. Our data also suggested that the loop domains of S-HBs play an essential role in entry of HBV into human hepatocytes, although we could not determine the binding site on hepatocytes. HBV vaccination using S-HBs is now widely used in clinical settings, and our results provide new insights into the mechanism of prevention of HBV infection with S-HBs vaccination.

Next, we examined how the antibodies against S-HBs are induced and the mechanism of their preventive effect against HBV infection in humans. We examined the subclass of mAbs elicited by HBsAg vaccination and showed firstly at the single B-lymphocyte level that the predominant subclass produced by HBsAg vaccination in humans is IgG1 (Fig. 1). This finding is in accordance with previous reports using serum (Borzì et al., 1992; Huang et al., 2006) indicating that the IgG1 subclass is predominantly found in the serum of HB-vaccinated subjects and infected patients. We then determined the epitopes of the mAbs produced by HBsAg vaccination using synthesized peptides, and showed that 70% of the mAbs bound to the extracellular loop domain in S-HBs, generally called the “a” determinant, while the rest bound to the non-loop region. With regard to the epitopes of mAbs in S-HBs, the conformation involving the eight cysteines preserved between different genotypes should be considered (Carman, 1997; Stirk et al., 1992; Vyas

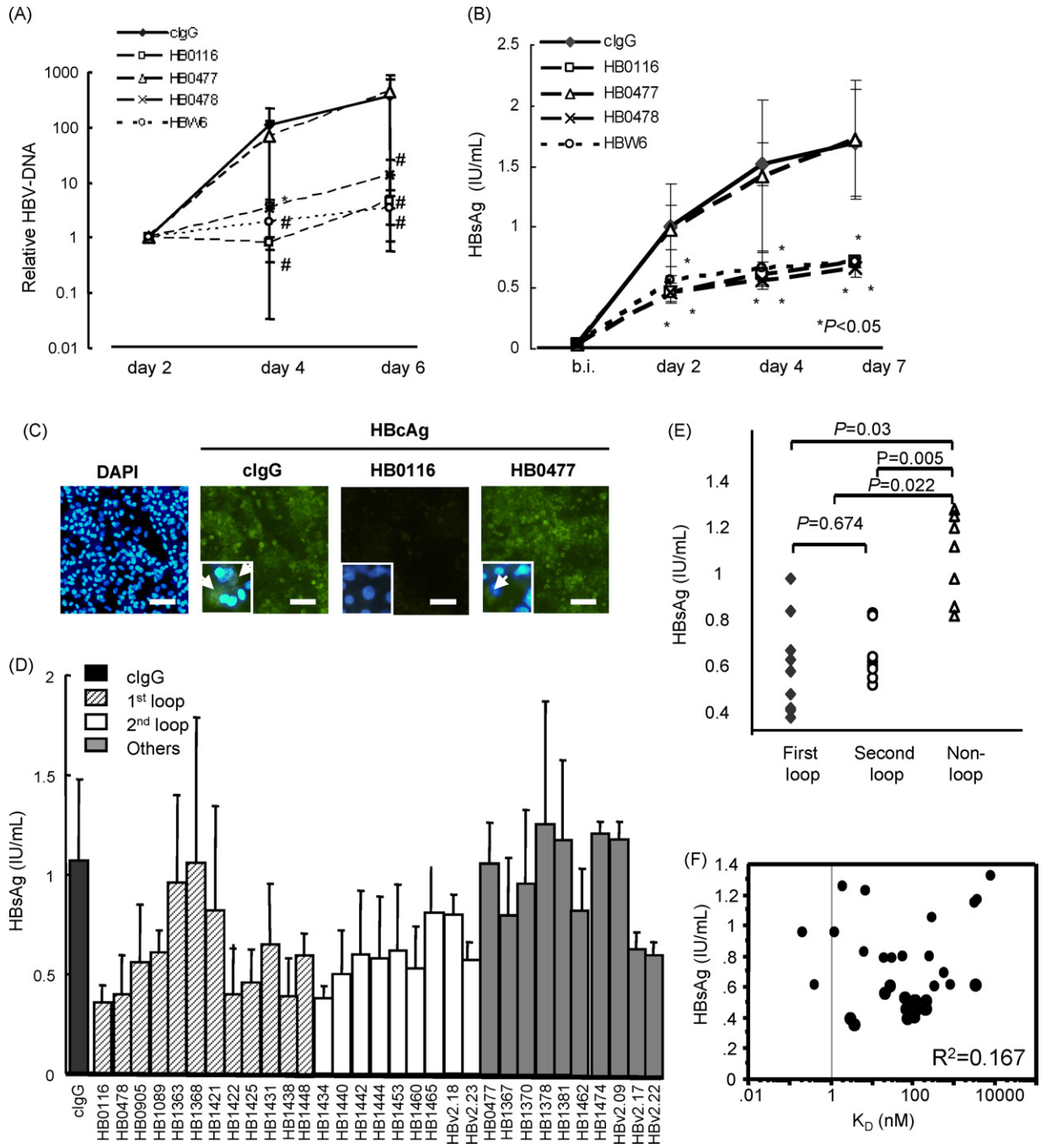


Fig. 4. HBV-neutralizing activities evaluated using HepaRG cells. (A and B) Quantification of HBV-DNA in HepaRG cells or HBsAg in the supernatant of HepaRG on before infection (b.i.) and various days after infection with HBV pretreated with mAbs. In part (A), the *Y*-axis shows that the relative HBV-DNA load as HBV-DNA level at day 2 is 1. In part (B), the *Y*-axis shows the concentrations of HBsAg. Means ± SD of three independent experiments are shown. (C) Immunohistochemical study with anti-HB core (HBc) protein antibody. HepaRG cells were infected with HBV pretreated with control human monoclonal IgG1 (cIgG), HB0116 or HB0477 mAbs and cells were stained with anti-HBc antibody after 7 days in culture. DAPI was used for nuclear staining. The bar in each panel represents 100 μm. Representative data of three independent experiments are shown. Merged images at higher magnification are shown in the left lower corner of each panel. White arrow means infected cells. (D) HBV-neutralizing capacities of each mAb. HBsAg levels in the supernatant of HepaRG on day 4 after infection with HBV pretreated with each of the mAbs were assessed. The black bar indicates the level of HBsAg with cIgG. Dashed bars, open bars, and gray bars indicate the levels of HBsAg with mAbs that bound to the first loop, the second loop, and the non-loop regions, respectively. Means ± SD of three independent experiments are shown. (E) Relationship between HBV-neutralizing activity and the antibody epitope. Epitopes were divided into three groups: loop 1 (aa 123–137, ♦), loop 2 (139–148, ○), and others (△). (F) Relationship between antibody affinity and HBV-neutralizing capacity. One dot indicates the level of HBsAg in culture supernatant and the mAb affinity. The *X*-axis indicates the affinity of antibody (K_D), whereas the *Y*-axis shows the HBsAg level at day 4 post-infection. Lower HBsAg level represents higher HBV-neutralizing capacity.

et al., 1972). Located from amino acid 101 to 159 in the extracellular domain in S-HBs is a major hydrophilic region, and residues 121 to 147 are especially important for the binding of antibodies to S-HBs (Carman, 1997; Mangold and Streeck, 1993; Stirk et al., 1992). Residues 121–124 are especially important for the epitope of B-cells (Alves Vianna et al., 2006; Chen et al., 1996; Qiu et al., 1996; Sa'adu et al., 1991; Waters et al., 1992). However, these previous studies were performed using animal-derived mAbs or B-cells immortalized with Epstein-Barr virus. Our data support the basic concept that the conformational structure of the loop is essential as the

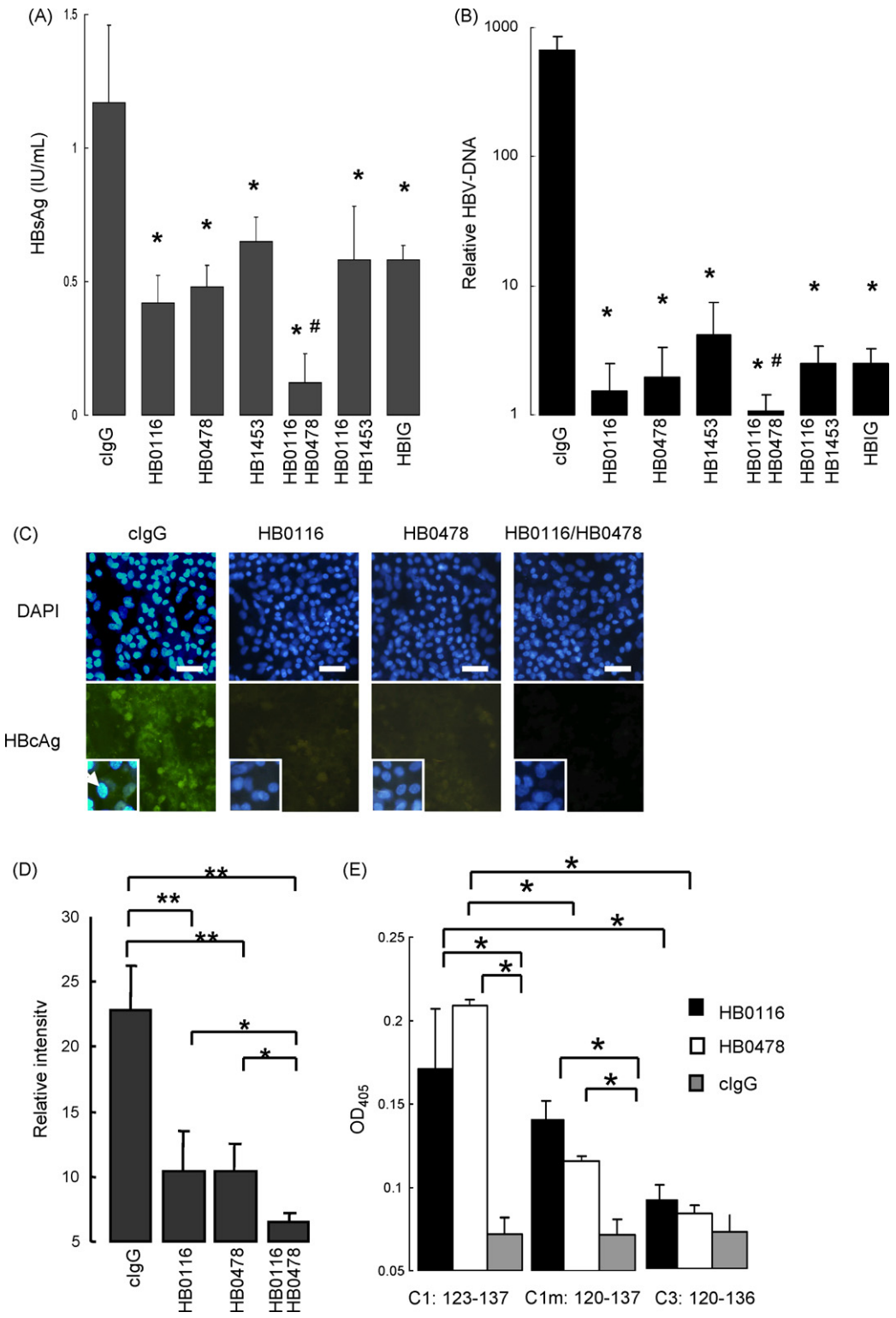


Fig. 5. Synergistic effects of mAbs on HBV-neutralizing capacity. (A) Level of HBsAg in the supernatant of HBV-infected HepaRG cells treated with mAbs. HepaRG cells were cultured for 4 days with HBV pretreated with each of the mAbs alone or various mAbs combinations. Means ± SD of three independent experiments are shown. Statistical significance between cIgG and mAbs was set as $^{*}P < 0.05$. $^{\#}P < 0.05$ in comparison with HB0116. (B) Quantification of HBV-DNA in HepaRG cells at day 7 after infection with HBV pretreated with mAbs or their combination. The *Y*-axis shows that the relative HBV-DNA load as HBV-DNA level at day 2 is 1. Statistical significance between cIgG and mAbs was set as $^{*}P < 0.05$. $^{\#}P < 0.05$ in comparison with HBIG. (C) Immunohistochemical study with HB0116 and a combination of HB0116 and HB0478. In part (C), the upper three panels show DAPI staining and the lower three panels show the results of staining for HB core protein with FITC-labeled anti-HBc antibody. The bar in each panel represents 100 μm. Merged images at higher magnification are shown in left lower corner of each panel. White arrow means infected cells. Representative data of three independent experiments are shown. (D) Relative quantitative intensity of HB core protein expression determined using NIH Image software. Pixel intensity in the same area of each mAb was quantified. $^{**}P < 0.01$, $^{*}P < 0.05$. (E) Binding of representative mAbs to modified first loop domain peptides assessed by ELISA. The binding of mAbs to C1 (aa 123–137), C1m (aa 120–137; mutation of 121C to A), and C3 (aa 120–136; CKTC motif without disulfide bond between C124 and C137). Average values of three independent experiments are shown. The *Y*-axis shows the OD at 405 nm.

Table 3
The epitope and neutralizing capacity of mAbs.

mAb	Epitope	HBV-neutralizing capacity
HB1367	L1: 104–120	–
HBV2.17	L2: 108–123	+
HB0116	C1: 123–137	++[a]
HB0478	C1: 123–137	++[a]
HB0905	C1: 123–137	+
HB1089	C1: 123–137	+
HB1363	C1: 123–137	–
HB1368	C1: 123–137	–
HB1421	C1: 123–137	–
HB1422	C1: 123–137, C3: 120–136	++
HB1425	C1: 123–137	++
HB1431	C1: 123–137	+
HB1438	C1: 123–137	++
HB1448	C1: 123–137	+
HB1434	L3: 135–144, C2: 139–148	++
HB1440	C2: 139–148	+
HB1442	C2: 139–148	+
HB1444	C2: 139–148	+
HB1453	C2: 139–148	+
HB1460	C2: 139–148	+
HB1465	C2: 139–148	–
HBV2.18	C2: 139–148	–
HBV2.23	C2: 139–148	+
HB1378	L4: 149–163	–
HBV2.22	L4: 149–163	+
HB0477	Undefined	–[b]
HB1370	Undefined	–
HB1381	Undefined	–
HB1462	Undefined	–
HB1474	Undefined	–
HBV2.09	Undefined	–

We evaluated the HBV-neutralizing capacities as the difference of HBsAg in the supernatant at day 4 post-infection from that with cIgG. –, $P > 0.05$; +, $P < 0.05$; ++, $P < 0.01$.

[a] These mAbs also showed HBV-neutralizing ability in chimeric mice study.

[b] This mAb did not neutralize HBV in chimeric mice study (Tokimitsu et al., 2007).

mAbs HB0116 and HB0478 bound to C1 peptides (aa 123–137), but not C3 peptide (aa 120–136 without the disulfide bond between C124 and C137) (Fig. 5(E)). This was confirmed using mAbs that bound to the C1 peptide (Fig. 3(B)) and found that none of them, except for HB1422, bound to C3 (data not shown).

The present study provided new insight regarding the efficacy of neutralization of human mAbs against HBV. First, although it has been reported that mAbs with high affinity have more potent HBV-neutralizing activity compared to those with low affinities in animal models or using humanized antibodies (Maynard et al., 2002; Wu et al., 1998), our data clearly demonstrated that HBV-neutralizing activity is more dependent on the epitope of the antibodies rather than their affinities (Figs. 2–4 and Table 3). Second, we have demonstrated that a combination of mAbs for the first loop domain showed more efficient HBV-neutralizing capacities than those for different loop domains (Fig. 5). It has been reported that combination therapy with two mAbs is effective for the prevention of HBV infection (Eren et al., 2000; Galun et al., 2002), although the mechanism was not verified. Our data indicate that different mAbs that recognize adjacent epitopes of the KTC motif of the first loop domain exhibit synergistic HBV-neutralizing capacities, probably because of the KTC motif structure of the first loop. Furthermore, our data demonstrated that the neutralization capacity of the combination of two mAbs exceed that of conventional HBIG, suggesting that a combination of human mAbs that bind to the functional epitopes in the viral entry may be substituted for HBIG in the prevention and therapy of HBV infection in future.

In conclusion, we showed that IgG1 with kappa chain is induced and confers protection against HBV with S-HBs vaccination in human subjects. The first loop domain of the "a" determinant region in S-HBs may be essential for HBV infection, and the neutralization activity is more dependent on the epitopes of mAbs than their affinities. Further investigation of functional epitopes in the first loop domain of S-HBs may lead to the development of efficient anti-HBV antibody therapeutics capable of replacing HBIG.

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References

Abou-Jaoudé, G., Sureau, C., 2005. Role of the antigenic loop of the hepatitis B virus envelope proteins in infectivity of hepatitis delta virus. J. Virol. 79, 10460–10466.

Abou-Jaoudé, G., Sureau, C., 2007. Entry of hepatitis delta virus requires the conserved cysteine residues of the hepatitis B virus envelope protein antigenic loop and is blocked by inhibitors of thiol-disulfide exchange. J. Virol. 81, 13057–13066.

Alter, M.J., 2003. Epidemiology and prevention of hepatitis B. Semin. Liver Dis. 23, 39–46.

Alves Vianna, C.O., da Silva, E., Mouta Júnior, S., da Glória Teixeira Martins, M., Batoreu, N.M., Queiroz, J.L., Gomes, S.A., Magalhães de Andrade Góes, A.C., Garcia Armoa, G.R., Marques, C.H., Baroni de Moraes, M.T., 2006. Evaluation of murine monoclonal antibodies targeting different epitopes of the hepatitis B virus surface antigen by using immunological as well as molecular biology and biochemical approaches. J. Immunol. Methods 313, 38–47.

Beasley, R.P., Hwang, L.Y., Stevens, C.E., Lin, C.C., Hsieh, F.J., Wang, K.Y., Sun, T.S., Szmuness, W., 1983. Efficacy of hepatitis B immune globulin for prevention of perinatal transmission of the hepatitis B virus carrier state: final report of a randomized double-blind, placebo-controlled trial. Hepatology 3, 135–141.

Borzì, R.M., Dal Monte, P., Honorati, M.C., Facchini, A., 1992. IgG subclass distribution of anti-HBs antibodies following vaccination with cDNA HBsAg. J. Immunol. Methods 146, 17–23.

Carman, W.F., 1997. The clinical significance of surface antigen variants of hepatitis B virus. J. Viral Hepat. 4 (Suppl. 1), 11–20.

Cerec, V., Glaise, D., Garnier, D., Morosan, S., Turlin, B., Drenou, B., Gripon, P., Kremsdorf, D., Guguen-Guillouzo, C., Corlu, A., 2007. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. Hepatology 45, 957–967.

Chayama, K., Suzuki, Y., Kobayashi, M., Kobayashi, M., Tsubota, A., Hashimoto, M., Miyano, Y., Koike, H., Kobayashi, M., Koida, I., Arase, Y., Saitoh, S., Murashima, N., Ikeda, K., Kumada, H., 1998. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology 27, 1711–1716.

Chen, Y.C., Delbrook, K., Dealwis, C., Mimms, L., Mushahwar, I.K., Mandecki, W., 1996. Discontinuous epitopes of hepatitis B surface antigen derived from a filamentous phage peptide library. Proc. Natl. Acad. Sci. U.S.A. 93, 1997–2001.

Eren, R., Ilan, E., Nussbaum, O., Lubin, I., Terkieltaub, D., Arazi, Y., Ben-Moshe, O., Kitchinzky, A., Berr, S., Gopher, J., Zauberman, A., Galun, E., Shouval, D., Daudi, N., Eid, A., Jurim, O., Magnius, L.O., Hammas, B., Reisner, Y., Dagan, S., 2000. Preclinical evaluation of two human anti-hepatitis B virus (HBV) monoclonal antibodies in the HBV-trimera mouse model and in HBV chronic carrier chimpanzees. Hepatology 32, 588–596.

Galun, E., Eren, R., Safadi, R., Ashour, Y., Terrault, N., Keeffe, E.B., Matot, E., Mizrachi, S., Terkieltaub, D., Zohar, M., Lubin, I., Gopher, J., Shouval, D., Dagan, S., 2002. Clinical evaluation (phase I) of a combination of two human monoclonal antibodies to HBV: safety and antiviral properties. Hepatology 35, 673–679.

Ganem, D., Prince, A.M., 2004. Hepatitis B virus infection – natural history and clinical consequences. N. Engl. J. Med. 350, 1118–1129.

Glebe, D., Urban, S., 2007. Viral and cellular determinants involved in hepadnaviral entry. World J. Gastroenterol. 13, 22–38.

Gripon, P., Rumin, S., Urban, S., Le Seyec, J., Glaise, D., Cannie, I., Guyomard, C., Lucas, J., Trepo, C., Guguen-Guillouzo, C., 2002. Infection of a human hepatoma cell line by hepatitis B virus. Proc. Natl. Acad. Sci. U.S.A. 99, 15655–15660.

Huang, C.F., Lin, S.S., Ho, Y.C., Chen, F.L., Yang, C.C., 2006. The immune response induced by hepatitis B virus principal antigens. Cell. Mol. Immunol. 3, 97–106.

Jin, A., Ozawa, T., Tajiri, K., Obata, T., Kondo, S., Kinoshita, K., Kadowaki, S., Takahashi, K., Sugiyama, T., Kishi, H., Muraguchi, A., 2009. A rapid and efficient single-cell manipulation method for screening antigen-specific antibody-secreting cells from human peripheral blood. Nat. Med. 9, 1088–1092.

Kim, S.H., Shin, Y.W., Hong, K.W., Chang, K.H., Ryoo, K.H., Paik, S.H., Kim, J.M., Brotman, B., Pfähler, W., Prince, A.M., 2008. Neutralization of hepatitis B virus (HBV) by human monoclonal antibody against HBV surface antigen (HBsAg) in chimpanzees. Antiviral Res. 79, 188–191.

Le Seyec, J., Chouteau, P., Cannie, I., Guguen-Guillouzo, C., Gripon, P., 1998. Role of the pre-S2 domain of the large envelope protein in hepatitis B virus assembly and infectivity. J. Virol. 72, 5573–5578.
Lucifora, J., Durantel, D., Testoni, B., Hantz, O., Levrero, M., Zoulim, F., 2010. Control of hepatitis B virus replication by innate response of HepaRG cells. Hepatology 51, 63–72.
Mangold, C.M., Streeck, R.E., 1993. Mutational analysis of the cysteine residues in the hepatitis B virus small envelope protein. J. Virol. 67, 4588–4597.
Maynard, J.A., Maassen, C.B., Leppla, S.H., Brasky, K., Patterson, J.L., Iverson, B.L., Georgiou, G., 2002. Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. Nat. Biotechnol. 20, 597–601.
Neurath, A.R., Kent, S.B., Strick, N., Parker, K., 1986. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 46, 429–436.
Ozawa, T., Kishi, H., Muraguchi, A., 2006. Amplification and analysis of cDNA generated from a single cell by 5′-RACE: application to isolation of antibody heavy and light chain variable gene sequences from single B cells. Biotechniques 40, 469–470, 472, 474.
Qiu, X., Schroeder, P., Bridon, D., 1996. Identification and characterization of a C(K/R)TC motif as a common epitope present in all subtypes of hepatitis B surface antigen. J. Immunol. 156, 3350–3356.
Sa'adu, A., Bidwell, D., Locniskar, M., Howard, C., McAdam, K.P., Voller, A., 1991. Epitope mapping of HBsAg using a panel of human anti-HBs antibodies. J. Virol. Methods 35, 199–206.
Sawada, H., Iwasa, S., Nishimura, O., Kitano, K., 1995. Efficient production of anti-(hepatitis B virus) antibodies and their neutralizing activity in chimpanzees. Appl. Microbiol. Biotechnol. 43, 445–451.
Seeger, C., Mason, W.S., 2000. Hepatitis B virus biology. Microbiol. Mol. Biol. Rev. 64, 51–68.
Shin, Y.W., Ryoo, K.H., Hong, K.W., Chang, K.H., Choi, J.S., So, M., Kim, P.K., Park, J.Y., Bong, K.T., Kim, S.H., 2007. Human monoclonal antibody against hepatitis B virus surface antigen (HBsAg). Antiviral Res. 75, 113–120.
Stirk, H.J., Thornton, J.M., Howard, C.R., 1992. A topological model for hepatitis B surface antigen. Intervirology 33, 148–158.
Tajiri, K., Kishi, H., Tokimitsu, Y., Kondo, S., Ozawa, T., Kinoshita, K., Jin, A., Kadowaki, S., Sugiyama, T., Muraguchi, A., 2007. Cell-microarray analysis of antigen-specific B-cells: single cell analysis of antigen receptor expression and specificity. Cytometry A 71, 961–967.
The EASL Jury, 2003. EASL International Consensus Conference on Hepatitis B. Consensus Statement (short version), 13–14 September 2002, Geneva, Switzerland. J. Hepatol. 38, 533–540.
Tokimitsu, Y., Kishi, H., Kondo, S., Honda, R., Tajiri, K., Motoki, K., Ozawa, T., Kadowaki, S., Obata, T., Fujiki, S., Tateno, C., Takaishi, H., Chayama, K., Yoshizato, K., Tamiya, E., Sugiyama, T., Muraguchi, A., 2007. Single lymphocyte analysis with a microwell array chip. Cytometry A 71, 1003–1010.
Vyas, G.N., Rao, K.R., Ibrahim, A.B., 1972. Australia antigen (hepatitis B antigen): a conformational antigen dependent on disulfide bonds. Science 178, 1300–1301.
Waters, J.A., Brown, S.E., Steward, M.W., Howard, C.R., Thomas, H.C., 1992. Analysis of the antigenic epitopes of hepatitis B surface antigen involved in the induction of a protective antibody response. Virus Res. 22, 1–12.
Wu, H., Beuerlein, G., Nie, Y., Smith, H., Lee, B.A., Hensler, M., Huse, W.D., Watkins, J.D., 1998. Stepwise in vitro affinity maturation of Vitaxin, an alphav beta3-specific humanized mAb. Proc. Natl. Acad. Sci. U.S.A. 95, 6037–6042.