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DONOR INFECTIOUS DISEASE TESTING

TRANSFUSION

Development of a recombinant hepatitis B immunoglobulin derived from B cells collected from healthy individuals administered with hepatitis B virus vaccines: A feasibility study

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Abstract

Background: In Japan, plasma with a high concentration of Hepatitis B Virus (HBV) antibodies for hepatitis B immunoglobulin (HBIG) is almost entirely imported. We aimed to produce recombinant HBIG by isolating immunoglobulin cDNAs against the HBV surface antigen (HBsAg).

Study Design and Methods: B cells expressing HBsAg antibodies were obtained from blood center personnel who had been administered HB vaccine booster and then isolated by either an Epstein–Barr virus hybridoma or an antigen-specific memory B cell sorting method. Each cDNA of the heavy and light chains of the target antibody was cloned into an IgG_1 expression vector and transfected into Expi293F cells to produce a recombinant monoclonal antibody (mAb), which was screened by ELISA and in vitro HBV neutralizing assays. The cross-reactivity of the mAbs to normal human molecules was evaluated by ELISA and immunohistochemistry.

Results: Antibody cDNAs were cloned from 11 hybridoma cell lines and 204 HBsAg-bound memory B cells. Three of the resulting recombinant mAbs showed stronger neutralizing activity in vitro than the currently used HBIG. All three bind to the conformational epitope(s) of HBsAg but not to human DNA or cells.

Discussion: We successfully isolated HBV-neutralizing monoclonal antibodies from B cells collected from healthy plasma donors boosted against the HBV. To obtain an alternative source for HBIG, HBV-neutralizing monoclonal

Abbreviations: EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; HBIG, hepatitis B immunoglobulin; HBsAb, antibody against HBsAg; HBsAg, HBV surface antigen; HBV, Hepatitis B virus; IHC, Immunohistochemistry; JRC, Japanese Red Cross; mAb, monoclonal antibody; PCR, polymerase chain reaction; vge, viral genome equivalents.

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antibodies from B cells collected from healthy plasma donors boosted against the HBV may be useful.

KEYWORDS
antibody drug, hepatitis B virus, human monoclonal antibody, neutralizing antibody

1 | INTRODUCTION

Hepatitis B immunoglobulin (HBIG) is prepared from human plasma containing a high concentration of antibodies (HBsAb) to the hepatitis B surface antigen (HBsAg). It was first developed in the 1970s for passive immunization against infection by the hepatitis B virus (HBV).[1,2] Anti-virals, such as nucleos(t)ide analogs, are widely used as a treatment for HBV-related diseases and as prophylactics against HBV reactivation,[3,4] while HBIG is used to prevent mother-to-child transmission and for post-exposure prophylaxis.[5–7] In Japan, HBIG is also approved for use as a prophylactic agent against HBV reactivation during liver transplantations related to HBV infection, where either the recipients or donors are positive for HBV.

The HB-vaccinated adult population in Japan had been limited to mainly health care or other essential workers with occupational infection risk. Therefore, the candidate population for plasma donors for HBIG has remained very small, which has led to the importation of nearly all plasma required for HBIG production. To solve this plasma shortage problem, we aimed to isolate monoclonal antibodies (mAbs) against HBsAg to develop recombinant HBIG as an alternative to imported plasma for HBIG production.

Through work done in Japanese Red Cross (JRC) blood centers, we have developed test reagents consisting of mouse and human mAbs that recognize red cell alloantigens.[8,9] This previous work was accomplished partly due to a fast and inexpensive large-scale screening method, the hemagglutination assay, which we applied in this study in combination with a conventional Epstein–Barr virus (EBV) hybridoma technique, to establish hybridoma-producing mAbs against HBsAg. We also adopted an antigen-specific single memory B cell sorting method that has been widely used in recent years to isolate antibody-producing B cells to obtain neutralizing mAbs that recognize antigens of pathogenic microorganisms, including HIV-1[10,11] and SARS-CoV-2.[12,13] Here, we describe the characteristics of mAbs that we isolated using either method and discuss some technical and practical issues that we encountered during the development of mAbs in blood centers.

2 | MATERIALS AND METHODS

2.1 | Blood collection and ethics statement

A national HB vaccine boosting program was undertaken in 2013 by the Ministry of Health, Labor, and Welfare in Japan, with the aim of raising the amount of plasma for HBIG production. Among the participants were three staff members working at the JRC Kanto-Koushinetsu and Kinki Block Blood Centers (KTKS-BBC and KK-BBC, respectively), who had previously received the HB vaccine and acquired humoral immunity against HBsAg. We collected 30 mL of whole blood from these participants at 35 (donor 1), 23 (donor 2), and 6 and 28 (donor 3) days after receiving boosters for either the Heptavax II (genotype A, MSD, NJ) or Bimmugen (genotype C, KM Biologics, Kumamoto, Japan) vaccines. The records of the vaccines administered for the first time were unavailable for these donors. Donors 1 and 2 are KTKS-BBC staff, and hybridomas producing HBsAb were established from their blood at that facility. Donor 3 was a KK-BBC staff member, and IgG genes of HBsAb were isolated by single-cell sorting at the facility. This study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Ethics Review Board of the Blood Service Headquarters, JRC (#2015–004-5 and #2018–012-3). All participants provided written informed consent for the sample collection and subsequent analyses.

2.2 | HBIG

In Japan, JRC does not manufacture plasma fractionated products, and two domestic manufacturers produce HBIG from donated plasma from JRC and imported plasma from overseas. The production method is based on ethanol fractionation, but details have not been disclosed. The HBIG used as a control in this study was an approved dry HBIG drug (DRIED HB GLOBULIN for IM injection 1000 units NICHIYAKU, Takeda Pharmaceutical Company Limited, Osaka, Japan). The concentration

of protein/immunoglobulin in the dissolved HBIG as 200 Unit/mL, an administration concentration to patients, was approximately 25 mg/mL, which slightly varied from lot to lot.

2.3 | Hybridoma cell lines

Peripheral blood mononuclear cells (PBMC) were isolated by standard density gradient centrifugation, transformed by the EBV, and hybridized with the myeloma cell line JMS-3.[14] Detailed protocol to establish hybridomas was described in the Appendix S1. Automated chemiluminescence tests using ARCHITECT i2000SR Immunoassay Analyzer (Abbott Laboratories, IL) confirmed the secretion of HBsAb from each hybridoma.

2.4 | Antigen-specific single memory B cell sorting

B cells were purified from the donor's whole blood using a magnetic selection method (MACSxpress B cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation with recombinant HBsAg (HBsAg-XT, Beacle Inc., Kyoto, Japan) at a concentration of 200 ng/μL for 30 min on ice, the cells were washed twice with PBS containing 1% bovine serum albumin (BSA, Merck Millipore, MA) and 0.1% sodium azide and stained with anti-HBs polyclonal rabbit antibody (Beacle Inc.), anti-rabbit IgG-Alexa488 (Jackson ImmunoResearch Laboratories, PA), anti-human IgG-APC, anti-CD27-PE, anti-CD19-ACP-Cy7, 7-AAD (BD Bioscience, NJ), and anti-CD38-PE-Cy7 (BioLegend, CA). All antibodies were titrated in advance and used at optimal concentrations for flow cytometry and cell sorting (BD FACSAria, BD Biosciences, NJ). HBsAg-bound IgG+ memory B cells gated in CD19+, CD27 hi, CD38 lo-neg, human IgG+, HBsAb+, and 7AAD-neg were sorted into 96-well PCR plates containing 8 μL/well of reverse transcription (RT) reaction buffer that included 2 μL of 5× buffer, 4 μL of 2 mM dNTP, 0.1 μL of 40 U/μL RNase inhibitor (ReverTra Ace, TOYOBO, Osaka, Japan), 1 μL of 2.5% NP-40, 0.2 μL of 10 μM RT primer, and 0.7 μL dH_2O. The plates with the sorted cells were stored at −80°C until RT-PCR.

2.5 | cDNA cloning of immunoglobulin

After single-cell sorting, the cDNA of immunoglobulin heavy- and light-chain variable regions (V_H and V_L) and corresponding light-chain constant regions (C_κ and C_λ) were amplified by RT-nested PCR using a modification of a previously reported method.[15] The detailed information of IgG cloning is described in the Appendix S1.

2.6 | Expression and purification of recombinant mAbs

Expi293F Expression System (Thermo Fisher Scientific, MA) was used for the recombinant IgG_1 expression, following the manufacturer's instructions. Seven days after transfection, the culture supernatants were harvested by centrifugation at 1500 *g* for 10 min and 3000 *g* for 30 min. After filtration through a 0.22-μm filter, the supernatant was directly applied to an affinity column for IgG (HiTrap Protein G HP column, Cytiva, Tokyo, Japan), gel-filtered (PD-10 desalting column, Cytiva), and concentrated by ultrafiltration (Amicon Ultra, Merck Millipore).

2.7 | ELISA

The concentration of each mAb to HBsAg was examined using antigen sandwich ELISA (Enzygnost Anti-HBs II, SIEMENS, München, Germany) according to the manufacturer's instructions. Binding of mAbs to HBsAg (HBsAg-XT, Beacle Inc.), human insulin (Fujifilm Wako Pure Chemical Corporation), and human genomic DNA extracted from PBMCs was also examined using an in-house ELISA (Antigen-Down ELISA Development Kit, ImmunoChemistry Technologies, MN) in accordance with the kit instructions. Briefly, the plates were coated with 1 μg/mL of each target molecule. After blocking the plates with a blocking buffer (Cat #651, ImmunoChemistry Technologies) at room temperature overnight, the mAb was incubated for 1 h at 37°C, washed with a washing buffer (Cat #63, ImmunoChemistry Technologies), and incubated with 1:6000 diluted HRP-conjugated anti-human IgG antibody (Jackson ImmunoResearch Laboratories). The optical density (OD) of the substrate (3,3′,5,5′-tetramethylbenzidine) solution was measured at a wavelength of 450 nm with a reference wavelength of 650 nm.

2.8 | Western blotting

One microgram of HBsAg (HBsAg-XT, Beacle Inc.) was loaded in a lane of either a 3–12% Bis-Tris native-PAGE

(Thermo Fisher Scientific) or 10% SDS-PAGE (Thermo Fisher Scientific) gel with or without 50 mM dithiothreitol (DTT). The proteins were transferred onto a polyvinylidene difluoride membrane, probed with 1 μg/mL (native-PAGE) or 2 μg/mL (SDS-PAGE) of each mAb, and detected with 1:10,000 diluted HRP-conjugated anti-human IgG (H+L) (Thermo Fisher Scientific). Anti-HBsAg horse polyclonal antibody (pAb) and rabbit anti-horse IgG H&L pAb were used as positive controls (ab9193 and ab6921; Abcam, Cambridge, UK).

2.9 | In vitro HBV neutralization assay

HBV neutralization activity was screened in human hepatocellular carcinoma-derived HepG2 cells overexpressing human sodium taurocholate cotransporting polypeptide (HepG2-hNTCP-C4).[16] HepG2-hNTCP-C4 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, MO), supplemented with 10% fetal bovine serum (FBS; Sigma), 0.1 mM sodium pyruvate, and MEM non-essential amino acids (Gibco, MD), at 37°C in a 5% CO_2 humidified incubator. Cells (2×10^5 cells per well) were seeded in 24-well plates 1 day before infection and infected at 1000 viral genome equivalents (vge) /cell of HBV (genotype D) in a culture medium containing 2% dimethyl sulfoxide (DMSO) and 4% PEG 8000, which had been preincubated with each mAb at either 1, 0.1, or 0.01 μg/mL for 30 min at 37°C. Two hours after inoculation, the cells were washed three times with DMEM and cultured in the growth medium containing each mAb at each concentration. The medium with each mAb was replaced every 2 or 3 days. Fourteen days after infection, the relaxed form (rc) DNA of HBV in the infected cells was quantified by PCR using conditions described in the Appendix S1.

The mAbs were also examined in primary human hepatocytes (PXB cells) that were derived from chimeric mice with hepatocyte-humanized livers.[17,18] The cells were maintained in DMEM supplemented with 2% FBS, 20 mM HEPES, 44 mM $NaHCO_3$, 15 μg/mL L-proline, 0.25 μg/mL insulin, 50 nM dexamethasone, 5 ng/mL epidermal growth factor (Merck Millipore), 0.1 mM L-ascorbic acid, and 2% DMSO (DMSO-supplemented hepatocyte clonal growth medium, dHCGM). The neutralization assay was conducted in a 24-well plate containing 4.0×10^5 PXB cells per well. Before infection, 2.0×10^6 vge HBV (genotype C) were preincubated with one of the three mAbs or HBIG at three concentrations of 2, 0.2, and 0.02 μg/mL in dHCGM, respectively, at 37°C for 30 min. To inoculate HBV, the culture medium of cells in each well was replaced with the preincubation mixture. Two hours after inoculation at 37°C, the cells were washed three times with dHCGM and cultured for 3 weeks with medium changes every 3 or 4 days in the presence of each mAb at a concentration of 2, 0.2. or 0.02 μg/mL. HBV infection was monitored by quantifying the HBV e antigen (HBeAg) in the culture medium with a commercial ELISA (UickTiterTM Hepatitis B "e" Antigen ELISA Kit, CELL BIOLABS, CA).

2.10 | Immunohistochemistry (IHC)

Paraffin arrays of HBV human liver cancer and liver tissue (LV1401, US Biomax, MD) and normal human tissue arrays (T8234708-5, BioChain, CA) were rehydrated using a series of xylene and ethanol, followed by inactivation of endogenous catalase activity by incubation with 3% H_2O_2 for 15 min. Arrays were blocked with 5% BSA in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) at 4°C overnight, and then they were incubated with 10 μg/mL mAb mixture in 1% BSA in TBS-T at 37°C for 45 min. After washing with TBS-T, the arrays were incubated with 1:2500 diluted HRP-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories) at room temperature for 30 min. After washing with TBS-T, the arrays were visualized by staining with diaminobenzidine (ImmPACT DAB; VETOR Laboratories, CA) and counterstaining with Meyer's hematoxylin (Thermo Fisher Scientific) and captured using a CCD camera mounted on a microscope and its imaging software (BX53 and cellSense, Olympus, Tokyo, Japan). Organs on the normal human tissue array examined were listed in the Appendix S1.

2.11 | Statistics

Curve fitting (nonlinear regression) of dose–response data in ELISA was processed using Prism version 8 (GraphPad Software, CA).

3 | RESULTS

In experiments to establish hybridoma, the plasma HBsAb concentrations of donors 1 and 2 during blood collection were 5489 and 7906 IU/L, respectively (Table 1). After selection, we obtained 249 hybridoma cell populations from B cells collected from donors 1 and 2. After screening for cell populations that secreted antibodies against HBsAg (HBsAb) and clonal selection, we established 11 HBsAb-positive cell lines (Table 1). Sequencing analysis revealed that the antibodies produced by all these hybridomas belonged to the IgG_1

TABLE 1 Characteristics of EBV-hybridoma lines established from two donors in JRC KTKS-BBC.

Donor ID	Duration between boosting and blood sampling (days)	HBsAb concentration in plasma (IU/L)[a]	The number of HBsAb (+) Hybridoma culture well	Established clone ID	Subclass of IgG	Light chain	HBsAb concentration in hybridoma culture (IU/L)[a]
1	35	5489	38	5A4	IgG_1	Kappa	626
				3D1	IgG_1	Lambda	951
2	23	7906	211	3B6	IgG_1	Kappa	44,445
				4H7	IgG_1	Kappa	41,079
				2F6	IgG_1	Kappa	275
				4G1	IgG_1	Kappa	285
				2G8	IgG_1	Kappa	219
				4B12	IgG_1	Kappa	165
				1G2	IgG_1	Kappa	23
				3F4	IgG_1	Kappa	420
				3C2	IgG_1	Kappa	887

[a]Measured by ARCHITECT i2000SR immunoassay.

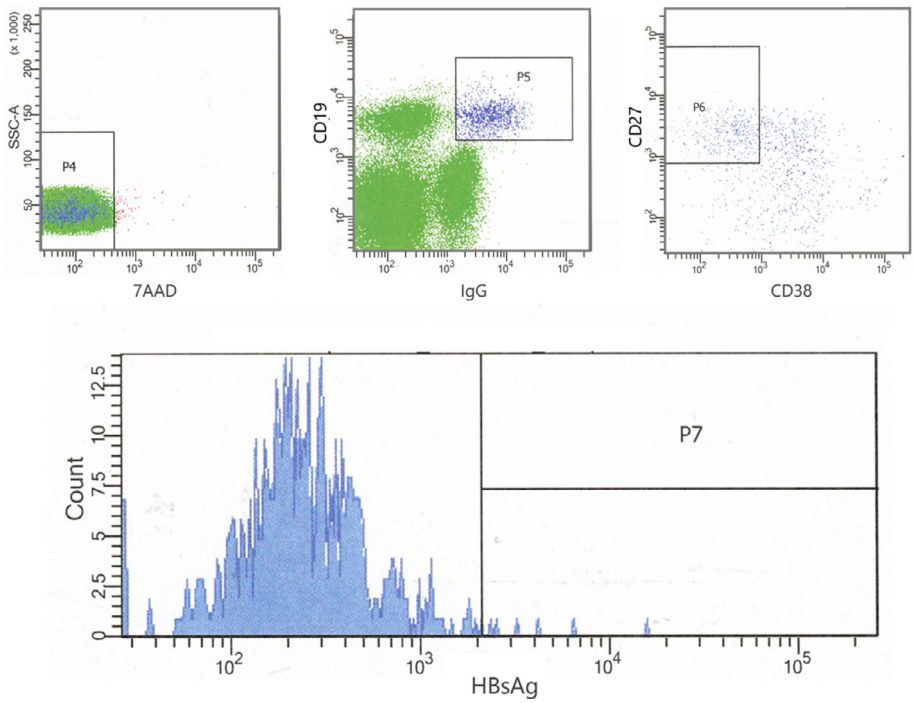


FIGURE 1 Antigen-specific single memory B cell sorting. B cells purified from whole blood collected from an HB vaccine boosted donor 3 were incubated with Hepatitis B Virus surface antigen (HBsAg) and stained for cell surface markers. To isolate viable IgG-switched memory B cells, 7AAD-negative fractions were gated as live cells, in which CD19-positive and IgG expressing on their surface were gated as IgG class-switched cells, in which CD27-positive CD38-negative fraction was considered as IgG-switched memory B cells. Among them, we selected cells with a high signal of HBsAg binding (fraction P7).

subclass. The corresponding light chains of all but one clone were kappa chains. In donor 2, the sequences of variable regions were identical in two (clone ID: 3B6 and 4H7) and five (clone ID: 2F6, 4G1, 2G8, 4B12, and 1G2) out of nine clones, suggesting that these clone groups were derived from B cells of the same chronotype. Thus, we subcloned six unique cDNAs from two donors into an IgG_1 expression vector and transiently transfected them into a cell line to produce recombinant mAbs.

In the antigen-specific single memory B cell sorting method, the immunogenicity of recombinant HBsAg is enhanced by heat denaturation followed by gradual cooling to form virus-like particles (VLPs, also referred to as subviral particles),[19–21] before cell sorting. B cells were purified from the whole blood of donor 3 collected 7 days and 4 weeks after HBV vaccine boosting. The plasma HBsAb concentration of donor 3 was 4812 IU/L 7 days after boosting. The cells were incubated with VLP-induced

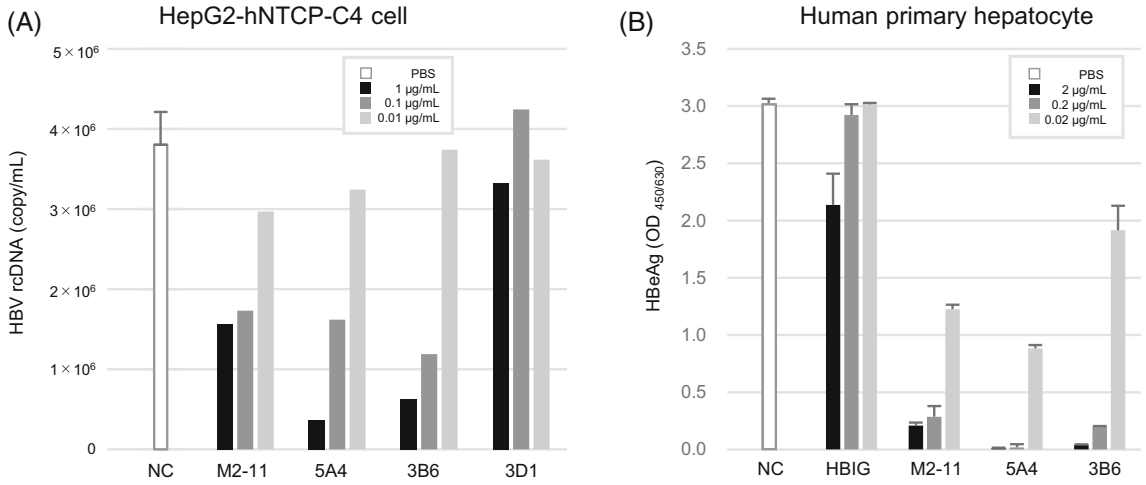
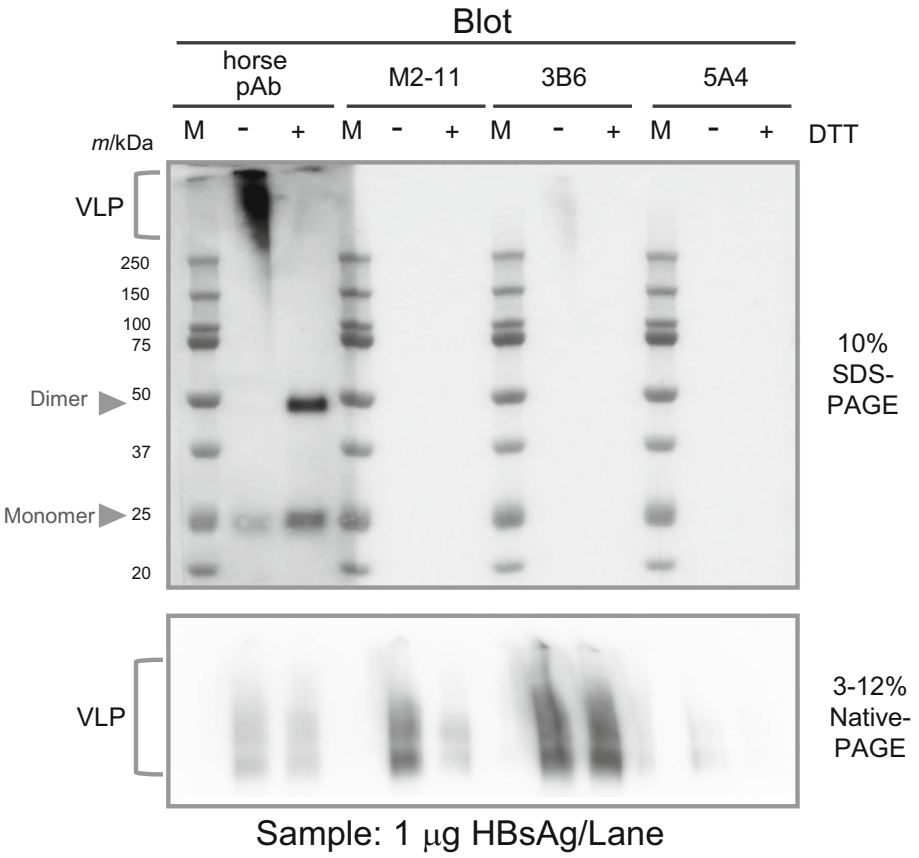


FIGURE 2 Neutralizing activities of mAbs. (A) HepG2-hNTCP-C4 cells were infected with HBV (genotype D) at 1000 vge/cell in the presence of each mAb (or PBS as a control) at a concentration indicated in the inset box. Fourteen days after infection, the relaxed form (rc) DNA of HBV in the infected cells was quantified by qPCR. The neutralization assay with this cell line was performed twice independently under the same conditions with comparable results. The first result was shown here as representative of the results. NC (PBS) data represents a mean and standard deviation of the results of cells in three wells infected without mAb. (B) A primary human hepatocyte (PXB cells) was infected triplicated with HBV (genotype C) at 5 vge/cell in the presence of each mAb or HBIG at a concentration indicated in the inset box. After 3 weeks, HBeAg in the culture supernatant was measured by ELISA. Bar; standard deviation. NC; negative control (PBS), M2-11; mAb isolated from donor 3 by the antigen-specific single memory B cell sorting methods, 5A4 and 3D1; mAbs isolated from donor 1 by EBV-hybridoma method, 3B6; mAb isolated from donor 2 by EBV-hybridoma method, HBIG; hepatitis B immunoglobulin.

FIGURE 3 Characterization of binding profiles of mAbs. One microgram of heat-denatured and refolded HBsAg was separated by SDS-PAGE (upper panel) or native-PAGE (lower panel), with or without 50 mM dithiothreitol (DTT). The proteins were transferred to polyvinylidene difluoride membranes that were cut into strips and blotted with the indicated antibodies. M: size marker (Precision Plus Protein Standards, Bio-Rad Laboratories, Inc., CA), VLP: virus-like particle.



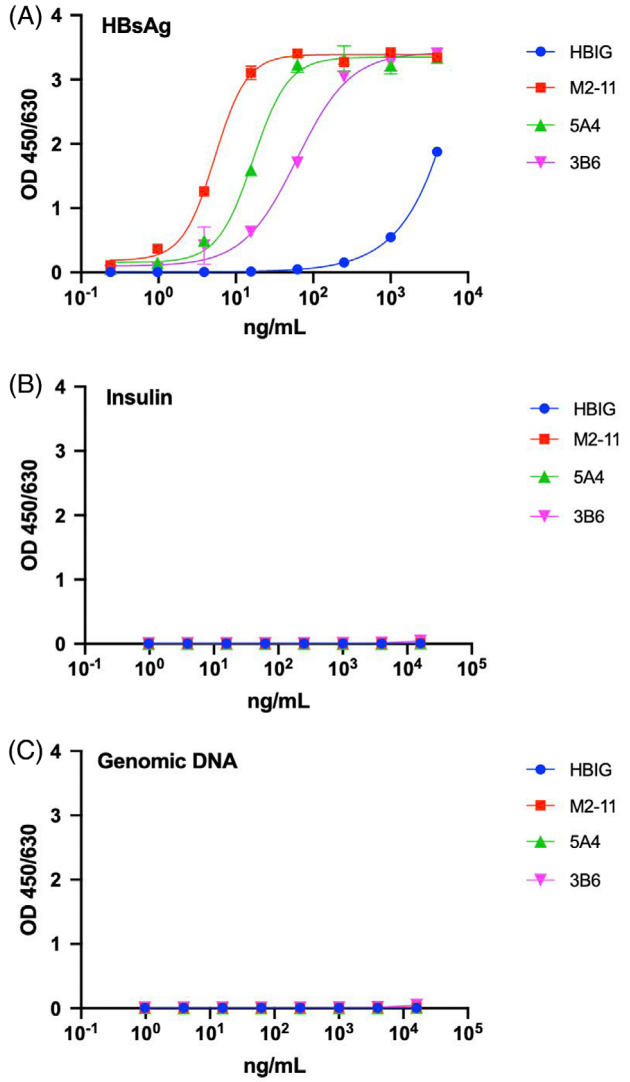


FIGURE 4 Binding specificities of mAbs determined by ELISA. A 96-well plate was coated with the indicated molecules (A, HBsAg; B, human insulin; C, human genomic DNA) at a concentration of 1 μg/mL. Serially diluted mAbs or HBIG were added to each well, incubated for 1 h, and then antigen binding was detected using a horseradish peroxidase-conjugated anti-human antibody.

HBsAg and stained by the surface markers of IgG-switched memory B cells. We sorted 282 cells, or 0.16% of the IgG-class-switched memory B cells, as binding to the VLP-induced HBsAg (fraction P7 in Figure 1). Among them, we isolated 204 cDNA pairs of heavy and light chains of IgG, which we subcloned into the same expression vector used in the EBV-hybridoma method.

During ELISA screening of the recombinant mAbs collected from culture supernatants of cells transiently transfected with the vectors, we identified three clones isolated from the EBV-hybridoma (clone ID: 5A4, 3B6, and 3D1) and one clone from single-cell sorting (clone ID: M2-11 isolated from a B cell collected 4 weeks after HBV vaccine boosting) that tested strongly positive (>3.0 of $OD_{450/630}$). We determined the neutralizing activities of these clones by in vitro HBV neutralization assays using two different cell cultures. In a human hepatoma cell line overexpressing NPCT2 (one of the receptors for HBV),[22,23] three clones (M2-11, 5A4, and 3B6) of the four mAbs inhibited HBV replication even at a high virus load of 1000 vge/cell (Figure 2A). Clone 3D1 showed no neutralizing activity in this experiment and was not analyzed further. In human primary hepatocytes, these three mAbs potently inhibited HBV replication, even at the lowest concentration investigated. This potency was superior to the HBIG currently used in Japan; however, it must be noted that a quantitative comparison of monoclonal reagents to polyclonal immunoglobulin (with unknown concentration of HBV-neutralizing antibodies) is limited (Figure 2B).

To determine the epitope(s) recognized by these three mAbs, the refolded HBsAg was separated by SDS- or native-PAGE under reducing or non-reducing conditions and then blotted with each of these mAbs (Figure 3). The control (a horse pAb against HBsAg) under reducing conditions detected two predominant forms of HBsAg of the expected sizes, which most likely correspond to monomers and dimers.[24] Meanwhile, under non-reducing conditions, the same pAb mainly bound to HBsAg at a higher molecular mass position in SDS-PAGE, which is likely a VLP that is composed of HBsAgs that form multiple intra- and intermolecular disulfide bonds.[25,26] The VLP form of HBsAg was also detected by pAb under both reducing and non-reducing conditions in native-PAGE. Western blots using our mAbs revealed no binding to either monomers or dimers in SDS-PAGE. The mAbs M2-11 and 5A4 preferentially bound to VLPs in non-reducing native-PAGE, whereas 3B6 bound to VLPs to the same degree in native-PAGE under both reducing and non-reducing conditions. The 3B6 clone also bound slightly to VLPs on non-reducing SDS-PAGE. These results strongly suggest that the three mAbs bind to some conformational epitopes and the epitope of 3B6 differs from that of the other two.

As it is undesirable for therapeutic antibodies to bind to human components other than the target molecule, we next examined the binding of these mAbs to human molecules. ELISA revealed no binding to insulin (representing human plasma protein) or to human genomic DNA (representing autoantigens of many autoimmune diseases), even at high concentrations (Figure 4). The binding specificities of our mAbs were also revealed by IHC using paraffin arrays containing tissue collected from individuals with or without HBV infection. The mixture of our three mAbs strongly stained parts of the

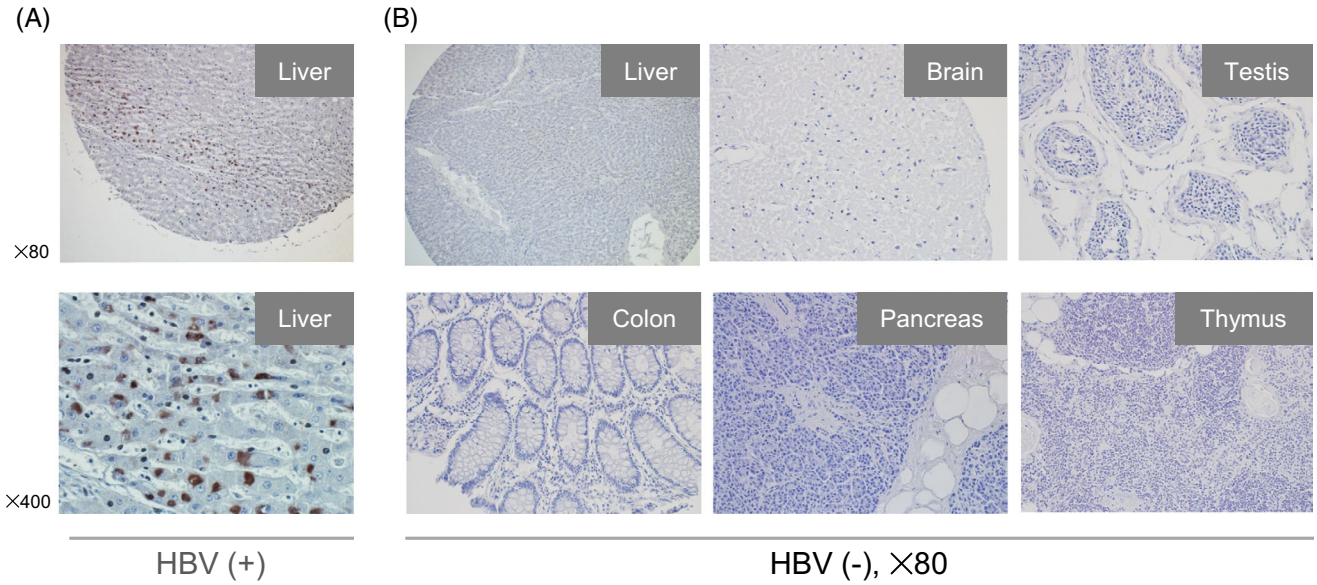


FIGURE 5 Binding specificities of mAbs determined by Immunohistochemistry (IHC). Paraffin human liver cancer and liver tissue arrays collected from HBV-infected individuals (A) and various normal tissue arrays collected from HBV-uninfected adults (B) were rehydrated and incubated with a mixture of the three mAbs (M2-11, 3B6, and 5A4 at 10 μg/mL each) at 37°C for 45 min. After washing, the arrays were incubated with 1:2500 diluted HRP-conjugated anti-human IgG. After washing, the arrays were visualized and captured after staining with diaminobenzidine (ImmPACT DAB) and counterstaining with Meyer's hematoxylin (Thermo Fisher Scientific).

TABLE 2 Isolation rates of target mAbs using two isolation methods.

	Number of						
Isolation method	**Donors**	**PHA (+) hybridoma**	**Sorted cells**	**Pairs of cloned cDNA**	**ELISA (+++)[a] Clones**	**In vitro neutral-lization (+) clones**	**Amino acid sequence of CDR3 in heavy chain**
EBV-hybridoma	2 (donor ID 1 and 2)	11	NA	6	3	2	ARGDATYGYW (Clone 5A4) AREDPAIVLPVIDSW (Clone 3B6)
Single cell sorting	1 (donor ID 3)	NA	282	204	1	1	AREQGTRRRGRYYYYGLDVW (Clone M2-11)

[a]>3.0 of $OD_{450/630}$ by enzygnost anti-HBs II.

cytoplasm of hepatocytes in HBV-infected patients (Figure 5A). In contrast, tissue arrays collected from HBV-uninfected individuals showed no staining signals in 31 organs tested, in which we showed some representative images in Figure 5B and Figure S1. These results indicate that our antibodies are unlikely to bind to non-target human molecules.

4 | DISCUSSION

The main aim of this study was to evaluate the feasibility of isolating anti-HBsAg mAbs in blood centers to ultimately develop as an alternative source of HBIG in Japan. We have identified three potential candidate mAbs obtained from three healthy donors who had been purposefully boosted with the HB vaccine for HBIG production. Repeated vaccine boosting of immunized donors is necessary to ensure a continuous supply of plasma for HBIG, for which the antibody concentration standard in Japan is 20,000 IU/L or higher. Alternatively, an mAb of interest can be isolated from the donor pool, so that it can be permanently available thereafter. Here, we isolated and characterized mAbs that satisfy the quality requirements for a virus neutralizing antibody, such as neutralizing activity and binding specificity (Figures 2–5). The neutralizing activity of our mAbs needs to be evaluated more rigorously because we only showed its

superiority over HBIG, a polyclonal antibody that contains many antibodies other than the target-neutralizing antibody. In addition, it is also necessary to evaluate whether the obtained mAbs have a sufficient neutralizing effect against drug resistance-associated mutations and vaccine escape mutations, which are an increasing concern worldwide.[27] To develop these mAbs as pharmaceuticals will require further optimization of qualities such as solubility and viscosity at high concentrations, glycosylation status, clearance rates, and others.[28–30]

The greatest advantage of using pathogen-neutralizing mAbs isolated from B cells of healthy human individuals is their unlikely cross-reactivity with normal human components or tissues as we demonstrated in this study (Figures 4 and 5). In addition, such mAbs are expected to have virtually no immunogenicity to humans. From this perspective, during the development of mAbs for medical use, isolation of mAbs from healthy individuals rather than from animals, humanized animals,[31,32] or phage display libraries[33,34] is preferable.

In this study, mAbs were isolated using the EBV-hybridoma method and the antigen-specific single memory B cell sorting method. Although both approaches proved to be successful, they were also inefficient. During the process of establishing hybridomas, antibody production from cloned cells obtained from an HBsAb-producing mixed population frequently stopped. This problem can be overcome by implementing large-scale, single-cell RNA sequencing without cell culture,[35,36] although this approach requires high-performance sequencing and large data analysis capacities. On the other hand, the single-cell sorting method should, in theory, improve the isolation efficiency of the target mAb. However, most of the recombinant mAbs produced from the immunoglobulin cDNAs isolated from memory B cells and sorted based on their binding to HBsAg tested negative in the HBsAg-sandwich ELISA. This unexpected result can be attributed to HBsAg binding to other molecules such as heparin sulfate proteoglycans[37,38] that are located on the cell surface. The antigen-specific single memory B cell method has been successfully used to isolate many neutralizing mAbs against pathogens of public concern,[39–41] however, screening recombinant antibodies cloned from each B cell still requires substantial labor and time during the evaluation process. This problem can be addressed by adopting fully automated laboratory platforms.

To avoid the emergence of pathogen mutants capable of escaping from a neutralizing mAb, the neutralizing mAb drug should be developed as a cocktail of multiple clones.[42–45] Although we obtained several immunoglobulin cDNAs from the donors, only a single clone from each donor had neutralizing activity, and the amino acid sequences of the variable regions of these clones were completely different (Table 2). These results are consistent with those of a recent study that found that the circulating IgG_1 repertoire in human plasma of healthy donors and septic patients is dominated by a limited number of clones and that the plasma IgG_1 repertoire of each donor is unique.[46] Therefore, we are more likely to raise a larger number of mAb clones against a certain antigen by screening B cells collected from a large number of individuals rather than screening a large number of B cells from a few individuals. During the drug development phase, a sufficient number of mAb clones of interest are required as starting seeds because a single mAb with the desired function often lacks the physical properties required for antibody drugs, such as a high solubility and thermal stability.

Our experiences indicate that mAb drug development in blood centers may remain challenging, particularly in the late stage of development, mainly because of the limited access to technical and financial resources. However, blood centers offer a unique advantage during the early stage of the development, i.e., they offer a large blood donor population with diverse immunological properties. If comprehensive informed consent could be obtained from blood donors regarding the use of lymphocytes as a resource for developing biologics, then it would be possible to isolate large numbers of mAbs or lymphocytes of interest from blood donors within a short period of time. Therefore, it is worth considering that the next generation of work at blood centers will include the development of mAb drugs as well as other therapeutic biologics such as T cell-derived cellular medicine.

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CONFLICT OF INTEREST STATEMENT

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

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