

Development of a Highly Sensitive Bioluminescent Enzyme Immunoassay for Hepatitis B Virus Surface Antigen Capable of Detecting Divergent Mutants

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Hepatitis B virus (HBV) infections are sometimes overlooked when using commercial kits to measure hepatitis B virus surface antigen (HBsAg) due to their low sensitivities and reactivities to mutant strains of various genotypes. We developed an ultrasensitive bioluminescent enzyme immunoassay (BLEIA) for HBsAg using firefly luciferase, which is adaptable to a variety of HBsAg mutants, by combining four monoclonal antibodies with a polyclonal antibody against HBsAg. The measurement of seroconversion panels showed trace amounts of HBsAg during the early infection phase by the BLEIA because of its high sensitivity of 5 mIU/ml. The BLEIA detected HBsAg as early as did PCR in five of seven series and from 2.1 to 9.4 days earlier than commercial immunoassay methods. During the late infection phase, the BLEIA successfully detected HBsAg even 40 days after the disappearance of HBV DNA and the emergence of antibodies against HBsAg. The HBsAg BLEIA successfully detected all 13 recombinant HBsAg and 45 types of HBsAg mutants with various mutations within amino acids 90 to 164 in the S gene product. Some specimens had higher values determined by the BLEIA than those by a commercial chemiluminescent immunoassay; this suggests that such discrepancies were caused by the dissociation of preS1/preS2 peptides from the particle surface. With its highly sensitive detection of low-titer HBsAg, including various mutants, the HBsAg BLEIA is considered to be useful for the early diagnosis and prevention of HBV infection because of the shorter window of infection prior to detection, which facilitates early prediction of recurrence in HBV-infected individuals.

The hepatitis B virus (HBV) is a causal agent for acute and chronic liver diseases, including fulminant hepatic failure, liver cirrhosis, and hepatocellular carcinoma. Since the discovery of HBV Australia antigen (Au) in 1965 (1), later named hepatitis B virus surface antigen (HBsAg), it has been detected by various methods, leading to improved diagnosis, prevention, and treatment of the disease. It is estimated that over two billion people worldwide are currently infected with HBV, of which >350 million people are chronically infected; therefore, HBV infection is a worldwide issue in public health (2–4).

HBV is a DNA virus in the family *Hepadnaviridae* and has a circular genome composed of approximately 3,200 nucleotides (nt) containing four open reading frames for the polymerase (P), preC/C, preS1/preS2/S, and X genes (5). Ten genotypes (A to J) of HBV have been identified so far, which differ by >8% over the entire genome (6–11). The HBV genome exhibits a high rate of mutations due to its reverse transcription process (12, 13), leading to the generation of a wide variety of mutations (14, 15) that may alter viral protein conformation and antigenicity. These mutations include those within the major hydrophilic region (MHR) of the S protein, including the T/I126S, T123N, C124R, Q129H, D144A, and G145R mutations (3, 16–20). It is known that some of the existing diagnostic reagents used for the detection of HBsAg cannot detect such HBsAg mutants even when a sufficient antigen concentration is present in the blood samples.

Occlude HBV infection (OBI) is characterized by the presence of very low levels of HBV DNA in the serum and/or liver with undetectable HBsAg using the most sensitive assays outside the pre-seroconversion window (21, 22). Several mechanisms have been proposed to underlie OBI, including multiple amino acid substitutions in the S protein affecting HBsAg detection with commer-

cial immunoassays (23, 24) and mutations in the HBV genomes that regulate S protein expression (22, 25–27).

Since an overlooked diagnosis of HBV infection can cause a loss of therapeutic opportunity and lead to the spread of infection, the development of a diagnostic reagent for HBsAg detection that can detect a variety of strains and overlook mutant strains less frequently is strongly desired (28–33).

Firefly luciferase luminescence is a bioluminescent system that uses a set of unique substrates and enzymes and is known to have a significant quantum yield (34, 35). Antibodies with streptavidin and thermostable biotinylated luciferase bind through an avidin-biotin interaction, allowing for the conjugation of luciferase on an antibody without decreasing the reactivity of luciferase. Some measurement systems using luciferase as a labeling enzyme have already been reported (36–38). In a previous study, we developed a measurement system for HBsAg with a high sensitivity of 10 mIU/ml on the basis of a bioluminescent enzyme immunoassay (BLEIA) using firefly luciferase with a high quantum yield (36, 37). However, this preliminary assay system failed to detect a G145R mutant due to the low reactivity of the immobilized antibodies with the mutant. Therefore, in the present study, we devel-

Received 26 March 2013 Returned for modification 5 May 2013

Accepted 5 June 2013

Published ahead of print 12 June 2013

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doi:10.1128/CVI.00186-13

TABLE 1 Detection of recombinant HBsAg with various mutations by BLEIA and CLIA1

Amino acid substitution(s)	CLIA1 (S/CO)	BLEIA (S/CO)
I126S	17	328
Q129H	21	434
M133L	21	331
D144A	20	325
G145R	19	230
I126S + G145R	20	228
P142L + G145R	16	253
P142S + G145R	16	155
D144A + G145R	19	470
R122I	18	702
C124R	19	1,049
T123N	20	3,094
122RA123 ^a	16	1,353

^a Insertion of arginine (R) and alanine (A) between aa 122 and 123.

oped an extensively improved HBsAg BLEIA as a clinically applicable measurement system for quantitative HBsAg detection with an increased sensitivity of 5 mIU/ml, which can detect even HBsAg mutants that have a wide variety of mutations within the S gene.

MATERIALS AND METHODS

Ethics statement. The present study, which used serum samples from patients with mutated HBsAg, rheumatoid arthritis or autoimmune hepatitis, and occult HBV infection as panels was approved by the ethics review committee of Jichi Medical University.

HBsAg-positive samples. The HBsAg-positive samples used for the selection of anti-HBs monoclonal and polyclonal antibodies for immobilization or enzyme labeling and for the correlation study with a commercially available kit (chemiluminescent immunoassay, called the CLIA1 in the present study) were purchased from ProMedDx (Norton, MA) and Trina Bioreactives AG (Nänikon, Switzerland). All of the HBsAg-positive samples had HBV DNA. The samples were diluted with normal human serum (Aries Diagnostika GmbH, Baden, Switzerland) to give measurement values within the measurement range. For the HBsAg samples that did not have information about its genotype listed in an attached document, the genotypes were determined by restriction fragment length polymorphism (SRL, Inc., Tokyo, Japan), an analysis of the 396-nt sequence within the S gene (39), or the 1,550-nt sequence covering nearly the entire preS1/preS2/S gene (40), or by the Immunis HBV genotype EIA (Institute of Immunology Co. Ltd., Tokyo, Japan).

HBsAg-negative samples. A total of 236 normal blood plasma samples that were prepared from citrate phosphate dextrose adenine (CPDA-1)-treated human whole blood were purchased from Tennessee Blood Services (Memphis, TN). Serum samples from 47 patients with rheumatoid arthritis and 48 patients with autoimmune hepatitis were also used as disease panel samples.

HBsAg seroconversion panels. Six series of seroconversion panels, including PHM926, PHM928, PHM929, PHM931, PHM935(A), and PHM935(B), were purchased from SeraCare Life Sciences (Milford, MA), and two series, HBV6279 and HBV6292, were purchased from Zepto-Matrix Corporation (Buffalo, NY).

Recombinant HBsAg mutants. After extraction of DNA from a genotype C HBsAg-positive blood plasma sample obtained from ProMedDx (Norton, MA), the entire S gene sequence was amplified by PCR and cloned into the pcDNA3.1 or pcDNA3.3 vector (Life Technologies, Carlsbad, CA). Using this plasmid as a template, 13 recombinant S gene sequences, representing naturally occurring mutations (Table 1), were generated using the KOD -Plus- mutagenesis kit (Toyobo, Osaka, Japan). To produce mutant HBsAg particles by transient expression, 2.5 µg of each

mutant plasmid DNA was transfected into 2×10^5 COS-7 or HEK293 cells/well in a six-well culture plate with 7 µl *TransIT-LT1* transfection reagent (Mirus, Madison, WI), and the cells were cultured at 37°C in 5% CO₂. The HBsAg particles secreted into the culture supernatant were harvested after 3 days and were stored at 4°C until use.

HBsAg mutant samples from HBV-infected patients. Our study included a total of 45 HBsAg mutant samples possessing mutations within amino acids (aa) 90 to 164 of the S gene product. All samples tested positive for HBsAg by the CLIA1. The HBV DNA quantitation and sequence analysis of the S gene region were performed according to previously described methods (41, 42).

OBI panel. Ten serum samples that were negative for HBsAg by the CLIA1 but positive for HBV DNA were used as an OBI panel. The quantitation of HBV DNA and a sequence analysis of the amplicons were performed using previously described methods (41, 42).

Anti-HBs MAbs. A total of 55 candidate anti-HBs monoclonal antibodies (MAbs) (commercially available or generated in-house) were immobilized on magnetic particles and labeled with luciferase as described below, and their reactivities with wild-type HBsAg (genotypes A to D) and a G145R mutant were evaluated. Five MAbs for labeling and four MAbs for the solid phase were selected based on this screening process. Based on

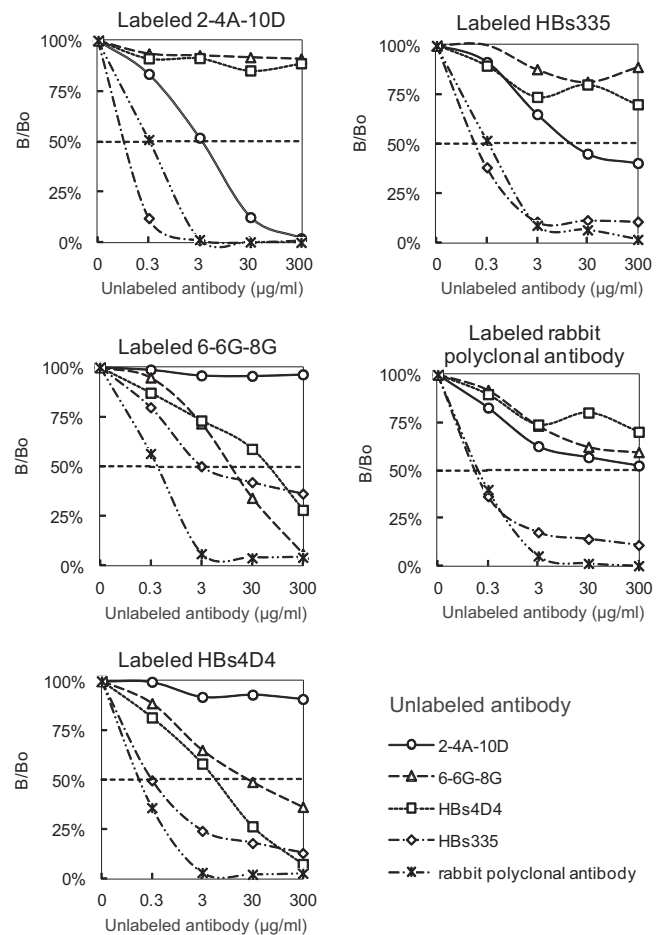


FIG 1 Characterization of the anti-HBs MAbs and the rabbit polyclonal antibody by the inhibition test. Captured HBsAg on rabbit anti-HBs polyclonal antibody-coated magnetic particles was reacted with each unlabeled antibody. Enzyme-labeled anti-HBs antibody was then added to compare the inhibition by each unlabeled antibody. The percent inhibition is shown as the B/B₀ ratio on the y axis, where B is bioluminescent intensity when unlabeled antibody is added, and B₀ is the bioluminescent intensity when unlabeled antibody is not added.

the results of inhibition tests (Fig. 1), three MABs for the solid phase and one MAB for labeling were chosen. Among the four MABs selected, two anti-HBs MABs, HBs335 and HB4D4, were obtained from the Advanced Life Science Institute, Inc. (Saitama, Japan). The other two anti-HBs MABs, 2-4A-10D and 6-6G-8G, were established by Eiken Chemical Co., Ltd. (Tokyo, Japan), following the standard method (43) and using purified HBsAg from the huGK-14 human cell line (Meiji Holdings Co., Ltd., Tokyo, Japan) as an immunogen. Subclasses of MABs were determined by an IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostics, Indianapolis, IN). The antibodies were purified using an Affi-Prep protein A MAPS II kit (Bio-Rad Laboratories, Inc., Hercules, CA).

Immobilization of anti-HBs MABs on magnetic particles. Anti-HBs MAB-coated magnetic particles were prepared using the previously described method (37), with slight modifications. In brief, 4 ml of 10% (wt/vol) magnetic particles (Dynabeads M-280 Tosyl activated; Life Technologies, Carlsbad, CA) were washed with distilled water, resuspended in 20 ml of 0.1 M carbonate buffer (pH 10.0) containing 0.15 mg/ml of anti-HBs MABs (2-4A-10D, 6-6G-8G, and HBs4D4), and incubated for 21 h with agitation. After the particles were washed with 0.1 M phosphate buffer (PB), they were stored in 50 mM 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES) (Dojin, Kumamoto, Japan) containing 0.15 M sodium chloride at 4°C until measurement. The magnetic particles used in the inhibition test were coated with 0.15 mg/ml of anti-HBs rabbit polyclonal antibodies (Biokit, Barcelona, Spain) and prepared in the same way as the MABs described above.

Preparation of the biotinylated luciferase (bL248)-streptavidin-Fab' conjugate. An enzyme-labeled anti-HBs rabbit polyclonal antibody (I.L. Japan, Tokyo, Japan) and an anti-HBs MAB, HBs335, were prepared using a previously described method (36, 37). In brief, through the reaction with maleimide-activated streptavidin (MP Biomedicals, Solon, OH), the Fab' fragments conjugated with thermostable biotinylated luciferase (bL248; Kikkoman, Chiba, Japan) were purified using Superdex 200 HR 10/30 (GE Healthcare Japan, Tokyo, Japan) and then by affinity absorption again to remove nonspecifically bound materials, using a gel coated with normal human serum (37). The conjugated anti-HBs rabbit polyclonal antibody and anti-HBs MAB solution were mixed to give equal immunoreactivity, and the mixture was stored at 4°C until measurements were performed.

Development of the fully automated BLEIA for detecting HBsAg. The HBsAg BLEIA was based on the utility of firefly luciferase as a labeling enzyme and a two-step sandwich immunoassay procedure. The measurement of HBsAg by this method was performed with a fully automated bioluminescent enzyme immunoassay analyzer, the BLEIA-1200, developed by Eiken Chemical Co., Ltd., which used 100 μ l of reaction buffer 1 (0.05 M PB, 0.25% bovine serum albumin [BSA], 0.1% bovine gamma globulin [BGG], 0.05% sodium azide, 20% rabbit serum, 10% mouse serum [pH 7.2]), 100 μ l of sample, and 30 μ l of anti-HBs antibody-coated magnetic particles, which were mixed and incubated at 37°C for 15 min. After washing with 0.5 ml of BLEIA wash buffer (0.05% Tween 20 in Tris-HCl [pH 7.2]), 80 μ l of the labeled antibody dilution solution (0.05 M PB, 0.5% BSA, 20% rabbit serum, 10% mouse serum [pH 7.2]) and 40 μ l of enzyme-labeled anti-HBs were added, and the samples were incubated at 37°C for 15 min. After another wash, 50 μ l of magnetic particle suspension buffer (0.1 M Tris-HCl, 0.02 M magnesium sulfate [pH 8.6]) and 50 μ l of BLEIA substrate solution were added and mixed. The bioluminescent intensity from the luciferin-luciferase reaction was measured. The HBsAg concentrations were calculated based on a standard curve drawn prior to the measurement using serial dilutions of purified HBsAg (Meiji Holdings) whose concentrations were determined by the WHO 2nd International Standard for HBsAg (National Institute for Biological Standards and Control [NIBSC] code 00/588) expressed as international units (IU)/ml.

Inhibition test for classifying epitopes on antibodies. Twenty-five microliters of anti-HBs rabbit IgG-coated magnetic particles was added to 75 μ l of HBsAg-positive plasma (genotype C, from ProMedDx) that had

been diluted to 1 IU/ml with normal human serum, and the mixture was incubated for 15 min at 37°C. After the particles were washed four times with BLEIA wash buffer, they were suspended in 25 μ l of a solution with unlabeled anti-HBs MAB or a rabbit polyclonal antibody (0.3 to 300 μ g/ml each), or a solution without antibody as a control to check if the unlabeled antibodies competed against labeled antibodies and inhibited the binding. The reaction was allowed to progress at 25°C for 30 min after mixing. To this reaction mixture, 25 μ l of enzyme-labeled anti-HBs MAB or rabbit polyclonal antibody was added, mixed, and left for another reaction at 25°C for 15 min, followed by washing with BLEIA wash buffer. Magnetic particle suspension buffer and BLEIA substrate solution (100 μ l each) were added and mixed. The bioluminescent intensity from the luciferin-luciferase reaction was measured using a tube luminometer, the Lumat LB 9507 (Berthold Japan, Tokyo, Japan). The percent inhibition was calculated by taking the ratio of the bioluminescent intensity measured when an additional unlabeled antibody was added to that measured when only the labeled antibody was added. The 50% inhibitory concentration (IC_{50}) was determined by plotting the inhibitory ratio.

BLEIA analysis of samples with discrepant measured values. To assess whether the discrepant values determined by the HBsAg BLEIA and the CLIA1 were due to the absence of the preS1/preS2/S proteins that make up HBsAg, the discrepant samples, as well as some that showed the same values (called nondiscrepant specimens), were analyzed by a modified BLEIA method using magnetic particles that were coated with each of the three different MABs, including the anti-preS1 MAB (Hyb-T0606; Institute of Immunology Co., Ltd.), anti-preS2 MAB (Hyb-5520; Institute of Immunology Co., Ltd.), and anti-HBs MABs (the mixture of three clones for the HBsAg BLEIA, 2-4A-10D, 6-6G-8G, and HBs4D4). The labeled anti-HBs antibodies and other reagents used in the analysis were the same as those used in the established HBsAg BLEIA.

Western blot analysis of samples with discrepant measured values. To confirm the absence of the preS1/preS2 proteins in the HBsAg particles with discrepant values from the two measurement systems (BLEIA and CLIA1), Western blotting was performed. For the discrepant and nondiscrepant specimens, the HBsAg was extracted by micro-affinity adsorption based on the method reported by Takahashi et al. (44) and were subjected to SDS-PAGE in a 10 to 20% acrylamide gel (ePage; Atto, Tokyo, Japan), followed by transfer onto a nitrocellulose membrane (0.45 μ m) (Bio-Rad). A horseradish peroxidase (HRP)-labeled anti-HBs rabbit polyclonal antibody was used to detect the large (p39/gp42), middle (gp33/gp36), and small (p24/gp27) HBs proteins. Anti-preS1 and anti-preS2 MABs were used as the primary antibodies to detect large HBs proteins and large and middle HBs proteins, respectively, and polyclonal goat anti-mouse immunoglobulin-HRP (Dako, Glostrup, Denmark) was added as a secondary antibody. Detection of proteins was performed using the Western Lighting Plus-ECL kit (PerkinElmer, Inc., Waltham, MA).

BLEIA analysis of samples treated with protease. To further examine whether the discrepancy could be reproduced by an intentional separation of the preS1 and preS2 proteins, nondiscrepant specimens (between the BLEIA and the CLIA1) were treated with a protease. The specimens were diluted 100-fold with 10 mM PBS, 10 mM EDTA (pH 8.0), and then an equal volume of 0.25% trypsin with 1 mM EDTA-4Na (Life Technologies, Carlsbad, CA) was added. The samples were incubated at 37°C for 1 h or overnight to allow the reaction to take place. The measurements with BLEIA and the CLIA1 were performed after the addition of four times the volume of the reaction solution.

RESULTS

Characterization of the four anti-HBs MABs and the anti-HBs polyclonal antibody used in the HBsAg BLEIA. Among the 55 available anti-HBs MABs, four MABs for the solid phase and five MABs for labeling were selected based on their reactivities with wild-type HBsAg (genotypes A to D) and a set of mutants (data not shown). These nine MABs were subjected to inhibition tests. The data on the three MABs, 2-4A-10D, 6-6G-8G, and HBs4D4

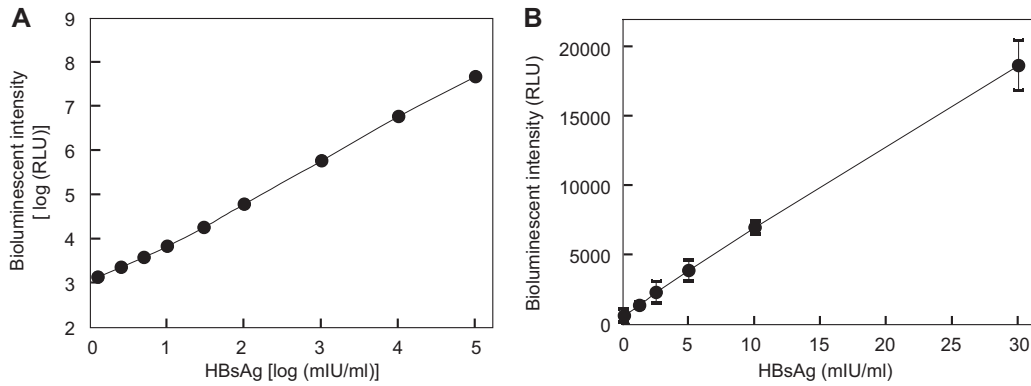


FIG 2 Standard curve and limit of detection of the HBsAg BLEIA. The bioluminescent intensity is shown in relative light units (RLU). (A) Standard curve was made using WHO standard 00/588. (B) Limit of detection was determined by taking the means of 10 assays. Error bars show ± 2.6 standard deviations (SD).

(all IgG1/ κ), which were selected for the solid phase, one MAb, HBs335 (IgG2a/ κ), and a rabbit anti-HBs polyclonal IgG used for labeling for the HBsAg BLEIA established in the present study, are shown in Fig. 1. The IC_{50} s were compared among the selected antibodies. When 2-4A-10D was used as a labeled antibody, the IC_{50} was $>300 \mu\text{g/ml}$ upon the addition of unlabeled MAb, 6-6G-8G, or HBs4D4, while the IC_{50} was $3.3 \mu\text{g/ml}$ upon the addition of 2-4A-10D as an unlabeled antibody. When 6-6G-8G or HBs4D4 was used as a labeled antibody and 2-4A-10D was used as an unlabeled antibody, the inhibition effect by 2-4A-10D was low and the IC_{50} was $>300 \mu\text{g/ml}$, suggesting that the epitopes recognized by 6-6G-8G and HBs4D4 are located far from that recognized by 2-4A-10D. When 6-6G-8G was used as the labeled antibody and 6-6G-8G or HBs4D4 was used as the additional antibody, similar IC_{50} s of $12 \mu\text{g/ml}$ and $51 \mu\text{g/ml}$ were obtained, respectively. When HBs4D4 was used as the labeled antibody, the IC_{50} was $5.2 \mu\text{g/ml}$ with HBs4D4 used as an additional antibody and was $25 \mu\text{g/ml}$ with 6-6G-8G as an additional antibody, suggesting that 6-6G-8G and HBs4D4 may recognize similar epitopes. However, these two MAbs were clearly different in that the 6-6G-8G antibody, but not

the HBs4D4 antibody, reacted with the G145R mutant (data not shown). The inhibition effect of HBs335 was seen for all three of the other labeled MAbs. However, when HBs335 was used as a labeled antibody, the IC_{50} s of both 6-6G-8G and HBs4D4 were $>300 \mu\text{g/ml}$, whereas the IC_{50} s of HBs335 and 2-4A-10D were $0.23 \mu\text{g/ml}$ and $14.2 \mu\text{g/ml}$, respectively, suggesting that the epitopes recognized by HBs335 and 2-4A-10D are close to each

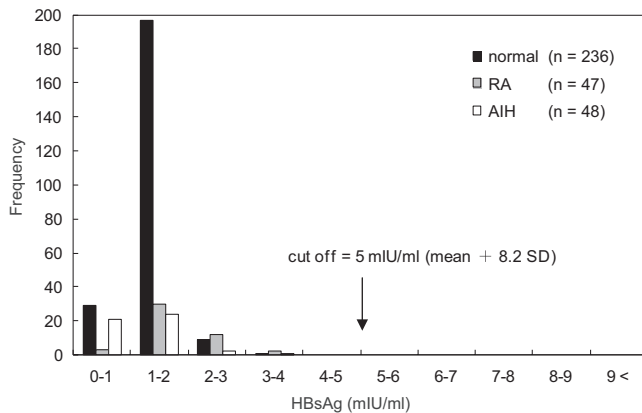


FIG 3 Distribution of the HBsAg concentrations (in mIU/ml) measured by the HBsAg BLEIA in HBsAg-negative samples. The level of HBsAg was measured by the HBsAg BLEIA in a total of 331 HBsAg-negative samples, including 236 blood plasma samples from apparently healthy individuals, 47 serum samples from patients with rheumatoid arthritis (RA), and 48 serum samples from patients with autoimmune hepatitis (AIH); the cutoff value was set at the mean plus 8.2 SD (5 mIU/ml).

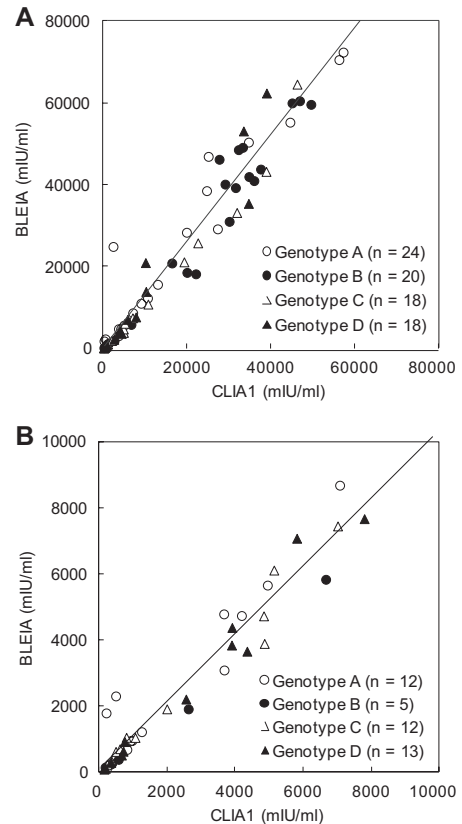


FIG 4 Correlation between the HBsAg BLEIA and a commercially available kit (CLIA1) in the quantitative HBsAg detection. The level of HBsAg was measured by the BLEIA and the CLIA1 in 80 HBsAg-positive samples and the results across genotypes A to D were compared. (A) HBsAg level was within the range of 0 to 80,000 mIU/ml. (B) HBsAg level was within the range of 0 to 10,000 mIU/ml.

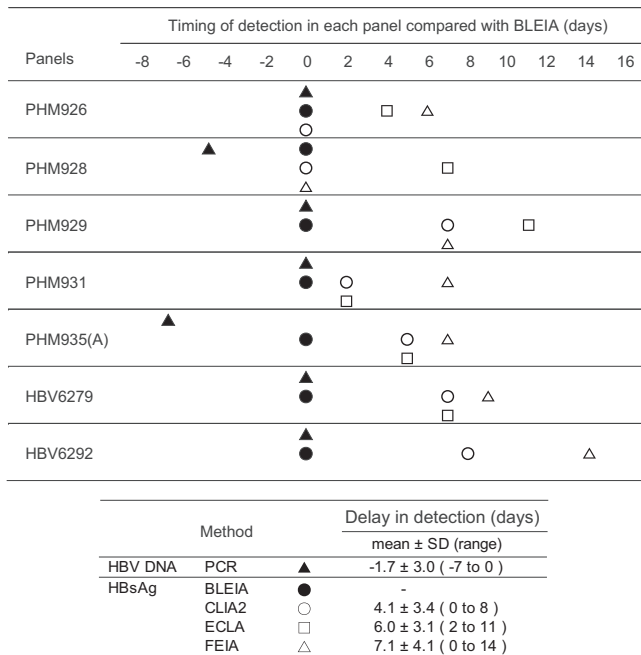


FIG 5 Comparison of timing of detection during the early phase of infection between the BLEIA and other commercial kits. Top, HBsAg was detected by the HBsAg BLEIA in seven series of seroconversion panels, PHM926, PHM928, PHM929, PHM931, PHM935(A), HBV6279, and HBV6292. The first days of continuous positive detection were compared among the HBsAg BLEIA, HBV DNA PCR, and commercial HBsAg detection kits. Bottom, the assay results obtained by HBV DNA PCR and commercial HBsAg detection kits (days delay in detection [mean ± SD] [range]) were referred from the data included in the seroconversion panels. ▲, HBV DNA PCR; ○, CLIA2; □, ECLA (electrochemiluminescence immunoassay); △, FEIA1 (fluorescent enzyme immunoassay).

other. Of note, a 6-fold difference in the reactivity against the C124R mutant was observed between the two MAbs (data not shown). Therefore, it is likely that HBs335 and 2-4A-10D recognize the nearby but distinct epitopes.

Another comparison was made between the anti-HBs rabbit polyclonal antibody and HBs335, both of which were used as labeled antibodies in the HBsAg BLEIA. When the anti-HBs rabbit polyclonal antibody was used as the labeled antibody, the IC₅₀s of the anti-HBs polyclonal antibody and HBs335 were 0.26 µg/ml and 0.16 µg/ml, respectively. In comparison, when HBs335 was used as a labeled antibody, the IC₅₀s of HBs335 and the anti-HBs polyclonal antibody were 0.23 µg/ml and 0.31 µg/ml, respectively. These results suggest that the anti-HBs polyclonal antibody can recognize the same epitope as HBs335. However, once these antibodies were used on the solid phase in the HBsAg BLEIA to measure the G145R mutant, HBs335 exhibited 4-fold higher reactivity than the anti-HBs rabbit polyclonal antibody (data not shown), suggesting that the combined use of the anti-HBs rabbit polyclonal antibody and HBs335 might lead to increased recognition of HBsAg.

Standard curve and sensitivity in the HBsAg BLEIA. The standard curve was prepared in conformity with WHO standard 00/588 and indicated linearity up to 5 log mIU/ml (Fig. 2A). The measurement sensitivity was assessed by measuring each standard sample (0, 1.25, 2.5, 5, and 10 mIU/ml) 10 times. The sensitivity was estimated to be 2.5 mIU/ml based on the results obtained when the mean - 2.6 standard deviations (SD) of the biolumines-

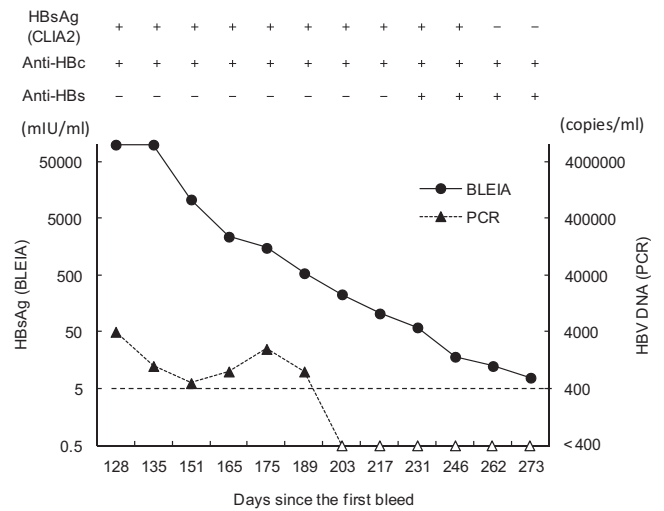


FIG 6 Comparison of the detectability during the late phase of infection (days since first bleed) between the BLEIA and PCR. The seroconversion panel PHM935(B) was used to detect HBsAg by the BLEIA. The measurement results of the HBsAg BLEIA (5 mIU/ml) and were compared against various HBV markers, including HBsAg by CLIA2, anti-HBc, anti-HBs, and HBV DNA by PCR, whose data were obtained from the data sheet included with the seroconversion panel. The horizontal dotted line indicates the cutoff value for both the BLEIA (5 mIU/ml) and PCR (400 copies/ml). ▲, HBV PCR-positive data; △, HBV PCR-negative data; ● HBsAg BLEIA-positive data. Top, HBsAg results by CLIA2, anti-HBc, and anti-HBs are shown as + and -.

cent intensity (relative light units [RLU]) of a sample containing 2.5 mIU/ml of HBsAg was > the mean + 2.6 SD RLU of a sample without HBsAg (0 mIU/ml) (Fig. 2B).

To further assess the distribution of measurement values in the HBsAg-negative samples and to verify the specificities of the antibodies, we measured the level of HBsAg by the BLEIA in a total of 331 HBsAg-negative serum and blood plasma samples, including those from 236 apparently healthy individuals, 47 patients with rheumatoid arthritis, and 48 patients with autoimmune hepatitis (Fig. 3). False-positive results were not recognized in any of the potentially interfering specimens from patients with rheumatoid arthritis or autoimmune hepatitis. The average measured value for the 331 subjects was 1.3 mIU/ml, and the validity when the sensitivity was set at 5 mIU/ml was verified, while using 5 mIU/ml as a cutoff was equal to mean + 8.2 SD.

Correlation between the HBsAg BLEIA and a commercial CLIA kit. For the correlation study, we measured 80 HBsAg-positive specimens using both the BLEIA and a commercially available CLIA kit (CLIA1), and measurement values were compared between the two methods. The specimens tested consisted of 24 samples with genotype A HBV (HBV/A), 20 HBV/B samples, 18 HBV/C samples, and 18 HBV/D samples. A favorable correlation coefficient of 0.99 was obtained. The BLEIA indicated approximately 30% higher values, with the correlation equation being $y = 1.28x + 66$ (Fig. 4A). When the comparison was restricted to 42 samples with an HBsAg titer of <10,000 mIU/ml, a high correlation coefficient of 0.98 was also obtained (Fig. 4B).

Detectability of HBsAg by the BLEIA during the early phase of infection. To assess the detectability of HBsAg by the BLEIA during the early phase of infection, seven series of HBV seroconversion panels were used (Fig. 5). The HBV DNA PCR data of panels PHM926, PHM928, PHM929, PHM931, and PHM935(A)

TABLE 2 Detection of mutated HBsAg in sera obtained from HBV-infected patients by CLIA1 and BLEIA^a

Sample no.	Amino acid substitution(s)	Gt	HBV DNA (copies/ml)	CLIA1 (S/CO)	BLEIA (S/CO)
1	C90F + T116S + T118R + Y134H	D	6.2×10^1	35	510
2	L95W + Y100C + F134V + T140I	B	1.0×10^6	>5,000	>20,000
3	Y100F + Q101R + M133L + Y161F	B	3.7×10^3	>5,000	>20,000
4	Q101H + T123I	D	2.1×10^2	424	6,298
5	Q101R + M133L + G145A	B	1.5×10^3	3,864	>20,000
6	Q101R + E164G	B	6.6×10^5	1,789	15,471
7	V106A + P127T	C	6.1×10^5	>5,000	>20,000
8	L108V	C	1.6×10^7	3,669	>20,000
9	L109I + M133I + F161Y	D	1.9×10^2	1.6	21
10	I110L + F134S	B	9.8×10^2	750	8,185
11	I110L	B	6.1×10^9	>5,000	>20,000
12	L110I	C	2.8×10^5	18	264
13	S117T + P120T + C139G	B	5.6×10^4	1,960	15,363
14	S117T	C	2.5×10^9	>5,000	>20,000
15	T118A + P127T	D	1.5×10^5	>5,000	>20,000
16	P120T	B	8.9×10^5	>5,000	>20,000
17	P120S	D	<20 (+)	4,777	>20,000
18	T123A	B	5.4×10^9	>5,000	>20,000
19	I126S	C	1.7×10^7	>5,000	>20,000
20	I126V	C	2.4×10^9	>5,000	>20,000
21	P127T + Q129H	C	2.0×10^9	>5,000	>20,000
22	P127T + F161Y	C	2.9×10^9	>5,000	>20,000
23	P127T	B	5.3×10^7	3,334	>20,000
24	P127S	B	4.2×10^7	2,161	>20,000
25	Q129H	D	2.8×10^4	4,026	>20,000
26	T131N + Y134S	D	2.4×10^4	2,244	>20,000
27	T131N	B	9.4×10^6	443	6,272
28	T131P	C	3.1×10^2	>5,000	>20,000
29	T131I	D	7.2×10^4	931	13,912
30	M133L + T143 M	B	1.4×10^8	2,240	>20,000
31	M133L	B	9.3×10^4	>5,000	>20,000
32	M133T	B	2.3×10^3	>5,000	>20,000
33	F134I	B	3.5×10^2	3,300	>20,000
34	T140I	B	8.5×10^7	>5,000	>20,000
35	T143 M + E164G	B	1.5×10^8	>5,000	>20,000
36	T143 M	B	3.4×10^9	>5,000	>20,000
37	D144A	D	3.2×10^4	>5,000	>20,000
38	D144E	D	1.0×10^4	>5,000	>20,000
39	G145R	B	4.2×10^7	3,348	>20,000
40	A157G	B	1.7×10^4	>5,000	>20,000
41	A159V	B	8.8×10^5	409	2,986
42	Y161S	B	4.7×10^3	947	12,658
43	Y161F	B	3.8×10^4	937	12,079
44	F161Y	C	9.3×10^5	>5,000	>20,000
45	E164G	B	5.9×10^8	>5,000	>20,000

^a Gt, genotype; (+), positive for HBV DNA.

were taken from those by the Roche Amplicor HBV monitor, and those of panels HBV6279 and HBV6292 were from Roche CAP-G/CTM-HBV, and all data were transcribed from the package insert of each seroconversion panel. The differences in the number of days from the first sampling to the first day of continuous positive detection were compared between the HBsAg BLEIA and HBV DNA PCR. Compared to PCR, the BLEIA showed a 5-day and a 7-day delay in two out of seven seroconversion panels. In the remaining five series of panels, the BLEIA and PCR detected positive samples with the same detection rate. There was little or no difference in the number of days to the first detection of a positive

sample between the BLEIA and PCR (0 versus -1.7 ± 3.0 days), in agreement with the previous study reporting that HBV DNA and HBsAg detections have a good correlation in the early phase of infection (45). Of note, no other HBsAg measurement methods, including the CLIA2 (another commercial CLIA kit), the ECLIA (an electrochemiluminescent immunoassay), or the FEIA1 (a commercial fluorescence enzyme immunoassay kit) were able to detect HBsAg earlier than the BLEIA, and the BLEIA detected HBsAg 5.8 ± 3.7 days earlier than these three HBsAg measurement methods.

Detectability of HBsAg by the BLEIA during the late phase of infection. To assess the detectability of HBsAg by the BLEIA

TABLE 3 Detection of HBsAg by BLEIA in 10 samples with occult HBV infection that tested negative for HBsAg by CLIA1^a

Sample no.	Amino acid substitution(s)	Gt	HBV DNA (copies/ml)	BLEIA (S/CO) ^b
1	G130N	C	<20 (+)	15.0
2	I126N + G145R	C	<20 (+)	1.3
3	T140S + P142S + G145R	C	<20 (+)	0.2
4	T118K + T131N + M133I + E164D + S167L + V168A	C	<20 (+)	0.5
5	G130N + M133T	C	<20 (+)	0.2
6	I126 M	C	<20 (+)	0.3
7	P111L	C	<20 (+)	0.2
8	S113T	C	5.7 × 10 ²	0.2
9	Q129R	D	2.8 × 10 ²	1.4
10	Y134H + T140I	D	8.8 × 10 ¹	0.6

^a Gt, genotype; (+), positive for HBV DNA.

^b Samples positive for HBsAg by BLEIA are indicated in bold type.

during the late phase of infection, a seroconversion panel, PHM935(B), which is a panel of blood withdrawn continuously after HBV infection, was used. HBsAg was detectable by the BLEIA throughout the observation period of 128 to 273 days since the first bleed, while HBsAg became undetectable by the CLIA2 after 262 days since the first bleed, and the HBV DNA was also below the level of detection (400 copies/ml) in the specimens taken beginning 203 days since the first bleed (Fig. 6). These findings were in agreement with those of the previous study by Kuhns et al. (46), where a lack of correlation between HBV DNA and HBsAg detection in the late phase of infection was demonstrated. Of note, the BLEIA detected the HBsAg for >40 days after the occurrence of anti-HBs antibodies.

Reactivity with recombinant HBsAg with mutations. The measurement value (signal to cutoff ratio [S/CO]) was compared between the BLEIA and the CLIA1 for the detection of 13 recombinant HBsAg with various mutations that are known to be undetectable by some commercially available HBsAg detection kits (3, 20). The S/CO value of the BLEIA was 10- to 155-fold higher than that of the CLIA1 (Table 1).

Reactivity with mutant HBsAg from HBV carriers. For the 45 CLIA1-positive and HBV DNA-positive specimens with mutated HBsAg (mutations within residues 90 to 164 of the S protein), HBsAg was detectable exclusively by the BLEIA (Table 2). Notably, the S/CO value of the BLEIA was seven to 15 times higher than that of the CLIA1.

Reactivity with OBI samples. Among the 10 serum samples collected from patients who are chronically infected with HBV with HBsAg that is undetectable even using the most sensitive commercial assay (the CLIA1), and who have low levels of HBV DNA ($\leq 5.7 \times 10^2$ copies/ml), three samples were positive for HBsAg by the BLEIA despite the presence of various amino acid substitutions in the MHR of the S protein (Table 3).

Characterization of three specimens with discrepant results between the BLEIA and the CLIA1. Among the 80 HBsAg-positive samples tested (Fig. 4), three samples (P10, P38, and P179) had discrepancies (in mIU/ml) of >5-fold between the BLEIA and the CLIA1. To verify whether this discrepancy was due to HBV genomic mutations, we determined the entire preS1/preS2/S gene sequence for the three specimens. All three specimens belonged to genotype A, and no mutations that could cause a deviation were seen in two of the three specimens (P10 and P38). In the remaining specimen, P179, the ATG (Met) codon of the preS2 region was mutated to ATA (Ile). This was a mutant strain incapable of producing the middle HBs (preS2 + S) protein but which was still capable of producing the large HBs (preS1 + preS2 + S) protein. Furthermore, the P179 specimen had a deletion mutation so that it lacked five amino acids (aa 4 to 8) in the preS2 protein. In this case, the low titer of HBsAg detected by the CLIA1 kit might have been due to the use of an antibody recognizing this deleted region as either a solid phase antibody or a labeled antibody.

To further clarify the cause of the discrepancies, the three discrepant samples, as well as three nondiscrepant samples (P16, P23, and P35) (Table 4), were subjected to the HBsAg BLEIA established in the present study, as well as a BLEIA using different magnetic beads coated with the anti-preS1 MAb or anti-preS2 MAb in place of the three anti-HBs MAbs used for the HBsAg BLEIA, and the measured bioluminescent intensities (in RLU) are shown in Table 4. The RLU value for the anti-preS1 antibody divided by the value for the anti-HBs antibody (anti-preS1/anti-HBs ratio) was low, at 0.011 to 0.012% for the discrepant specimens, in contrast to the nondiscrepant specimen ratios of 3.7 to 12.4%. Similarly, the anti-preS2/anti-HBs ratio was low (0.008 to 0.014%) for the discrepant specimens but high (6.5 to 10.5%) for the nondiscrepant specimens, suggesting that a defect or significant decrease in the preS1 and preS2 antigens was a feature of the discrepant specimens.

HBsAg particles purified by micro-affinity adsorption from specimens with or without the discrepancy were subjected to Western blot analysis with the anti-preS1 MAb, anti-preS2 MAb,

TABLE 4 Results of the analysis of discrepant and nondiscrepant specimens by BLEIA

Specimen	HBsAg detection			BLEIA results using the indicated solid-phase antibody:				
	CLIA1 (mIU/ml)	BLEIA (mIU/ml)	Ratio (BLEIA/CLIA1) (%)	Anti-HBs (RLU)	Anti-preS1 (RLU)	Ratio (anti-preS1/anti-HBs) (%)	Anti-preS2 (RLU)	Ratio (anti-preS2/anti-HBs) (%)
Discrepant								
P10	790	7,153	905	59,455,412	6,397	0.011	4,868	0.008
P38	110	775	705	39,820,359	4,753	0.012	3,876	0.010
P179	150	1,706	1,137	47,211,848	5,517	0.012	6,397	0.014
Nondiscrepant								
P16	1,140	1,132	99	7,319,060	269,629	3.7	767,382	10.5
P23	660	666	101	36,971,057	4,601,863	12.4	2,386,321	6.5
P35	530	616	116	5,800,491	585,396	10.1	384,123	6.6

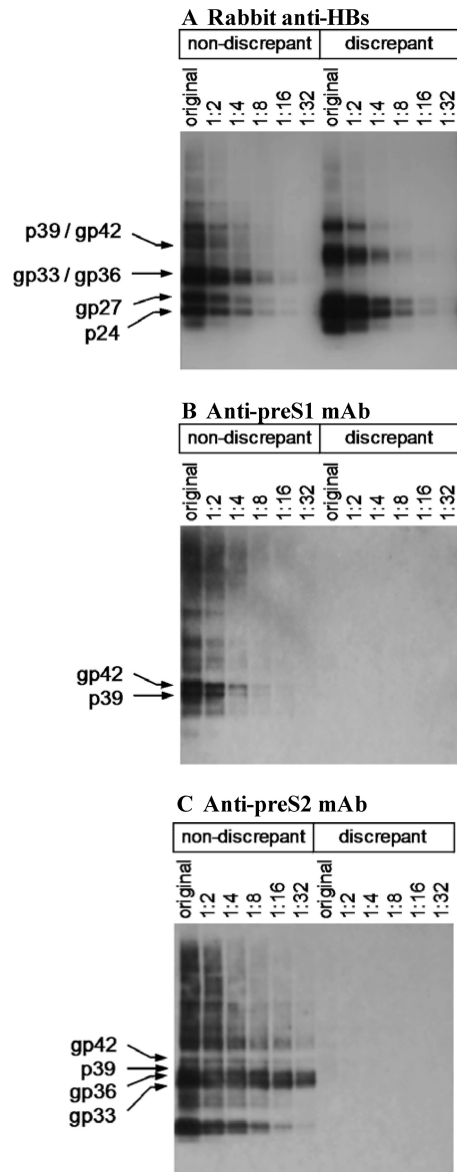


FIG 7 Detection of preS1/preS2/S proteins by Western blotting. Electrophoresis was conducted in 10 to 20% polyacrylamide gels. The nondiscrepant specimen (P35; see Table 4) was serially diluted 1:2 with 1× sample buffer. The concentration decreases by half going from left to right. The discrepant specimen (P38; see Table 4) was prepared in the same way. (A) A 10- μ g/ml preparation of HRP-labeled anti-HBs rabbit polyclonal antibody was used for detection. (B) A 10- μ g/ml preparation of anti-preS1 MAb was used as a primary antibody, and 500-fold-diluted HRP-labeled anti-mouse immunoglobulin (IgG) was used as a secondary antibody. (C) A 10- μ g/ml preparation of anti-preS2 MAb was used as a primary antibody, and 500-fold-diluted HRP-labeled anti-mouse IgG was used as a secondary antibody. The large HBs proteins (p39 and gp42), middle HBs proteins (gp33 and gp36), and small HBs (S) proteins (p24 and gp27) are marked with arrows.

or anti-HBs rabbit polyclonal antibody (Fig. 7). This analysis revealed that a nondiscrepant sample (P35) had all three species of large, middle, and small HBs proteins (p39/gp42, gp33/gp36, and p24/gp27, respectively). However, a discrepant sample (P38) lacked the large and middle HBs proteins. We obtained the same results in the remaining two specimens with discrepancies (P10 and P179), showing that the discrepant specimens either lacked or

had a significantly decreased amount of middle and large HBs proteins.

To further examine whether the nondiscrepant samples showed a discrepancy after protease treatment, nondiscrepant samples were digested with a protease and the HBsAg concentration was compared between the BLEIA and the CLIA1. After the treatment with trypsin, reactivities with the anti-preS1 and anti-preS2 MAbs decreased markedly for all four samples tested (P41, P43, P75, and P180) (Table 5). The rate of discrepancy (BLEIA/CLIA1) increased 1 h after treatment in two samples (P41 and P180) with lower reactivity against the anti-preS1 MAb, and 21 h after treatment in the remaining two samples (P43 and P75) with relatively high reactivity against anti-preS1 MAb, which may have been rich in large HBs protein.

DISCUSSION

Extensive efforts are being made to improve the performance of HBsAg assays by developing an assay system that is capable of reducing the window of infection prior to detection and capable of detecting OBI. Our present study aimed to develop a novel system for measuring HBsAg that is as sensitive as HBV DNA testing, without the complicated handling process. As a labeling enzyme, we adopted firefly luciferase, which has a high quantum yield, leading to the development of a highly sensitive detection system for HBsAg. With detection through the luciferin-luciferase reaction, highly sensitive measurement can be achieved during both the early and late phases of infection, in which the levels of HBsAg are low. In our previous study reporting the preliminary HBsAg BLEIA (37), there were several issues that needed to be resolved, including the detection of mutants, the stability of luciferase, and the sensitivity of the assay during the late phase of infection. The present study includes new developments that allow our method to provide a performance level that is applicable for clinical use.

It is necessary to minimize the risk of false-negative results due to mutations of the HBV genome, which occur at a high frequency. It has been reported that some of the previously developed HBsAg measurement kits could not detect HBsAg when such mutations were present; the kits using MAbs were found to be especially prone to being unable to detect mutants (17, 28). Our present method utilizing the BLEIA attempted to solve this problem by combining multiple MAbs with a rabbit polyclonal antibody. The HBsAg is known to contain multiple epitopes (14, 47). Among the three MAbs selected for the solid phase, the interactions of 2-4A-10D and 6-6G-8G MAbs and the 2-4A-10D and HBs4D4 MAbs did not influence one another, suggesting that the epitope recognized by 2-4A-10D is located remotely from that recognized by 6-6G-8G and HBs4D4. On the other hand, the epitopes recognized by 6-6G-8G and HBs4D4 were suggested to be located in close proximity to each other (Fig. 1). However, 6-6G-8G and HBs4D4 showed different reactivities to the G145R mutant (data not shown), suggesting that these two MAbs recognize closely related epitopes. Therefore, it was considered that each of the three MAbs used in the solid phase recognize different epitopes of HBsAg. With regard to the labeled antibodies, although the anti-HBs rabbit polyclonal antibody exhibited a strong inhibitory effect for HBs335 (Fig. 1), the reactivity to the G145R mutant was increased by 2.8-fold as a result of combining HBs335 with the rabbit polyclonal antibody compared to using the rabbit polyclonal antibody alone (data not shown). Therefore, to estab-

TABLE 5 Results of the analysis of nondiscrepant specimens following protease treatment

Specimen	Time after trypsin digestion (h)	BLEIA results using the indicated solid-phase antibody:				HBsAg detection		
		Anti-preS1 (RLU)	Anti-preS2 (RLU)	Anti-HBs (RLU)	Ratio (anti-preS1/anti-preS2) (%)	CLIA1 (mIU/ml)	BLEIA (mIU/ml)	Ratio (BLEIA/CLIA1) (%)
P41	0	11,255	5,246,331	12,704,848	0.2	17,500	20,330	116
	1	125	788	12,015,611		3,130	19,137	611
	21	394	349	4,063,816		460	6,272	1,363
P43	0	984,912	3,622,374	7,445,464	27.2	9,540	11,496	120
	1	479	2,068	11,054,376		14,400	17,491	121
	21	1,135	1,989	2,530,338		1,900	3,919	206
P75	0	3,387,259	4,579,081	8,319,900	74.0	14,700	12,923	88
	1	15,951	13,047	9,618,812		16,700	15,074	90
	21	21,316	18,542	3,047,562		1,350	4,709	349
P180	0	4,022	671,251	948,868	0.6	1,490	1,445	97
	1	62	353	657,138		420	1,010	240
	21	46	426	178,713		<50	304	>609

lish the HBsAg BLEIA in the current study, we used three MABs with distinct specificities for the solid phase and one MAB and the anti-HBs rabbit polyclonal antibody for labeling.

As illustrated in Fig. 2, we were able to achieve high sensitivity and a wide measurable range with the BLEIA (5 mIU/ml to 100,000 mIU/ml). The cutoff value of 5 mIU/ml corresponds to the mean + 8.2 SD of the measurement of 331 HBsAg-negative samples. Furthermore, no false-positive results were observed when testing HBsAg-negative specimens from patients with rheumatoid arthritis or autoimmune hepatitis, conditions that can sometimes elicit a false-positive result (48) (Fig. 3). These results indicate that the HBsAg BLEIA has a high sensitivity, with a detection limit of 5 mIU/ml, as well as a high specificity.

To compare the HBsAg BLEIA developed in the present study with the most sensitive assay kit that is currently available (CLIA1, which has a sensitivity of 50 mIU/ml), 80 specimens that were positive for HBsAg (genotypes A, B, C, and D) by the CLIA1 were subjected to HBsAg detection with the BLEIA (Fig. 4). The measurements obtained with the BLEIA were slightly higher (1.28-fold) than those obtained with the CLIA1 but with a high correlation coefficient ($r = 0.99$), despite the fact that HBsAg specimens of four distinct genotypes were tested. Therefore, it was confirmed that the BLEIA can detect HBsAg in all specimens that test positive for HBsAg by an existing measurement method.

However, three discrepant specimens were found in the 80 HBsAg-positive specimens tested. These three discrepant specimens had BLEIA measurement values more than five times higher than those measured with the CLIA1 (Table 4). Since the dilution test revealed a preferable linearity for these three discrepant specimens similar to that of nondiscrepant specimens, it was suggested that the influence of the matrix of the specimens was not the cause of the discrepancy (data not shown). In contrast to the nondiscrepant specimens, weak reactivities with anti-preS1 and anti-preS2 were evident for the discrepant specimens (Table 4). Western blotting confirmed that the discrepant specimens lacked the large and middle HBs proteins (Fig. 7), and a sequence analysis ruled out the possibility that there were defects in the preS1 and preS2 regions of the HBV genome in two of the three discrepant specimens; this suggests that the preS1/preS2 proteins were de-

tached from the surface of the HBsAg particles, likely due to protease activity during specimen preservation. In fact, Lu et al. (49) reported that there was a protease-hypersensitive region between preS2 and S in the large and middle HBs proteins and that proteins were digested by proteases from microorganisms that contaminated the specimens. In the clinical setting, aseptic manipulation is not usually practiced; therefore, bacterial contamination of the specimens may occur. If specimens are stored without freezing or adding a preservative, the BLEIA has advantages based on variations in specimen stabilities and is capable of detecting HBsAg even when it cannot be detected by other commercial kits because of deterioration.

The advantageous detection of the BLEIA of diversified HBsAg due to the existence of many genotypes and existence or nonexistence of the preS region is also illustrated by the results of mutant measurements. Based on the commercial HBsAg measurement kits already on the market, it has been pointed out that the reactivity with specific mutants is significantly lower than that with wild-type viruses and that the detection of some mutants can be overlooked by these kits (28, 50). The BLEIA showed a higher S/CO ratio than the CLIA1 in all specimens of 13 recombinant HBsAg with representative mutations and in 45 mutated HBsAg from HBV-viremic patients. Especially for the recombinant specimens, C124R, T123N, and 122RA123, the S/CO ratios were 50 times higher than those for the CLIA1, corresponding to a 10-fold or higher difference in detection sensitivity. Not only does our measurement system have high sensitivity, but it also has an increased ability to detect various mutant HBsAg (Table 1). Moreover, the BLEIA could detect HBsAg in clinical samples with a wide variety of mutations within aa 90 to 164 of the S protein (Table 2). The choice of multiple monoclonal or polyclonal antibodies with distinct specificities for the solid phase and labeling in the HBsAg BLEIA may have contributed to the successful detection of all of the types of mutant HBsAg tested.

The HBsAg BLEIA can measure the HBsAg during the majority of the infection period, ranging from early after infection to long after the development of anti-HBs antibodies. When seroconversion panels of the early phase of infection were subjected to the HBsAg BLEIA, the HBsAg was detectable in five out of seven series

as early as by the HBV PCR method. An inexpensive and easy-to-operate immunoassay is therefore now capable of early detection at around the same time as HBV DNA testing. Similarly, seroconversion panels of the late phase of infection revealed that the HBsAg BLEIA could detect HBsAg positivity >11 days later than the CLIA2, 70 days after HBV DNA was no longer detectable, and >40 days after the anti-HBs titer became positive. The HBsAg BLEIA therefore improved testing efficiency and increased the possibility of more-rapid detection of relapses or the development of resistance, due to the extensive mutant reactivity and high sensitivity in the late phase of infection.

There is an ongoing debate regarding whether an OBI reflects the low sensitivity of HBsAg assays or the inability of these assays to detect highly mutated strains in the MHR (29, 51). We assembled a panel of OBI samples and tested them with the BLEIA. It has been reported that OBIs have relatively frequent mutations of the critical MHR cysteines (aa 121, 137, and 139) that have been reported to affect the detectability of HBsAg (24). Although such mutations were not found in the 10 OBI samples studied, three samples with a G130N, I126N/G145R, or Q129R mutation tested positive for HBsAg by the BLEIA. This finding suggests that a highly sensitive HBsAg assay may change the definition and frequency of OBI. To draw a plausible conclusion about this issue, a greater number of OBI samples should be tested for HBsAg by the BLEIA in future studies.

In this study, we have overcome common obstacles associated with HBsAg detection, such as the mutations occurring at a high frequency, specimen deterioration during preservation, and low levels of HBsAg (or OBI status) in the late phase of infection, by combining four MAbs with distinct specificities with a rabbit polyclonal antibody and adopting luciferase for labeling of the antibody. Our HBsAg BLEIA reagents are expected to minimize the undetectability period during the early and late phases of infection and will thus decrease the loss of therapeutic opportunities, which can lead to more effective prevention of an expansion of HBV infection.

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