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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Novel and highly sensitive immunoassay for total hepatitis B surface antigen, including that complexed with hepatitis B surface antibody

Akihiro Matsumoto[1] · Masayasu Imaizumi[2] · Yasuhito Tanaka[3] · Shuhei Nishiguchi[4] · Hiroshi Yatsuhashi[5] · Takehiko Ishida[6] · Kazushige Moriyama[2] · Katsumi Aoyagi[2,6] · Eiji Tanaka[1]

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Abstract
Background Hepatitis B surface antigen (HBsAg) is used as a clinical marker of hepatitis B virus (HBV) infection. However, conventional HBsAg assays have so far failed to accurately detect HBsAg in blood because of interference by patient-derived antibodies against HBsAg (HBsAb).
Methods We developed a novel, fully automated assay system that can detect total HBsAg in blood, including antigens complexed with HBsAb. The immunoassay inactivates HBsAb via a simple pretreatment step to dissociate the HBsAg molecule from HBsAg–HBsAb complexes and thereby estimate total HBsAg. Accordingly, the test has been termed the "immunoassay for total antigen including complex via pretreatment (iTACT)-HBsAg."
Results The recovery rate of HBsAg in the presence of HBsAb was greater than 87 % at a cutoff value set at 5.0 mIU/mL on the basis of data from 545 healthy controls. Analyses using serial serum samples from 25 HBV carriers who became negative for HBsAg during follow-up showed that the iTACT-HBsAg could detect HBsAg over a period of years despite a loss in detection by conventional assays and was able to detect HBsAg in 39 (53 %) of 73 samples with HBsAb.
Conclusions The new iTACT-HBsAg assay appears to detect total HBsAg with high sensitivity, even in the presence of HBsAb, and may useful in identifying subclinical or occult HBV carriers.

Keywords Immunoassay · Hepatitis B surface antigen · Immunoassay for total antigen including complex via pretreatment

✉ Eiji Tanaka
etanaka@hsp.md.shinshu-u.ac.jp;
etanaka@shinshu-u.ac.jp

Akihiro Matsumoto
amatsumo@shinshu-u.ac.jp

Masayasu Imaizumi
ms-imaizumi@fujirebio.co.jp

Yasuhito Tanaka
ytanaka@med.nagoya-cu.ac.jp

Shuhei Nishiguchi
nishiguc@hyo-med.ac.jp

Hiroshi Yatsuhashi
yatsuhashi@nagasaki-mc.com

Takehiko Ishida
t-ishida@reno.co.jp

Kazushige Moriyama
kg-moriyama@fujirebio.co.jp

Katsumi Aoyagi
km-aoyagi@fujirebio.co.jp

[1] Department of Medicine, Shinshu University School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan

[2] New Product Development Department, Fujirebio Inc., Hachioji, Tokyo, Japan

[3] Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan

[4] Division of Hepatobiliary and Pancreatic Disease, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

[5] Clinical Research Center, National Hospital Organization, Nagasaki Medical Center, Omura, Nagasaki, Japan

[6] Research and Development Department, Advanced Life Science Institute Inc., Wako, Saitama, Japan

Abbreviations

ALP	Alkaline phosphatase
CLEIA	Chemiluminescent enzyme immunoassay
HBcAb	Hepatitis B core antibody
HBsAb	Hepatitis B surface antibody
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
iTACT	Immunoassay for total antigen including complex via pretreatment

Introduction

There are more 240 million long-term carriers of the hepatitis B virus (HBV) worldwide, and approximately 780,000 people die yearly of cirrhosis or liver cancer due to chronic and active HBV infection [1–3]. The measurement of hepatitis B surface antigen (HBsAg) is widely used to screen for HBV infection. HBsAg positivity indicates infection with HBV, whereas its subsequent clearance generally indicates a state of resolution [4]. Thus, high-sensitivity HBsAg assays are desired for the accurate diagnosis and treatment of patients with HBV infection.

Recent studies have demonstrated that low levels of HBV replication persist in the liver even in patients who clear HBsAg [5–7]. The presence of hepatitis B surface antibody (HBsAb) and/or hepatitis B core antibody (HBcAb) is usually positive in such individuals and is indicative of resolved HBV infection. Reactivation of hepatitis B is a severe complication not only in patients with current HBV infection but also in those with resolved infection receiving immunosuppressive therapy [8–12]. Accordingly, measurements of HBsAg, HBsAb, and HBcAb are all recommended in patients who undergo immunosuppressive treatment. As the risk of hepatitis B reactivation is higher in individuals with HBsAg than in those without it, assay systems that can detect HBsAg accurately and with high sensitivity may enhance prophylactic measures against the reactivation of hepatitis B.

A new HBsAg assay kit (Lumipulse HBsAg-HQ; Fujirebio, Tokyo, Japan) was recently developed with a sensitivity of 5 mIU/mL, which was six to times higher than that of other commercially available HBsAg assays. The Lumipulse HBsAg-HQ test partially denatures HBsAg particles in serum samples with detergents and then detects HBsAg using monoclonal antibodies not only against the epitopes located outside particles but also against those found inside them [13].

Seroconversion of HBsAg occurs during the clinical course of patients who enter the recovery phase [4]. However, HBsAg clearance may be affected by the production of HBsAb since the existence of HBsAb in test samples influences HBsAg detection by competitive binding with the antibodies used in the assay. Therefore, we presume that the status of HBsAg production in the liver can be more accurately assessed by elimination of interference by coexisting HBsAb.

In light of the above findings, we developed a new HBsAg assay that can detect total HBsAg, including antigens complexed with HBsAb. Termed an "immunoassay for total antigen including complex via pretreatment (iTACT)-HBsAg," the test includes a preliminary process that both dissociates HBsAg from HBsAg–HBsAb complexes by inactivating HBsAb and denatures HBsAg particles. The present study evaluated the general performance of the iTACT-HBsAg in 25 long-term carriers of HBV.

Materials and methods

Samples and materials

One hundred sixty-five serial serum samples collected from 25 HBV carriers who achieved HBsAg seroconversion were used to compare the performance of several HBsAg assay kits, including the iTACT-HBsAg. The carriers were followed up at Shinshu University Hospital from 1998 to 2012. Complicating liver cirrhosis was judged by histological examination or typical findings of cirrhosis, such as signs of liver failure. One patient developed hepatocellular carcinoma during follow-up. Stored serum samples were kept frozen at −20 °C or below until they were assayed. This study was approved by the Ethics Committee of Shinshu University (approval reference 521).

HBsAg-positive and HBsAg-negative samples were purchased from ProMedDx (Norton, MA, USA) and Vitrologic (Charleston, SC, USA) respectively. HBsAb-positive samples were purchased from ProMedDx. These samples were used to evaluate the basic performance of the iTACT-HBsAg. Alkaline phosphatase (ALP) was purchased from Oriental Yeast (Tokyo, Japan), anti-HBsAg monoclonal antibodies were purchased from Advanced Life Science Institute (Saitama, Japan), native HBsAg was purchased from Trina Bioreactives (Naenikon, Switzerland), blue dextran was purchased from Sigma-Aldrich (St Louis, MO, USA), and rabbit anti-HBsAg polyclonal antibodies were purchased from Fujirebio.

Conventional assays for HBV markers

The measurement of HBsAb concentration by the Lumipulse HBsAb-N assay was performed according to the manufacturer's instructions (Fujirebio). Detection of HBsAg by the Architect HBsAg-QT (Abbott Laboratories, North Chicago, IL, USA) [14], HISCL HBsAg (Sysmex,

Kobe, Japan) [15], and Lumipulse HBsAg-HQ (Fujirebio) [13] assays was conducted according to the directions of each manufacturer. The cutoff values for the Architect HBsAg-QT, HISCL HBsAg, and Lumipulse HBsAg-HQ assays were 50, 30, and 5 mIU/mL respectively.

iTACT-HBsAg

Monoclonal-antibody-coated ferrite particles were prepared as the solid phase by the coupling method with chemical linkers [16]. The particle solution was made by suspension of the antibody-coated ferrite particles in particle diluent [2 % bovine serum albumin, 2 % sucrose, 0.1 % ProClin® 300 (Sigma-Aldrich), 500 mM bicine–NaOH; pH 9.2]. For the detection reagent, ALP-linked monoclonal antibodies (ALP conjugate) were prepared by the hinge method [17] and purified by chromatography with a HiLoad 16/600 Superdex 200 pg column (GE Healthcare, Wauwatosa, WI, USA). The conjugate solution was prepared by dilution of ALP conjugate in the conjugate diluent [2 % bovine serum albumin, 0.1 % casein sodium, 0.1 % ProClin® 300, 50 mM 2-(*N*-morpholino)ethanesulfonic acid–NaOH; pH 6.8].

The iTACT-HBsAg was performed with a LUMIPULSE PRESTO II (Fujirebio) automated chemiluminescent enzyme immunoassay (CLEIA) system. Samples of 50 μL were first incubated with 30 μL of the acidic pretreatment solution (0.6 M HCl, 2.5 M urea, 0.75 % Triton X-100) for 6.5 min at 37 °C. Pretreated samples were then mixed with 50 μL of the particle solution and further incubated for 8 min at 37 °C. After they had been washed with Lumipulse Presto washing buffer (Fujirebio), the ferrite particles were incubated with the conjugate solution for 8 min at 37 °C, and then the relative luminescent intensity was measured by incubation for 4 min at 37 °C with 200 μL of substrate solution [3-(2′-spiroadamantan)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt; Life Technologies, Carlsbad, CA, USA].

To determine the lower limit of HBsAg quantification for the iTACT-HBsAg, HBsAg-positive samples were diluted to 2–4 mIU/mL with use of an HBsAg-negative and HBsAb-negative sample. The samples were mixed with 0.1 vol of the HBsAb-positive sample (final HBsAb concentration 534 mIU/mL) and then incubated for 2 h at 37 °C for the antigen–antibody reaction. All samples were measured ten times by the iTACT-HBsAg. The lowest HBsAg concentration that displayed a coefficient of variation of less than 10 % was set as the limit of quantitation.

Performance of the pretreatment process

The performance of the acidic pretreatment to inactivate HBsAb was confirmed with use of commercial HBsAb-positive samples. Each sample of 50 μL was mixed with 30 μL of the iTACT-HBsAg pretreatment solution and then incubated for 6.5 min at 37 °C. Thereafter, 50 μL of the iTACT-HBsAg particle diluent was added to pretreated samples and assayed by the Lumipulse HBsAb-N assay.

The recovery rate of immunoreactive HBsAg in the presence of HBsAb at approximately 1,000 mIU/mL was evaluated with use of three commercial HBsAg-positive samples. Each sample was mixed with 0.1 vol of the HBsAb-positive sample and incubated for 2 h at 37 °C. Then, the HBsAg concentration of each sample was measured by both the iTACT-HBsAg and the Architect HBsAg-QT assay. This procedure was performed ten times for each sample. As a reference, one HBsAb-negative specimen was mixed instead of an HBsAb-positive specimen. The recovery rate (%) was calculated as follows: (HBsAg concentration in HBsAb-positive specimen)/(HBsAg concentration in HBsAb-negative specimen) × 100.

Gel filtration chromatography analysis

Gel filtration chromatography analysis was performed with a HiPrep 16/60 Sephacryl S-500 HR column (GE Healthcare). Purified native HBsAg was diluted with tris(hydroxymethyl)aminomethane-buffered saline [150 mM NaCl, 0.1 % NaN_3, 50 mM tris(hydroxymethyl)-aminomethane–HCl; pH 7.2] and then mixed with an equal volume of rabbit anti-HBsAg polyclonal antibody solution (HBsAg concentration approximately 100 IU/mL; HBsAb concentration approximately 7 IU/mL). The HBsAg and HBsAb mixture was thereafter incubated for 2 h at 37 °C for the preparation of HBsAg–HBsAb complexes. As a control, purified native HBsAg was diluted with tris(hydroxymethyl)aminomethane-buffered saline without HBsAb. Samples were subjected to gel filtration chromatography with or without the acidic pretreatment, which was as follows: 5 vol of the sample and 3 vol of the acidic pretreatment solution were mixed and incubated for 6.5 min at 37 °C. After incubation, 5 vol of the particle diluent was added. Each fraction was then measured by the iTACT-HBsAg.

HBV genome sequencing

The nucleotide sequences of full-length HBV genomes were determined by a method reported previously [18]. Briefly, two overlapping fragments of an HBV genome were amplified by PCR, and then eight overlapping HBV DNA fragments were amplified by nested PCR. All necessary precautions to prevent cross-contamination were taken, and negative controls were included in each assay. The sequencing reaction was performed according to the manufacturer's instructions (ABI Prism BigDye

Terminator version 3.1 cycle sequencing ready reaction kits; Applied Biosystems, Foster City, CA, USA) with an automated ABI DNA sequencer (model 3100, Applied Biosystems, Carlsbad, CA, USA).

Statistical analyses

The proportion of each clinical factor was compared by Fisher's exact probability test, and group medians were compared by the Mann–Whitney *U* test. The positive rates of HBsAg measured by the Lumipulse HBsAg-HQ assay and the iTACT-HBsAg were compared for each HBsAb-positive and HBsAb-negative group by the McNemar test. Those analyses were performed with the IBM SPSS Statistics 23.0.0.0 statistical software package (IBM Japan, Tokyo, Japan). Correlation coefficient and Passing–Bablok regression analysis was adopted to examine the relationship between the HISCL HBsAg assay and the iTACT-HBsAg. This test was performed with the Analyse-it 4.10.1 statistical software package (Analyse-it Software, Leeds, UK). In the statistical analyses, *P* values less than 0.05 were considered statistically significant.

Results

Performance of the acidic pretreatment method

The performance of the new pretreatment process to inactivate HBsAb was first tested with four samples with high HBsAb concentrations ranging from 293 to 750 mIU/mL. All concentrations had decreased to below the cutoff value (10 mIU/mL) after the acidic pretreatment (data not shown).

The recovery rates of immunoreactive HBsAg from samples containing high HBsAb concentrations were 87–101 % as measured by the iTACT-HBsAg, as compared with 1–23 % as measured by the Architect HBsAg-QT assay, a conventional HBsAg assay (Table 1).

Table 1 Recovery rate of hepatitis B surface antigen (*HBsAg*) in the presence of high hepatitis B surface antibody (*HBsAb*) concentration as measured by the immunoassay for total antigen including complex via pretreatment (*iTACT*)-HBsAg and the Architect HBsAg-QT assay

Sample	HBsAg concentration (mIU/mL)[a]	HBsAg recovery rate	
		iTACT-HBsAg (%)	Architect HBsAg-QT assay (%)
1	9805	87–100	2–23
2	9965	94–100	1–17
3	11,205	89–101	3–11

Recovery tests were performed ten times for each sample

[a] The HBsAg concentration of each sample was measured in the absence of HBsAb as a reference

Gel filtration chromatography analysis of HBsAg complexed and not complexed with HBsAb

HBsAg that was either complexed or not complexed with HBsAb was analyzed by gel filtration chromatography. In non-pretreated samples, elution of noncomplexed HBsAg produced a gentle peak at fractions heavier than 2000 kDa, whereas the elution of complexed HBsAg showed a broad elution profile for higher molecular mass fractions (Fig. 1a). On the other hand, in pretreated samples, elution of both complexed and noncomplexed HBsAg produced sharp peaks clustered around a molecular mass between 44 and 150 kDa (Fig. 1b). The latter results suggested that circulating HBsAg particles as well as those complexed with HBsAb were degraded by pretreatment into smaller molecules containing HBsAg.

General performance of the iTACT-HBsAg

The mean HBsAg concentrations of the samples prepared to determine the detection limit of the iTACT-HBsAg were 2.1, 3.4, and 4.2 mIU/mL, with coefficients of variation of 9.5, 9.4, and 6.8 % respectively. Thus, the lower limit of HBsAg quantification in this assay was set as 2.1 mIU/mL.

Sufficient within-run reproducibility was confirmed by six measurements of each of the three HBsAg-positive specimens with HBsAb at 1,000 mIU/mL. The mean levels of HBsAg were 733, 7262, and 65,316 mIU/mL, with coefficients of variation of 2.6, 1.0, and 3.4 % respectively.

Five hundred forty-five HBsAg-negative normal serum samples were measured for HBsAg to evaluate the specificity of the iTACT-HBsAg. The mean value of the negative samples was 0.5 mIU/mL, the mean +3 standard deviation was 2.2 mIU/mL, and the mean +8 standard deviation was 5.1 mIU/mL. On the basis of these data, a cutoff value was tentatively set at 5.0 mIU/mL. As immunoreactivity was below this cutoff value in all negative controls, specificity was determined as 100 % at the 5.0 mIU/mL cutoff (data not shown).

Comparison of HBsAg assays using clinical samples

Detection ability was compared among the iTACT-HBsAg and the Lumipulse HBsAg-HQ and HISCL HBsAg assays with serial serum samples collected from 25 HBV carriers who became negative for HBsAg as measured by the HISCL HBsAg assay during follow-up. The Lumipulse HBsAg-HQ and HISCL HBsAg assays are widely used commercial HBsAg assays with detection limits of 5 and 30 mIU/mL respectively. HBsAb was measured by the Lumipulse HBsAb-N assay in all samples. The point at

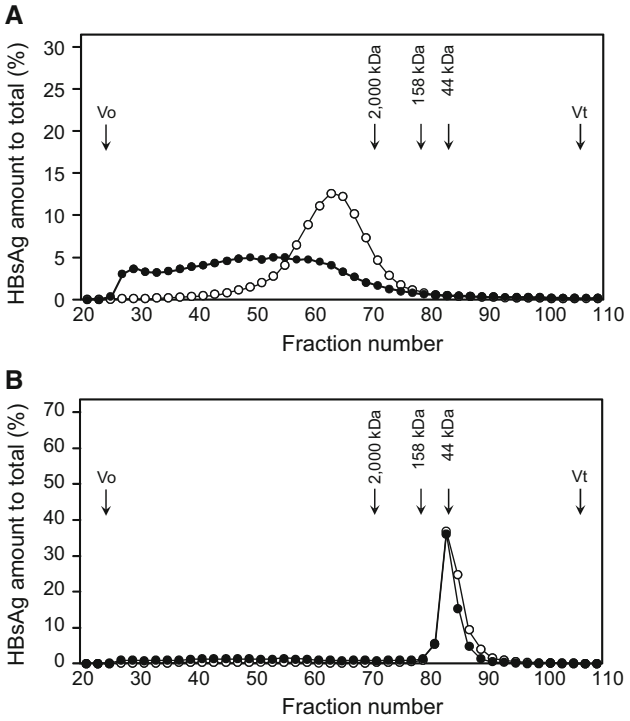

Fig. 1 Gel filtration chromatography analysis of hepatitis B surface antigen (*HBsAg*). *Closed circles* indicate samples in which HBsAg was complexed with hepatitis B surface antibody (HBsAb) and *open circles* indicate samples in which HBsAg was not complexed with HBsAb. Immunoreactive HBsAg was measured by the immunoassay for total antigen including complex via pretreatment–HBsAg after fractionation with a HiPrep 16/60 Sephacryl S-500 HR column. **a** Samples without acidic pretreatment. **b** Samples with acidic pretreatment. Elution points of molecular markers are indicated by *arrows*: blue dextran 2000 kDa, γ-globulin 158 kDa, and ovalbumin 44 kDa. *Vo* void volume, *Vt* total column volume

which HBsAg became negative as measured by the HISCL HBsAg assay was defined as the baseline. HBsAg and HBsAb were measured at 1 year before the baseline, at the baseline, and every year afterward.

The 25 carriers were classified into three groups according to the results of the HBsAg assays. Group A consisted of two patients (8 %) who became negative for HBsAg at the same time as measured by the three assays. Group B comprised seven patients (28 %) who became negative for HBsAg as measured by the HISCL HBsAg assay first, and then either became negative as measured by the Lumipulse HBsAg-HQ assay and the iTACT-HBsAg simultaneously or remained positive as measured by these assays at the end point. Group C included 16 patients (64 %) who became negative for HBsAg as measured by the HISCL HBsAg and Lumipulse HBsAg-HQ assays earlier than with measurement by the iTACT-HBsAg. Representative cases in each group are shown in Fig. 2, wherein the point at which patients became negative for HBsAg as measured by the HISCL HBsAg assay was set as time point zero.

The iTACT-HBsAg could detect HBsAg for 1–11 years longer than the HISCL HBsAg assay in 23 (92 %) of the 25 patients. Similar results were found in comparison with the Lumipulse HBsAg-HQ assay in 16 (64 %) of the 25 patients, with differences ranging from 1 to 8 years.

The clinical backgrounds of patients exhibiting time points with HBsAg detection by the iTACT-HBsAg only (group C) were compared with those who did not (groups A and B) and revealed a tendency for group C patients to experience complicating liver cirrhosis at a higher frequency (Table 2). Other characteristics, including age and results of liver function tests, did not differ markedly between these groups. Although patients with cirrhosis tend to exhibit hypergammaglobulinemia, it is generally considered that this condition does not cause nonspecific reactions in HBsAg detection [13–15, 19].

The positive rate of HBsAg in 165 serum samples collected from 25 HBV carriers who became negative for HBsAg during follow-up is compared among the iTACT-HBsAg, the HISCL HBsAg assay, and the Lumipulse HBsAg-HQ assay in Fig. 3. The positive rate in the total samples was lowest (22 %) for the HISCL HBsAg assay, intermediate (52 %) for the Lumipulse HBsAg-HQ assay, and highest (68 %) for the iTACT-HBsAg. In the 92 samples without HBsAb, the positive rate determined by the iTACT-HBsAg (79 %) was significantly higher ($P = 0.004$) than that determined by the Lumipulse HBsAg-HQ assay (71 %). Similarly, the positive rate determined by the iTACT-HBsAg (53 %) was significantly higher ($P < 0.001$) than that determined by the Lumipulse HBsAg-HQ assay (27 %) in 73 samples with HBsAb. It was noteworthy that the difference was significantly greater ($P < 0.001$) in samples with HBsAb than in samples without HBsAb. No sample with an HBsAg-negative result as measured by the iTACT-HBsAg was positive for HBsAg as measured by either the HISCL HBsAg assay or the Lumipulse HBsAg-HQ assay.

The correlation between HBsAg levels measured by the iTACT-HBsAg and the HISCL HBsAg assay is shown in Fig. 4. Of the 165 samples tested, 37 were positive as measured by both tests, with a correlation coefficient of 0.921 and slope of 2.681 according to Passing–Bablok regression analysis. Of those 37 positive samples, 9 had an HBsAg level ratio of iTACT-HBsAg/HISCL HBsAg assay greater than fivefold (range fivefold to 136-fold). These samples tended to exhibit HBsAg levels lower than 1,000 mIU/mL as by measured by the HISCL HBsAg assay. Seventy-five of the 165 samples were positive for HBsAg as measured by the iTACT-HBsAg but negative as measured by the HISCL HBsAg assay. It was noteworthy that 10 (13 %) of the 75 samples displayed an HBsAg level higher than 1,000 mIU/mL as measured by the iTACT-HBsAg. The positive rate of HBsAb was significantly

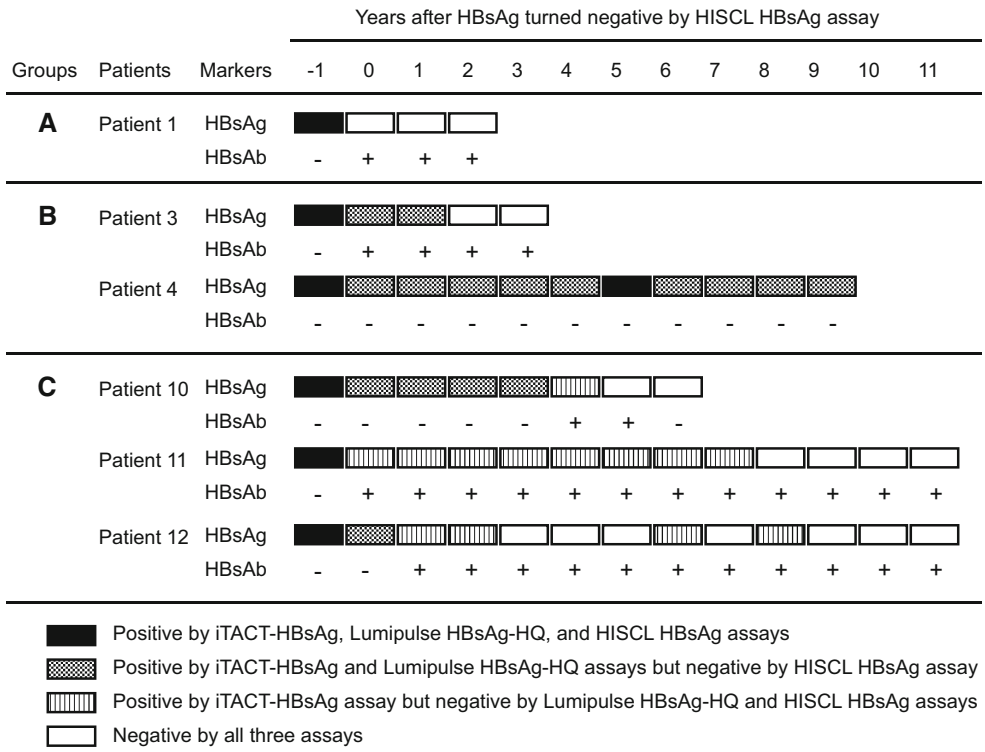

Fig. 2 Representative clinical courses of patients who became negative for hepatitis B surface antigen (HBsAg) during follow-up classified according to the patterns of HBsAg detectability by the immunoassay for total antigen including complex via pretreatment (*iTACT*)-HBsAg, the Lumipulse HBsAg-HQ assay, and the HISCL HBsAg assay. Group A corresponds to patients who became negative for HBsAg at the same time as measured by the three assays. Group B corresponds to patients who became negative for HBsAg as measured by the HISCL HBsAg assay first, followed by the Lumipulse HBsAg-HQ assay and the iTACT-HBsAg simultaneously or whose results were both positive at the end point. Group C corresponds to patients who became negative for HBsAg as measured by the HISCL HBsAg and Lumipulse HBsAg-HQ assays before they became negative as measured by the iTACT-HBsAg. *HBsAb* hepatitis B surface antibody

Table 2 Comparison of clinical backgrounds between group A and group B patients and group C patients in the analysis of carriers who lost hepatitis B surface antigen during follow-up

Factor	Group A and group B patients ($n = 9$)	Group C patients ($n = 16$)	P
Male sex	67 %	63 %	1.000
Age (years)[a]	61 (34–68)	57 (35–77)	0.350
Genotype C	89 %	88 %	1.000
NUC therapy	0 %	19 %	0.280
Complication of cirrhosis	0 %	31 %	0.066
AST (IU/L)[a]	21 (13–31)	24 (19–33)	0.079
ALT (IU/L)[a]	20 (9–61)	27 (13–58)	0.500
γ-GTP (IU/L)[a]	20 (11–38)	22 (10–105)	0.602

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *γ-GTP* γ-glutamyl transferase, *NUC* nucleoside/nucleotide analogue

[a] Data are expressed as the median, with the range in *parentheses*. All data were recorded at the baseline

higher ($P < 0.001$) in those samples (80 %) than in the remaining 65 samples (38 %). The genome sequence of HBV could be determined in six of the ten samples to find possible vaccine escape mutations that influence HBsAg recognition by HBsAb in the second "a" determinant loop [20]. On the basis of the sequencing results of complete HBV nucleotides from serum samples, no typical escape mutations of the S region were found in this series.

Discussion

Multiple reports have described HBV carriers who were positive for HBsAg and HBsAb simultaneously and possessed immune complexes of HBsAg and HBsAb in circulating blood [21–25]. Hence, the amounts of HBsAg in such patients may have been underestimated since conventional reagents for the detection of HBsAg are based on

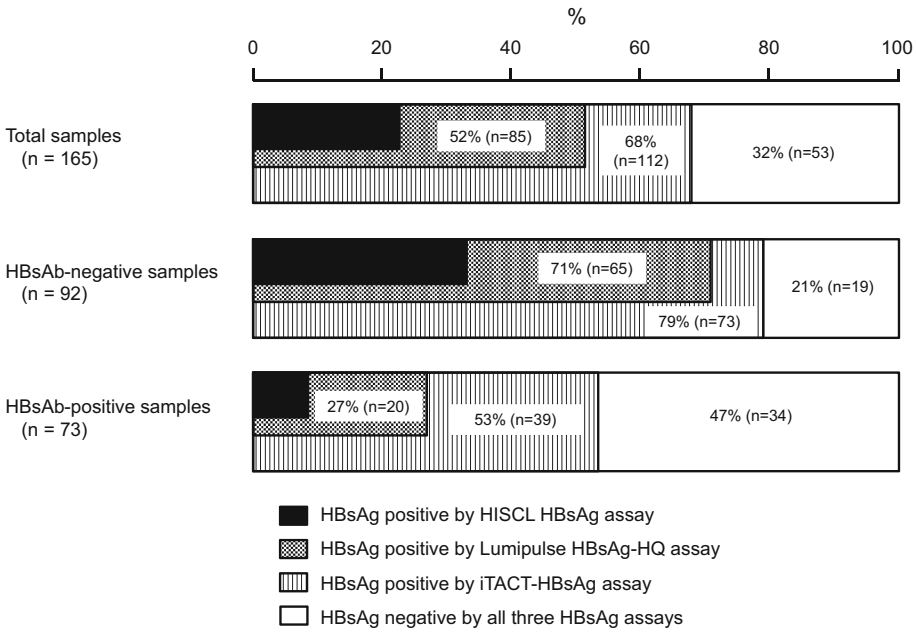

Fig. 3 Comparison of hepatitis B surface antigen (*HBsAg*)-positive rate among the immunoassay for total antigen including complex via pretreatment (*iTACT*)-HBsAg, HISCL HBsAg assay, and Lumipulse HBsAg-HQ assay in 165 samples obtained from 25 hepatitis B virus carriers who became negative for HBsAg as measured by the conventional HISCL HBsAg assay during follow-up. *HBsAb* hepatitis B surface antibody

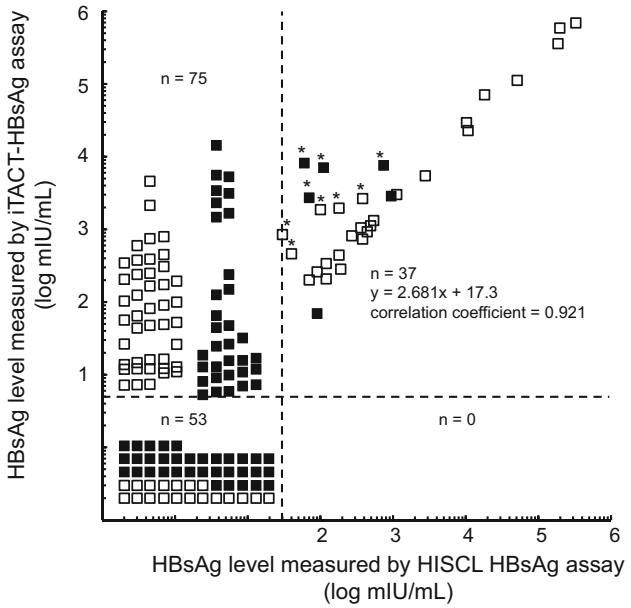

Fig. 4 Correlation between hepatitis B surface antigen (*HBsAg*) concentrations as measured by the immunoassay for total antigen including complex via pretreatment (*iTACT*)-HBsAg or the HISCL HBsAg assay. *Open squares* indicate hepatitis B surface antibody (HBsAb)-negative samples and *closed squares* indicate HBsAb-positive samples. *Broken lines* indicate the lower detection limit of the corresponding assays. The correlation was analyzed statistically in 37 samples having HBsAg concentrations that were measurable by both assays. The nine samples highlighted by *asterisks* had more than fivefold higher HBsAg concentrations as measured by the iTACT-HBsAg

an immunoreaction using antibodies to HBsAg. In the present study, we evaluated a new HBsAg assay, named the "iTACT-HBsAg," that incorporated an acidic pretreatment process to both inactivate HBsAb and detect total HBsAg, including complexed and noncomplexed forms.

Our group has been focusing on the development of sensitive assays for viral antigens through the inactivation of antibodies against target antigens using a simple and effective pretreatment process. For instance, we created a CLEIA for detecting hepatitis C virus (HCV) core antigen using a preliminary process of high concentrations of detergents and heating to above 56 °C [26, 27]. The assay was shown to detect HCV viremia and monitor the effects of antiviral therapy against HCV comparably to amplified HCV RNA testing. This pretreatment process also has been applied to a hepatitis B core-related antigen CLEIA that measures the amount of HBV core, e, and p22cr antigens simultaneously [28]. The hepatitis B core-related antigen assay estimates HBV activity even during nucleoside/nucleotide analogue therapy, and thus can be applied clinically.

However, the pretreatment processes adopted in those assays cannot be applied to a fully automated immunoassay system because of the need for higher temperatures. We have therefore developed a new pretreatment technique for the iTACT-HBsAg using an acidic solution that completes the pretreatment process in 6.5 min, is compatible with the fully automated LUMIPLUSE PRESTO II CLEIA system, and has a total immunoreaction time of approximately 20 min. We first confirmed the performance of the acidic pretreatment to inactivate HBsAb activity in HBsAb-positive samples, and next demonstrated the recovery of immunoreactive HBsAg in the presence of excess HBsAb. Gel filtration chromatography analysis showed that HBsAg

particles complexed with HBsAb were uncomplexed after acid pretreatment. Furthermore, this analysis suggested that HBsAg particles became degraded into uniformly small molecules that might have been beneficial for enhanced detection of HBsAg; the sensitivity of the iTACT-HBsAg was comparable (5 mIU/mL) to that of the currently highest-sensitivity Lumipulse HBsAg-HQ commercial HBsAg assay. Our results indicated that the acidic pretreatment process did not affect the sensitivity of the iTACT-HBsAg.

We analyzed the performance of the iTACT-HBsAg by comparisons with the HISCL HBsAg and Lumipulse HBsAg-HQ assays using serial serum samples collected from HBV carriers who had become negative for HBsAg as measured by the HISCL HBsAg assay during follow-up. More than 90 % of patients were HBsAg positive for longer (1–11 years) as by measured by iTACT-HBsAg than by the HISCL HBsAg assay. Similarly, more than 60 % of patients were HBsAg positive for longer (1–8 years) as measured by the iTACT-HBsAg than by the Lumipulse HBsAg-HQ assay, which indicated that the iTACT-HBsAg may be able to detect subclinical HBV carriers who were judged as HBsAg negative by conventional assays. Of the 165 samples analyzed, 92 were HBsAb negative and 73 were HBsAb positive. Although the positive HBsAg rate was similar between the iTACT-HBsAg and the Lumipulse HBsAg-HQ assay in samples without HBsAb, it was significantly higher as measured by the iTACT-HBsAg in samples containing HBsAb. Since the lower HBsAg detection limits were the same in both assays, our results suggested that the higher detection rate was attributable to the newer assay's HBsAb inactivation properties. Meanwhile, HBsAg was detected for longer by the iTACT-HBsAg than by the Lumipulse HBsAg-HQ assay in a purchased seroconversion panel of acute hepatitis B, but this difference was only 1 week (data not shown). Further studies are required to clarify whether the detection of HBsAg by the iTACT-HBsAg after HBsAg seroconversion observed in carrier patients is also observed in patients with acute hepatitis.

The amounts of HBsAg measured by the iTACT-HBsAg and the HISCL HBsAg assay were well correlated in samples that were positive in both assays. However, some samples deviated from this correlation, especially those with lower HBsAg levels as measured by the HISCL HBsAg assay. Such samples displayed more than fivefold (maximum 136-fold) HBsAg levels as measured by the iTACT-HBsAg. In addition, several samples with HBsAg levels greater than 1,000 mIU/mL were detected by the iTACT-HBsAg that showed HBsAg-negative results with the HISCL HBsAg assay. Samples exhibiting large differences in HBsAg detection between the two assays tended to possess a higher prevalence of HBsAb. As we could not detect any involvement of HBsAg vaccine escape mutations, the coexistence of HBsAb may play an important role in this discrepancy. The conventional HISCL HBsAg assay is widely used, and thus there may be many such patients with contradictory HBsAg assay results. The clinical significance of concealed elevated HBsAg levels requires further clarification.

Reactivation of hepatitis B occurs not only in HBsAg-positive carriers but also among HBsAg-negative individuals with past HBV infection who are HBsAb positive and/or HBcAb positive. However, the detection of HBsAg is particularly important because of a higher risk of reactivation [8–12]. The present study demonstrated that the iTACT-HBsAg could detect HBsAg in a considerable portion of patients who had become negative for HBsAg or who had seroconverted to HBsAb according to conventional assays. Detection of HBsAg by the iTACT-HBsAg in individuals with prior HBV infection may be of benefit to identify those with an elevated risk of reactivation because HBsAg positivity may suggest higher HBV replication activity.

Lastly, it is noteworthy that all patients with liver cirrhosis who lost HBsAg positivity as measured by conventional HBsAg assays during their clinical course exhibited assay points where HBsAg was positive as measured by the iTACT-HBsAg only. Since cirrhotic patients experience severer clinical manifestations during HBV reactivation, the accurate measurement of HBsAg by the iTACT-HBsAg seems especially important in these individuals.

The limitations of this study were that it was retrospective and had a small cohort. However, the promising results revealed in this investigation warrant further large-scale trials.

In conclusion, we have developed a new HBsAg test named the "iTACT-HBsAg" that features an effective acidic pretreatment process able to transform HBsAg particles or HBsAg–HBsAb complexes into smaller HBsAg molecules and inactivate excess amounts of patient-derived HBsAb in serum samples. Thus, the iTACT-HBsAg can detect HBsAg produced in the liver accurately and with high sensitivity, even in HBV carriers with coexisting HBsAb. This novel assay also may be useful for identifying subclinical carriers who have been judged as negative for HBsAg by conventional assays.

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