A novel hepatitis B virus surface antigen immunoassay as sensitive as hepatitis B virus nucleic acid testing in detecting early infection

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BACKGROUND: The aim was to considerably enhance the sensitivity of hepatitis B virus (HBV) surface antigen (HBsAg) detection and investigate whether the window period for HBV detection could be reduced.

STUDY DESIGN AND METHODS: A high-sensitivity chemiluminescent enzyme immunoassay (CLEIA) was developed for quantitative HBsAg detection by a combination of monoclonal antibodies, each one for a specific epitope of HBsAg, and by improving the conjugation technique. The sensitivity of the assay was compared with that of the existing chemiluminescent immunoassay (CLIA). Commercially available seroconversion panels and samples of HBV-infected chimpanzees were tested with the developed prototype to assess whether the window period for HBsAg detection could be reduced to that for DNA detection.

RESULTS: Compared to the existing CLIA, the CLEIA prototype detected HBsAg with approximately 230-fold higher sensitivity and showed a reduced window period. HBsAg detection by the CLEIA prototype and HBV DNA detection by polymerase chain reaction (PCR) occurred simultaneously. The mean time for the CLEIA prototype to first detect HBsAg was approximately 17.4 days less than that for the existing systems. Further, CLEIA prototype enabled HBsAg detection even in anti-HBs–positive seroconversion samples. In the inoculated chimpanzees the HBsAg and HBV DNA became detectable simultaneously and concentrations increased in parallel, whereas HBsAg remained detectable longer than HBV DNA in the declining phase of viremia.

CONCLUSION: The CLEIA prototype yielded results comparable with those of HBV DNA PCR. This novel high-sensitivity assay may be useful for early detection of HBV infection and monitoring patients with a history of infection.

Hepatitis B virus (HBV) infection is one of the main causes of chronic hepatitis. It is estimated that more than 2 billion people worldwide are infected with HBV, of which approximately 400 million people are chronically infected.[1-4] HBV is transmitted by exposure to infectious blood, for example, via accidental injection and transfusion of blood or sexual contact and from the mother to a newborn infant. The development of a persistent HBV infection depends on the age of the infection and immune status of the host.[1,3-12] It is reported that 10 to 30 percent of individuals persistently infected with HBV develop liver cirrhosis and hepatocellular carcinoma. HBV infection causes up to 80 percent of liver cancers.[1,3,4,13-15]

HBV is a double-stranded DNA virus, composed of a nucleocapsid core coated with an envelope containing the

ABBREVIATIONS: CLEIA = chemiluminescent enzyme immunoassay; CLIA = chemiluminescent immunoassay; ER = endoplasmic reticulum.

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Experiments using chimpanzees had been performed before 2006 and the ethical approval for each experiment had been gained which was applied according to the form of the facilities.

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surface antigen (HBsAg). The diagnosis of HBV infection involves the detection of specific antigens and/or antibodies such as HBsAg, antibody to HBsAg (anti-HBs), antibody to HBV core antigen (anti-HBcAg), hepatitis Be antigen (HBeAg), and antibody to HBeAg. HBV DNA is considered to be followed after a few weeks by HBsAg during HBV infection. Enzyme-linked immunoassay (ELISA) is currently used in the commercial HBsAg detection assays[16-19] because of its simplicity and cost-effectiveness.

Despite the fact that many countries perform HBV DNA and HBsAg screening tests, posttransfusion HBV infection continues to occur.[20-25] This is because a relatively long infectious window period remains before HBV DNA and HBsAg become detectable in serum.[19-22,24-30] HBV has been reported to exist in the serum in distinct morphologic forms, the most abundant of which are small or tubular HBsAg particles.[1,26] Empty HBsAg particles are composed of HBsAg and do not contain DNA. The number of these particles is 10,000 to 1,000,000 more than the number of Dane particles, which are infectious HBV particles containing HBV DNA. Because of the large number of empty HBsAg particles, the potential for theoretical detection of HBsAg may be greater.

Although the sensitivity of the HBsAg assay has been improved, reduction in the window period such that it is similar to that of DNA detection remains difficult.[16,19,24,25,27-29,31] The window period for HBV detection has been estimated to be 35 to 76 days by individual sample nucleic acid testing (NAT), 41 to 90 days by minipool NAT, and 50 to 97 days by high-sensitivity HBsAg chemiluminescent immunoassay (CLIA).[29,30] In this study, an unprecedented high-sensitivity prototype was developed and evaluated. The sensitivities of the newly developed prototype and the existing detection systems were compared. To investigate the prototype's ability to detect HBV infection in the early phase, we used the developed prototype to compare the time of appearance of HBsAg and HBV DNA in seroconversion panels. Subsequently, the detection ability of the developed prototype was compared with the detection abilities of the DNA detection and existing HBsAg detection systems.

MATERIALS AND METHODS

Samples

Seroconversion panels were purchased from BioClinical Partners, Inc. (Franklin, MA; Panels 6277, 6278, 6279, and 6281), and Boston Biomedica, Inc. (West Bridgewater, MA; Panels PHM 902, PHM 903, PHM 908, PHM 909, PHM 910, PHM 911, PHM 912, PHM 914, PHM 915, PHM 916, PHM 919, PHM 920, PHM 922, and PHM 935B). The HBsAg sensitivity panel PHA 808 (Boston Biomedica, Inc.) was used for comparing the in-house and international units. The ninth sample obtained from PHA 808 was diluted 5- to 100-fold with a pooled negative serum sample and used for the dilution study. Individual samples that were negative for all viral markers were purchased from ProMedex, Inc. (Flushing, NY).

Chimpanzees

Sequential samples obtained from six chimpanzees infected with HBV reported previously were tested.[30] Chimp 246 was inoculated intravenously with fresh-frozen plasma units from blood donors acutely infected with genotype A (6.9×10^4 copies/mL), and Chimp 272, with genotype C (5.3×10^5 copies/mL). Chimps 279 and 280 were inoculated with pre–acute-phase plasma of Chimp 246, and Chimps 269 and 285 were inoculated with pre–acute-phase plasma of Chimp 272. After the inoculation, serum samples were obtained at intervals of 2 to 7 days in the early phase of infection and as required. The collected samples were assessed for HBsAg by the chemiluminescent enzyme immunoassay (CLEIA) prototype and for HBV DNA by polymerase chain reaction (PCR; TaqMan, Roche Diagnostics KK, Tokyo, Japan) with a sensitivity of 100 copies per mL.

Recombinant HBsAg

Recombinant HBsAg for the in-house standard was purchased from HyTest Ltd. (Turku, Finland). The recombinant HBsAg was serially diluted to produce the in-house standard. The concentration was expressed in international units (IUs; IU/mL or mIU/mL) by calibrating it against the World Health Organization (WHO) reference standard. The measured unit values were compared with the values measured by the existing quantitative HBsAg detection system (ARCHITECT, Abbott Laboratories, Abbott Park, IL), which was based on CLIA method.

Monoclonal antibodies

Monoclonal antibodies (MoAbs) to HBsAg were obtained from established hybridomas by the following method. The recombinant antigen was mixed with an equal volume of Freund's adjuvant, and a dose of 10 to 20 μg of the resultant mixture was intraperitoneally administered to 4- to 6-week-old BALB/c mice. A booster was intraperitoneally administered every 2 to 4 weeks, and a final immunization dose of 10 μg was injected into the caudal vein. After 3 days, splenocytes were obtained from the immunized mice and fused with myeloma cells. The fusion cells were suspended in RPMI 1640 containing 10 percent fetal bovine serum, hypoxanthine, aminopterin, and thymidine and plated onto a 96-well cell culture plate. After approximately 10 days of culturing, when only the hybridomas were proliferated, clones producing the required antibody were selected.

Development of the CLEIA prototype for detecting HBsAg

A CLEIA prototype was constructed for testing the performance of the high-sensitivity HBsAg assay. This sandwich assay utilizes a 96-well microtiter plate (FluoroNunc Module Maxisorp surface; Nunc, Roskilde, Denmark) coated with MoAbs that recognize HBsAg. To detect HBsAg more effectively, MoAbs were selected for ELISA, since each antibody recognized a different HBsAg epitope. Three clones were used for solid antibodies, each of which recognized the determinant "a" as the constructive epitope, amino acids 111 to 130, and amino acids 31 to 50, respectively. For tracer antibodies, two clones were used, each of which recognized the determinant "a" as the constructive epitope in the different region of the solid antibody and amino acids 51 to 69, respectively. The conjugate formed comprised anti-HBs MoAbs labeled with alkaline phosphatase (Roche Diagnostics, Mannheim, Germany), which were linked to some mass molecules, for example, dextran, beta-galactosidase, and polymerized albumin by the modified method reported;[32] these alkaline phosphatase–labeled antibodies enhanced the signals, avoiding aggregation and minimizing the detection limit. In the case using albumin, albumin (32 mg) was mixed with 100 µL glutaraldehyde to produce the polymerized albumin. Next, 1.5 mol per L Tris buffer was added to stop the reaction, and it was incubated for at least 3 hours. The product was purified by gel permeation, and it was used as the linker between the enzyme and the antibody. The antibody was used after pepsin digestion to remove the Fc region, treated with 15 mmol per L mercaptoethylamine for 90 minutes, and conjugated with the polymerized albumin-linked enzyme using *N*-(6-maleimidocaproyloxy)succinimide (Dojindo Laboratories, Kumamoto, Japan). The product was purified by gel permeation and stored with preservative and carrier bovine serum albumin before use. For analyzing HBsAg in the serum or plasma, 75 µL of the sample and 25 µL of the reaction buffer (containing 100 mmol/L Tris buffer supplemented with 1% Triton X-100, 1% sodium dodecyl sulfate, and 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; pH 7.2) were added to the antibody-coated microwells and incubated at 37°C for 60 minutes. After the wells were washed six times with a washing buffer (phosphate-buffered saline containing 0.05% Tween 20), 100 µL of alkaline phosphatase–conjugated anti-HBsAg MoAbs was added to the wells and incubated at room temperature for 30 minutes. After incubation, the microwells were washed again six times and incubated with 100 µL CDP-Star with Emerald II (Tropix, Inc., Bedford, MA). After incubation at room temperature for approximately 15 minutes, the relative light units of the microwell samples were measured. The results were expressed in IU values, which were determined according to the in-house standard curve.

Assessment of the CLEIA prototype

The selected HBsAg-positive samples were diluted with pooled normal serum, and the dilution linearity was assessed. On the basis of the in-house standard curve, linearity was defined relative to the calculated amount of HBsAg.

RESULTS

Performance of CLEIA for HBsAg detection

The amount of recombinant HBsAg was expressed in IU by calibrating it against the WHO reference standard. The dilution series of the recombinant HBsAg were analyzed for linearity and used as the in-house standard. The lowest concentration of the in-house standard was 4.6 mIU per mL with a signal-to-noise ratio of 22.5 (Fig. 1A). In the same assay, the analytical sensitivity of the CLEIA prototype was 0.05 mIU per mL. To confirm the consistency of the IU values, which was determined from the in-house standard values, the PHA 808 panel was tested. A high correlation was observed between the IU value determined from the in-house standard and that of the international standard mentioned in the data provided (Fig. 1B). The unit values of the existing quantitative HBsAg assay and those of the CLEIA prototype were also compared. One HBsAg-positive serum sample was determined to have a titer of 11.08 IU per mL according to the existing quantitative HBsAg CLIA; it was diluted with pooled normal serum and measured using both the existing CLIA and the CLEIA prototype. The sample was diluted serially from 5- to 3125-fold: the expected value was in the range of 2.2 to 3.5 mIU per mL, although the cutoff value of the existing CLIA was 50 mIU per mL. The data of the comparison between the existing CLIA and the CLEIA prototype are shown in Fig. 1C. The approximation formulas for the unit value were determined by the existing CLIA or the CLEIA prototype, and the expected unit values were $Y = 1.078X^{0.695}$ and $Y = 1.405X^{0.997}$, respectively.

Dilution linearity test

Three individual HBsAg-positive samples were diluted with pooled normal serum and assessed using the CLEIA prototype. Table 1 shows the dilution linearity of the CLEIA prototype in the range of 97.6 to 115.3 percent. The ninth sample of PHA808, which showed a value of 0.05 IU per mL according to the data provided, was also diluted for analyzing the dilution linearity at a low titer of HBsAg. The recovered values calculated using the dilution factor ranged from 96.4 to 113.8 percent (Table 1),

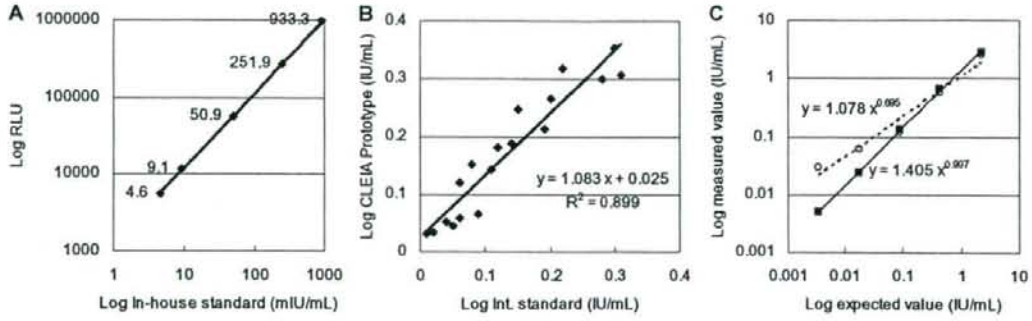

Fig. 1. The performance of the CLEIA prototype was evaluated and expressed in IU values. (A) The in-house standard (933.3-4.6 mIU/mL) determined using recombinant HBsAg was measured by the CLEIA prototype. (B) The concentrations of the HBsAg sensitivity panel PHA 808 (Boston Biomedica, Inc.) measured by the CLEIA prototype were compared with the provided data. The correlation coefficient is shown as $R^2 = 0.899$. (C) HBsAg-positive serum (11.08 IU/mL) was diluted with pooled normal serum and measured by the existing quantitative CLIA (○) and the CLEIA prototype (■).

TABLE 1. Dilution linearity for four HBsAg-positive samples

Sample	Dilution factor (DF)	Observed value (mIU/mL) × DF	Expected value (mIU/mL) × DF	Recovery rate (%)
HBsAg-positive 1*	Neat	35.0	35.0	100.0
	5	38.9		111.2
	25	37.2		106.4
HBsAg-positive 2*	Neat	67.8	67.8	100.0
	5	66.1		97.6
	25	78.2		115.3
HBsAg-positive 3*	Neat	264.7	264.7	100.0
	5	263.8		99.7
	25	281.0		106.2
No.9 from PHA808†	Neat	50.0	50.0	100.0
	5	51.2		102.4
	10	48.2		96.4
	20	61.4		122.7
	50	55.3		110.7
	100	56.9		113.8

* HBsAg-positive samples.
† The ninth sample obtained from the purchased panel PHA 808. The sample was calibrated against the international standard as 0.05 IU per mL by the panel vendor (Boston Biomedica, Inc.). The assay results were provided by the panel vendor.

while all the five points were below the cutoff value of the existing CLIA.

Normal sample distribution measured by the CLEIA prototype

Using the CLEIA prototype, we measured 230 serum and 273 plasma samples obtained from normal individuals. The results of the 503 normal samples were analyzed to set the cutoff value of the CLEIA prototype (Fig. 2). The mean and mean + 10 standard deviations (SDs) were 0.10 and 0.22 mIU per mL, respectively, and the cutoff point was set at 0.22 mIU per mL.

Performance in the commercially available seroconversion panels

A total of 18 commercially available seroconversion panels were tested using the CLEIA prototype. These panels were composed of serial specimens collected from individuals during a period of hepatitis B seroconversion. All the individuals became HBsAg-positive during the collection period, and two of the selected panels, namely, HBP 6281 and PHM 935B, were assessed as anti-HBs–positive according to the data supplied by the manufacturer. All panels were assessed as HBV DNA–positive before the appearance of detectable HBsAg when measured by the commercially available detection systems (data provided by the manufacturer). Serial specimens collected from 16 individuals during the hepatitis B seroconversion were tested using the CLEIA prototype. The detection time (days) for the CLEIA prototype was compared with that for the DNA detection and existing HBsAg detection systems (data provided). The mean number of days after which HBV DNA and HBsAg were first detected were 21.8 and 41.9 days, respectively, in the 16 panels; this implied a mean difference of 20.1 days (Table 2). The mean difference in the first HBsAg detection between the CLEIA prototype (24.5 days) and the existing systems (41.9 days) was 17.4 days. This showed that the CLEIA prototype was the most sensitive HBsAg detection assay compared with the other assays (data provided). The representative seroconversion panel with short sampling intervals was shown in Table 3. When

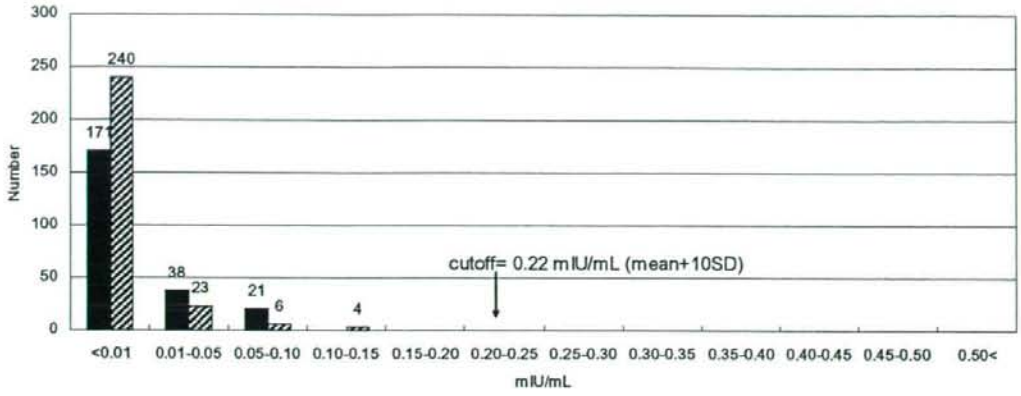

Fig. 2. The normal sample distribution was measured by the CLEIA prototype. To test the specificity of the CLEIA prototype, 230 serum samples (■) and 273 plasma samples (▨) obtained from HBV-negative individuals were measured. The highest values in the serum and plasma were 0.10 and 0.14 mIU per mL, respectively. The mean value in the 503 samples measured was 0.10 mIU per mL. The cutoff point was set at 0.22 mIU per mL as the mean + 10 SDs value.

TABLE 2. Comparison of the assay performances for HBV detection in seroconversion panels

	Days to detection from the first bleed			Window reduction by the CLEIA prototype compared with		
Panels	DNA	Existing HBsAg*	Prototype	Existing HBsAg detection system	DNA detection system	Earliest HBV detection
PHM 902†	57	71	57	14	0	DNA, prototype
PHM 903†	0	14	0	14	0	DNA, prototype
PHM 908†	0(+/–)	33	5	28	–5	DNA
PHM 909†	0	9	0	9	0	DNA, prototype
PHM 910†	18(+/–)	35	0	35	18	Prototype
PHM 911†	49	79	63	16	–14	DNA
PHM 912†	0	42	12	30	–12	DNA
PHM 914†	146	158	146	12	0	DNA, prototype
PHM 915†	0	33	0	33	0	DNA, prototype
PHM 916†	48	65	50	15	–2	DNA
PHM 919†	0(+/–)	19	0	19	0	DNA, prototype
PHM 920†	5(+/–)	26	26	0	–21	DNA
PHM 922‡	0	16	0	16	0	DNA, prototype
HBV 6277†	26	33	21	12	5	Prototype
HBV 6278†	0	12	0	12	0	DNA, prototype
HBV 6279†	0	26	12	14	7	Prototype
Mean	21.8	41.9	24.5	17.4	–2.7	

* The data provided for PHM 902 through PHM 922 (Boston Biomedica, Inc.) represent the results of an overnight assay performed by binding of the Auszyme MoAb to the HBsAg (Abbott Laboratories). The data for HBV 6277, HBV 6278, and HBV 6279 were provided by Zeptometrix Corp. (Buffalo, NY) and analyzed by PRISM HBsAg (Abbott Laboratories).
† The detection limit for the HBV DNA assay is 100 copies per mL.
‡ The detection limit for the HBV DNA assay is 10 copies per mL.

compared by signal-to-cutoff ratio, the CLEIA prototype showed approximately 110-fold higher sensitivity than PRISM (cutoff, 25 mIU/mL). In 8 of the 16 panels tested, HBsAg detection by the CLEIA prototype and HBV DNA detection by PCR (the limit of detection in the provided data was 100 copies/mL or less) occurred simultaneously. In 2 of the 16 panels tested, namely, PHM 910 and HBV 6277, the CLEIA prototype detected HBsAg earlier than PCR detected HBV DNA. In the remaining 6 panels, HBV DNA detection by PCR occurred earlier than HBsAg detection by the CLEIA prototype. The difference in the window period for HBV DNA and HBsAg detection was only 2.7 days.

The two panels that became anti-HBs–positive were also tested (Fig. 3). In PHM 935B, DNA was not detected (the limit of detection in the provided data was 400 copies/mL) in the late phase of infection. It was indicated that the PRISM HBsAg assay (Abbott Laboratories), which

TABLE 3. The result of HBV 6279

Panel	DNA PCR	HBsAg (S/CO) PRISM	HBsAg (S/CO) Prototype
HBV6279-01	278	0.36	0.6
HBV6279-02	NEG	0.33	1.7
HBV6279-03	Not tested	0.24	3.2
HBV6279-04	75.3	0.38	11.8
HBV6279-05	529	0.53	32.8
HBV6279-06	5110	1.63	107.8

S/CO = signal-to-cutoff ratio.

showed the highest sensitivity in the data provided, detected the 246th sample that had become anti-HBs–positive. The CLEIA prototype exhibited a higher sensitivity than PRISM; it detected all the samples in PHM 935B, even those in the anti-HBs–positive phase. In HBV 6288, DNA (the limit of detection in the provided data was 100 copies/mL) and HBsAg measured by the CLEIA prototype alone detected HBV in the first sample. In the late phase of HBV 6288, both DNA and HBsAg measured by PRISM did not detect HBV in the sample that became anti-HBs–positive (data provided). In contrast, the CLEIA prototype detected the last sample that was anti-HBs–positive.

Detection of HBsAg in chimpanzee HBV infectious models

The first detection day for Chimp 246 (genotype A) was Day 17 for HBV DNA at 120 copies per mL and Day 15 for HBsAg measured by the CLEIA prototype at 0.24 mIU per mL (Fig. 4A). In the late phase of infection, HBV DNA was detected at approximately 100 copies per mL by Day 334. In contrast, HBsAg persisted longer than HBV DNA, which was detected by Day 377. In Chimp 272 (genotype C), HBV DNA was detected at 300 copies per mL on Day 6 after inoculation, and HBsAg was detected by the CLEIA prototype on Day 10 from inoculation (Fig. 4B). In the latter phase of infection, HBV DNA was detected on 100 copies per mL at Day 189, and HBsAg measured by the CLEIA prototype was detected on Day 182. The first detection days of DNA measured by qualitative nested PCR and HBsAg measured by AxSYM (Abbott Laboratories) or the CLEIA prototype were compared in the samples from six chimpanzees (Table 4). The mean number of days which HBV DNA and HBsAg measured by AxSYM or the CLEIA prototype were 28.5, 56.7, and 38.2 days, respectively. The mean difference in the first HBsAg detection between AxSYM and the CLEIA prototype was 18.5 days. The difference in the window period for HBV DNA measured by qualitative PCR and HBsAg measured by the CLEIA prototype was 9.7 days.

DISCUSSION

NAT can detect a low-titer HBV infection. It is considered to be a superior technique for the early detection of HBV infection.[24,25,28] However, despite the regular use of minipool NAT for blood donor screening, the risk of posttransfusion HBV infection persists.[20-25] In this study, we developed an unprecedented high-sensitivity HBsAg detection prototype and investigated whether such an assay could reduce the risk of undesired HBV transmission.

For the development of the CLEIA prototype, we focused on the epitope that was the target region of the antibodies used for HBsAg detection. HBsAg is a four-transmembrane protein comprising 226 amino acid residues (amino acids 1-226). Although the transmembrane structure of HBsAg is not completely elucidated, it has been proposed that HBsAg is composed of an endoplasmic reticulum (ER) lumen region comprising amino acids at positions 1 to 11 from the N-terminal of HBsAg, a hydrophobic transmembrane region comprising amino acids at positions 12 to 28, a region inside the lipid bilayer comprising amino acids at positions 29 to 80, a hydrophobic transmembrane region comprising amino acids at positions 81 to 97, a hydrophilic ER lumen region comprising amino acids at positions 98 to 156, and two hydrophobic transmembrane regions comprising amino acids at positions 157 to 226.[33] A common determinant "a," which is used in the conventional methods for HBsAg detection, is contained in the amino acids at positions 110 to 156, particularly in the amino acids at positions 98 to 156 localized on the ER lumen side, that is, on the surface of the viral particle. It is indicated that the detection limit is improved when the MoAbs, each bound to a distinct epitope, are used in combination.[34] In this study, to detect the HBsAg more effectively, we used MoAbs that recognized HBsAgs localized inside the lipid bilayer, on the ER lumen side, and at the position of the common determinant "a." To enhance the effect of antibodies that recognize the inner epitope, detergents were added in the reaction buffer. It is confirmed that detergents were necessary for the reaction of such antibodies in the preliminary experiment. Detergents might help to exposure the epitope localized inside the lipid bilayer, and this is one of the characters of the CLEIA prototype. Furthermore, we tried to increase the detection limit by improving the tracer.[32] The improvement of the uniting ratio of the enzyme and antibody is the way to enhance the signal. However, especially for the high-molecular-weight molecule such as alkaline phosphatase, the high ratio conjugation often causes the aggregation. The aggregated tracers cause the high background and sometimes cause the low positive signal by covering the antigen-binding site of antibody with enzyme. In this report, we made the labeled tracer as a hydrophilic mass molecule and successfully prevented the aggregation. Although this is a simple method to improve sensitivity, which was only modified from the classical technique,[32] it can be applied to many types of conjugation.

The detection limit and cutoff value for the CLEIA prototype were investigated by standardizing the unit

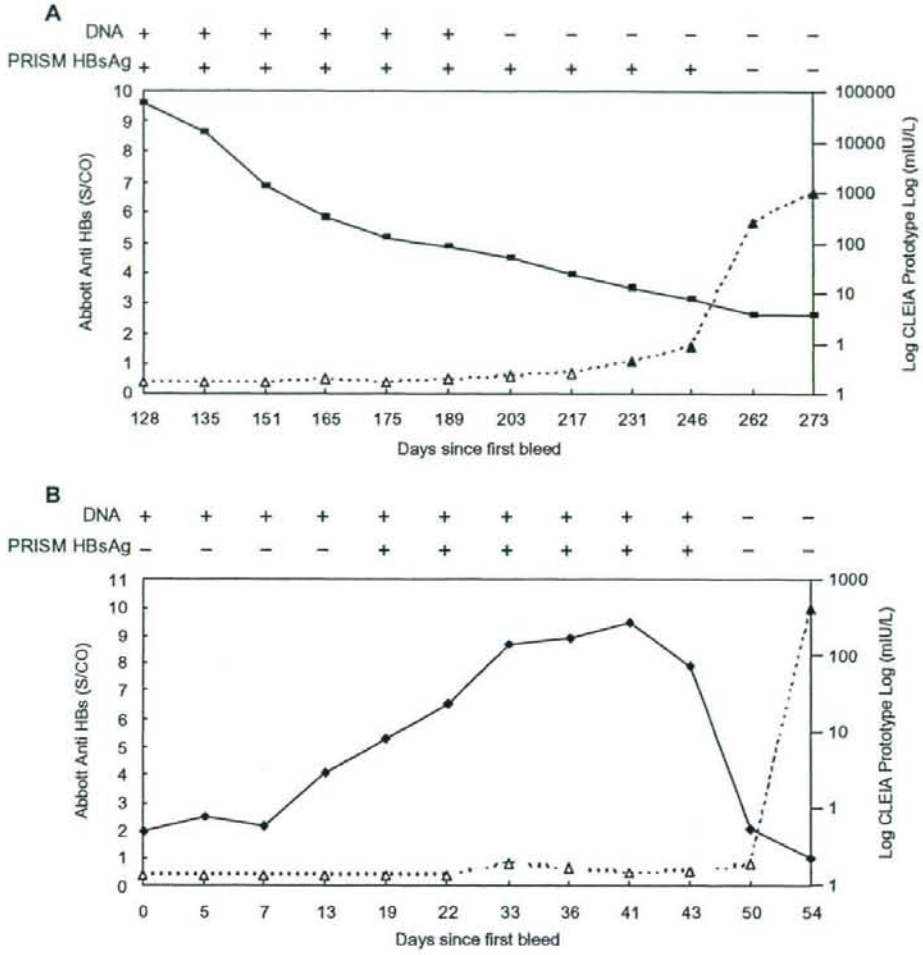

Fig. 3. The seroconversion samples that became anti-HBs–positive were measured by the CLEIA prototype. (A) PHM 935B (Boston Biomedica, Inc.). (B) HBV 6281 (Zeptometrix Corp.). The results of the CLEIA prototype (■) were compared with the DNA and PRISM HBsAg data provided (Abbott Laboratories). + = detected HBV DNA or PRISM HBsAg; − = under the detection limit for DNA or PRISM HBsAg. The appearances of anti-HBs in the serum samples were monitored using IMx AUSAB kits (Abbott Laboratories) supplied by the panel vendors. It is indicated as signal-to-cutoff ratio (S/CO). (△) Anti-HBs–negative; (▲) anti-HBs–positive. For detecting HBV DNA, the Roche Amplicor HBV Monitor assay with a detection limit of 400 copies per mL was used in PHM 935B, and the PCR conducted by the vendor with a detection limit of 100 copies/mL was used in HBV 6281.

against the WHO standard. In comparison of the cutoff point, the sensitivity of the CLEIA prototype (0.22 mIU/mL) was approximately 230-fold higher than that of the quantitative HBsAg detection system, ARCHITECT (50 mIU/mL), and 114-fold higher than that of the qualitative HBsAg detection system, PRISM (25 mIU/mL). It is suggested that the CLEIA prototype greatly improved the detection limit not only by the combination of MoAbs but also by the polymerization technique for conjugation. A dilution linearity test, which provides information about the precision of assay results for samples tested at different levels of dilution, was performed.

To evaluate whether the high-sensitivity HBsAg CLEIA prototype contributed to early detection of HBV, commercially available seroconversion panels were tested. The CLEIA prototype reduced the window period

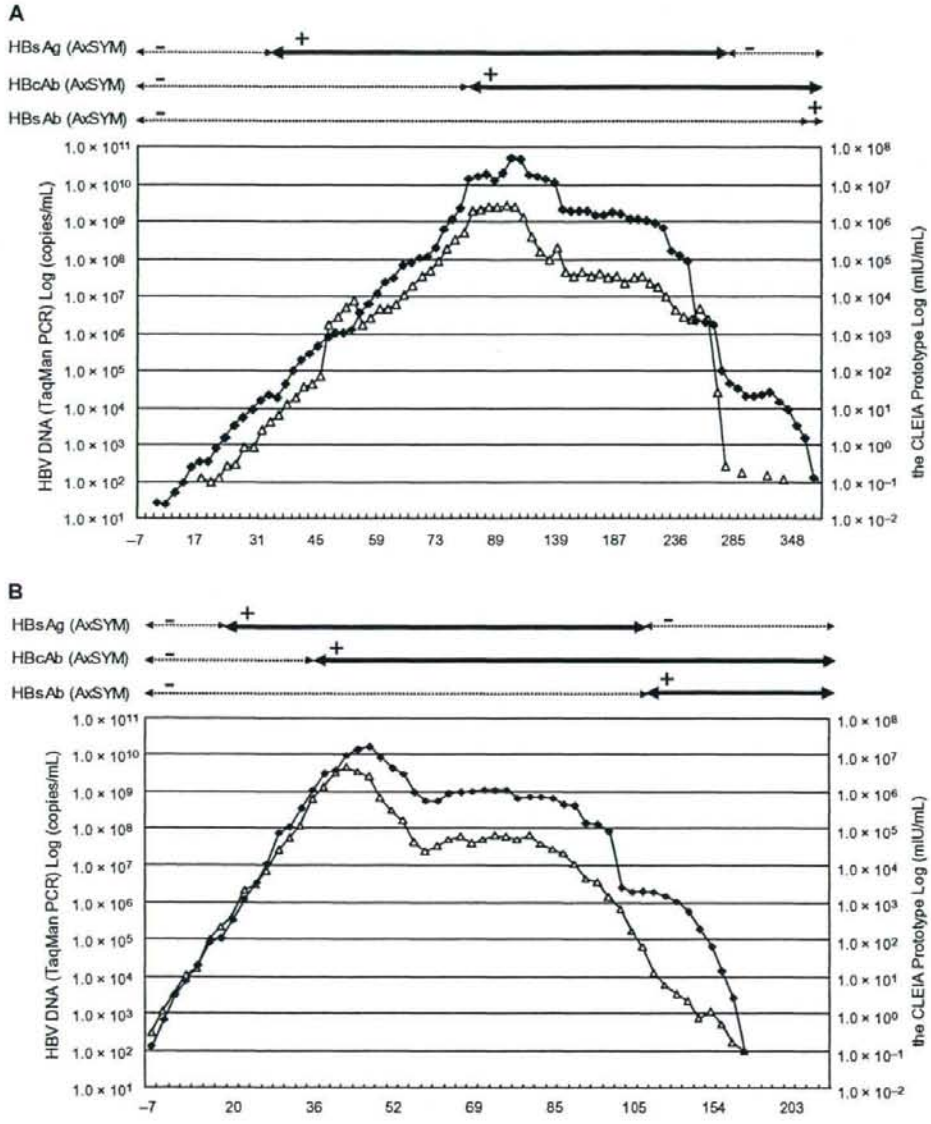

Fig. 4. The samples obtained from HBV-inoculated chimpanzees were tested by the CLEIA prototype. (A) The serum sample was collected from Chimp 246 between 1 week before inoculation and 410 days after inoculation of HBV. (B) The serum sample was collected from Chimp 272 between 1 week before inoculation and 238 days after HBV inoculation. (△) HBV DNA (TaqMan PCR); (◆) HBsAg measured by the CLEIA prototype. The data of HBsAg, HBcAb, and HBsAb measured by AxSYM (Abbott Laboratories) are shown above each graph (+ = detected; – = under the detection limit).

by 17.4 days compared with the existing CLIA; the reduced window period for HBsAg detection almost equaled that of DNA detection. In the tested 16 seroconversion panels, the results indicated that the CLEIA prototype detected HBsAg earlier than DNA in Panels PHM910 and HBV6277. However, for panel HBV6277, it was interpreted by the vendor that DNA was reactive from the first blood sample whereas it was below the level of quantitation. In addition,

TABLE 4. The first detection days of HBV DNA or HBsAg in six chimpanzees

Chimp	Genotype	Days to detection from first bleed			Windows reduction by prototype comparison with	
		DNA	AxSYM	Prototype	AxSYM	DNA
246	A	9	35	15	20	−6
280	A	55	97	69	28	−14
279	A	43	69	50	19	−7
272	C	6	22	10	12	−4
269	C	29	53	35	18	−6
285	C	29	64	50	14	−21
Mean		28.5	56.7	38.2	18.5	−9.7

since the detection limit of NAT is improved, it is considered that DNA will be detected earlier than the provided data in these two panels. This was indicated in the result of six chimpanzees compared with DNA measured by nested PCR.

The other characteristic of the CLEIA prototype was that it could detect HBsAg in the anti-HBs–positive seroconversion samples. The position of the determinant "a" is the major region detected by several commercially available HBsAg assays as well as by the naturally occurring anti-HBs after exposure to either the virus or the vaccine.[35-37] The CLEIA prototype was designed using many MoAbs for detecting various epitopes so that the HBsAg could be detected even in the anti-HBs–positive samples. It is reported that mutations occur at the many positions of "a"[17,38,39] and that individuals exposed to strains with mutations in the position of "a" may get infected despite possessing what are considered as protective titers of anti-HBs; such mutants may escape detection by some of the existing HBsAg assays.[40] Therefore, it is considered that the use of more than one MoAb to HBsAg is better.[39]

The CLEIA prototype was also assessed by measuring the serum samples derived from the chimpanzee infectious models. In the chimp infected with genotype A, HBsAg could be detected earlier than HBV DNA, both in the early and in the latter phases of infection. In contrast, in the chimp infected with genotype C, HBV DNA could be detected earlier than HBsAg both in the early phase and in the latter phases of infection. It is reported that the doubling time of genotype C was shorter than that of genotype A,[30] and the result suggested that HBsAg is affected by doubling of the HBV DNA and is correlated to the amplification. This ultrahigh-sensitive HBsAg detection is correlated to the HBV DNA, so it might be useful for monitoring patients under treatment. The utility of the CLEIA prototype for monitoring the patients in the latter phase of infection is currently being tested, and the possibility of its utility for monitor is being indicated at the moment (data not shown).

In this report, it is indicated that the sensitivity of the procedure that is commonly used for detecting HBsAg can be increased so that it reaches that of the procedure used for DNA detection. The high-sensitivity HBsAg CLEIA prototype could reduce the window period for HBV detection such that it almost equaled that of DNA detection, and it might also reduce the risk of HBV transmission caused by transfusion. Because the CLEIA prototype is not a specialized technique, it can also be performed in hospitals, and it may contribute toward reducing undesired HBV transmission. It has been reported that reactivation is induced when an individual's immune condition becomes suppressed as a result of chemotherapy, immunosuppressant treatment, or occult HBV infection.[41,42] It is suggested that the high-sensitivity HBsAg assay, which detects HBsAgs even in anti-HBs–positive samples, might be useful for monitoring chronic infection patients under treatment.

ACKNOWLEDGMENTS

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B型・C型ウイルス肝炎へのアプローチ
その1
実地医家に必要な基本的知識

ウイルス肝炎の疫学

わが国の肝炎ウイルス罹患状況と推定キャリア数

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広島大学大学院医歯薬学総合研究科疫学・疾病制御学／たなか・じゅんこ

わが国の肝癌死亡の年次推移・地理的分布

わが国の肝癌による死亡実数および人口10万人対死亡数を**図1**に示す(肝及び肝内胆管の悪性新生物，人口動態統計)．1970年代前半までは1万人以下であった死亡実数は増加の一途を辿り，1995年の国際疾病分類の改正(ICD-10)に伴い3万人を超えた後，さらに微増したが，2002年に至ってようやく頭打ちの状態となった(2002年34,637人，2004年34,510人，2006年33,662人)．人口10万人対死亡数を見ると，男女とも1970年代後半から増加傾向が認められること，男性は女性のほぼ2倍以上の高い死亡数を示していることが特徴的である(2006年，人口10万人対：男性36.7，女性17.2)．

日本肝癌研究会による調査成績[1]および人口動態統計資料を基に算出した成因別の肝癌死亡(人口10万人対)の年次推移をまとめて**図2**に示す．

HBV(B型肝炎ウイルス)の持続感染に起因する肝癌(B型の肝癌)による死亡は，1970年代から今日に至るまでほぼ増減がないまま，人口10万人対3～4の状態で推移しており，HBVの持

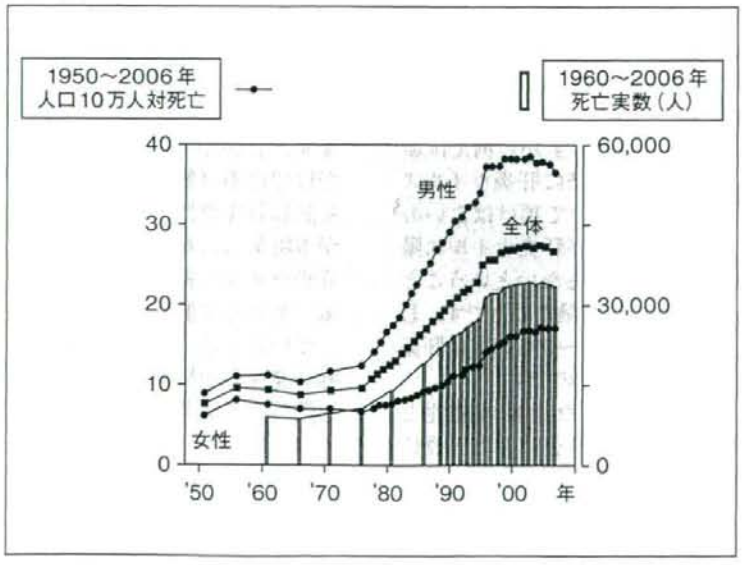

図1　わが国における肝癌*人口10万対死亡数および死亡実数の推移
*肝及び肝内胆管の悪性新生物(死因簡単分類：02106)

1345-1551/08/ ¥100/頁 /JCLS

- *わが国の肝癌による死亡実数は頭打ちの状態となった.*
- *わが国の「肝及び肝内胆管の悪性新生物」による死亡数は33,662人(2006年)(人口10万人対:男性36.7,女性17.2).*
- *B型の肝癌による死亡は,人口10万人対3～4.非B型の肝癌の約80～90%はC型の肝癌である.*

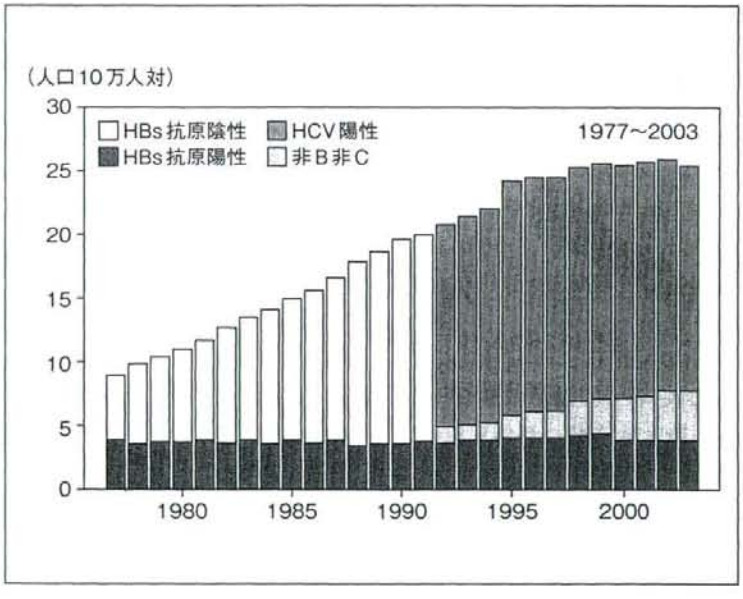

図2 わが国における成因別肝癌死亡の推移
(厚生労働省大臣官房統計情報部:人口動態統計.日本肝癌研究会:全国原発性肝癌追跡調査報告の数値を用いて概算)

続感染によらない肝癌(非B型の肝癌)による死亡が死亡率全体の増加に寄与していることがわかる.また,HCV(C型肝炎ウイルス)感染の確定診断が可能になった1992年以降についてみると,それまで非B型の肝癌とされてきた集団の約80～90%はHCVの感染に起因する肝癌(C型の肝癌)であることが明らかとなっている[2].

全国市町村別の肝癌標準化死亡比を三つの時期に分け図3に示す(年齢の偏りと小さい人口集団への偏りを補正したベイズ法による標準化死亡比推定量[3]).人口10万人あたりの肝癌死亡数が9人程度に止まり,B型の肝癌が全体の約半数を占めていた1970年代前半のSMR地理分布と比べ,人口10万人あたりの肝癌死亡が27人を超え,非B型の肝癌が全体の80%以上を占めるに至った2000年代前半のSMR地理分布では,非B型の肝癌(すなわちC型の肝癌)死亡の増加による時間的変化とともに,肝癌死亡の多発地域は駿河湾沿岸,大阪湾沿岸,中国地方の瀬戸内沿岸,そして九州北部を中心とした市町村への偏りが顕在化

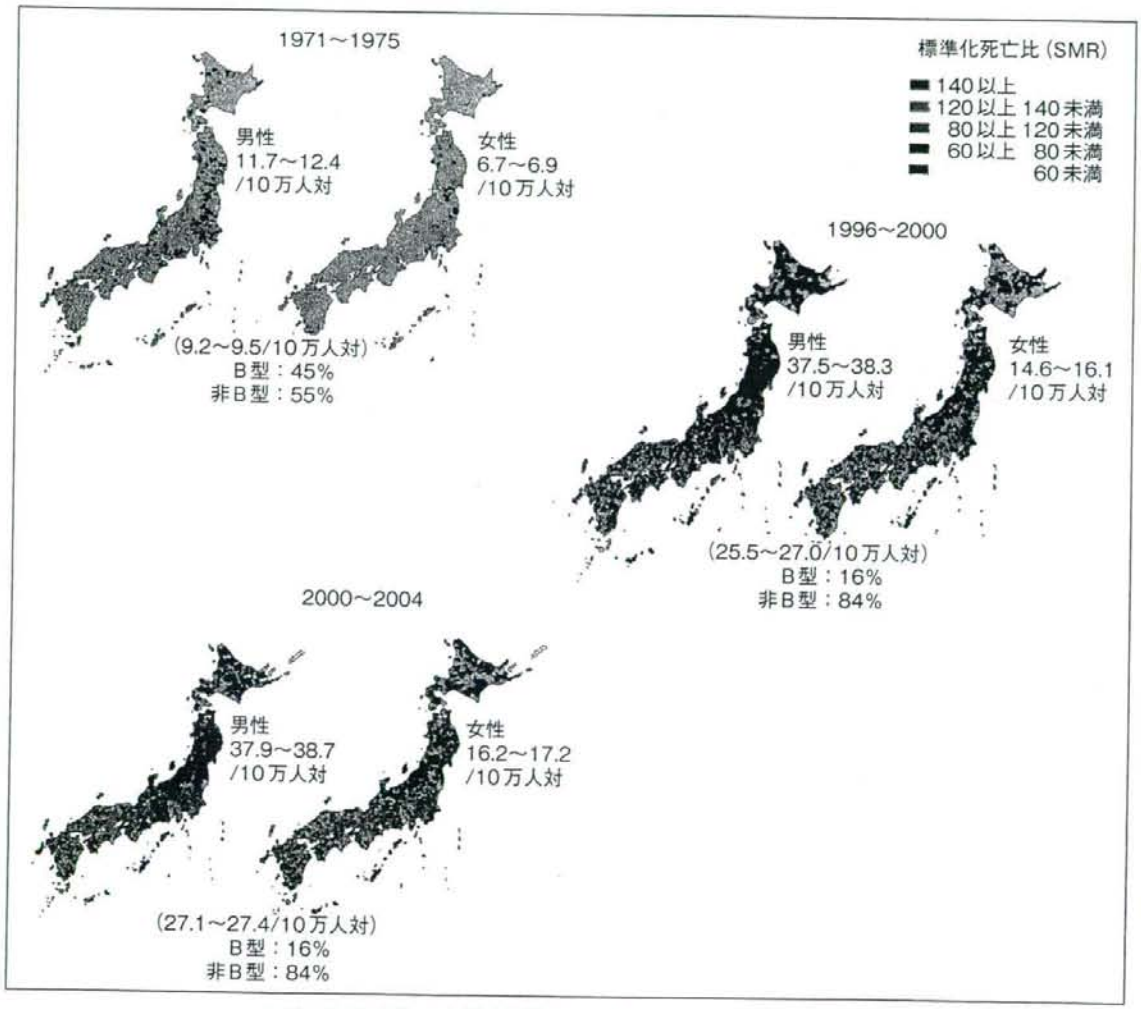

図3　市町村別にみた肝癌標準化死亡比(Bayesian method)の推移
(厚生労働省：肝炎疫学研究班より)

- *約350万人にのぼる初回供血者集団を対象とした調査から,*
  - *HBVキャリア率は全体では0.6%, 50歳代(2000年時点)の年齢集団では1.5%である.*
  - *北海道地区および, 近畿以西の西日本地区ではHBVキャリア率はやや高い値を示す.*
  - *HCV抗体陽性率は全体では0.5%, 年齢が高い集団で高い値を示す.*
  - *西日本地域の'40歳以上の年齢集団におけるHCV抗体陽性率は高い値を示す.*

していることがわかる.

一方, 肝癌による死亡率が高い都道府県順に1993年から2006年の14年間分を集計すると, 県ごとの年齢構成を調整していない粗死亡率での比較となるが, 10位以内にランクされる県の7割が中国・四国・九州地域に位置し, 近畿を含む西日本地域に拡大すればそのほとんどすべてを占めることがわかる. 肝癌による死亡が人口割合でみて高頻度に起こっている地域は西日本地域に偏在していることを示している.

性, 年齢, 地域別にみたHBs抗原陽性率とHCV抗体陽性率

わが国のいわゆる一般集団における肝炎ウイルス(HBV, HCV)の感染率は, 唯一, 日本赤十字血液センターの献血者の資料を元にして算出することができる. 血液センターでは, 毎年約600万本の献血された血液が輸血用血液の安全性を確保する目的で, 全国一律の基準により同一の試薬を用いて検査されている.

厚生労働省肝炎に関する疫学研究班と日本赤十字社との協力のもと, 1995年から2000年までの6年間に全国の血液センターで初めて献血した3,485,648人(初回献血者)を対象として, 地域別, 2000年の時点における年齢に換算した年齢階級別にHBs抗原陽性率, HCV抗体陽性率を算出した成績[4]を図4a, bに示す.

HBs抗原陽性率を全体でみると0.6%であるが, 年齢階級別にみると50歳代までは年齢が高い集団で高い値を示す傾向がみられる. 日赤基幹血液センターの所在地を中心に8つに分けた地域別にみると, 北海道地区では他の地域と比べて高いHBs抗原陽性率(1.5%)を示している. また, 近畿以西の西日本地区では関東・中部地域と比べて, やや高い値を示し(0.6～1.1%), 50歳代のみならず40歳代においてもHBs抗原陽性率が高値を示す傾向がみられる.

HCV抗体陽性率は全体でみると0.5%であり, 年齢が高い集団で高い値を示す傾向がみられる. 0～20歳代では0.2%以下の低い値を示すが, 年齢が高い集団, 特に, 60歳以上の年齢集団では3%を超える値を示している. 地域別にみると, 肝癌多発地域である西日本の各地域, すなわち九州, 中国・四国, 近畿地方の40歳以上の年齢層におけるHCV抗体陽性率が他の地域・年齢層に比べて高い値を示す傾向が認められる.

性, 年齢, 地域別にみたHBVキャリア数(推計)と, HCVキャリア数(推計)

現在, 社会に存在しているHBVキャリアおよびHCVキャリアは,「すでに患者として入院, または通院している」キャリアと,「自覚症状がないまま社会に潜在している」キャリアとに分けることができる. 前者については, 現時点におけるわが国では, 各種の患者調査成績を元に患者数の概数を推計しても, 実際の慢性肝疾患患者数の近

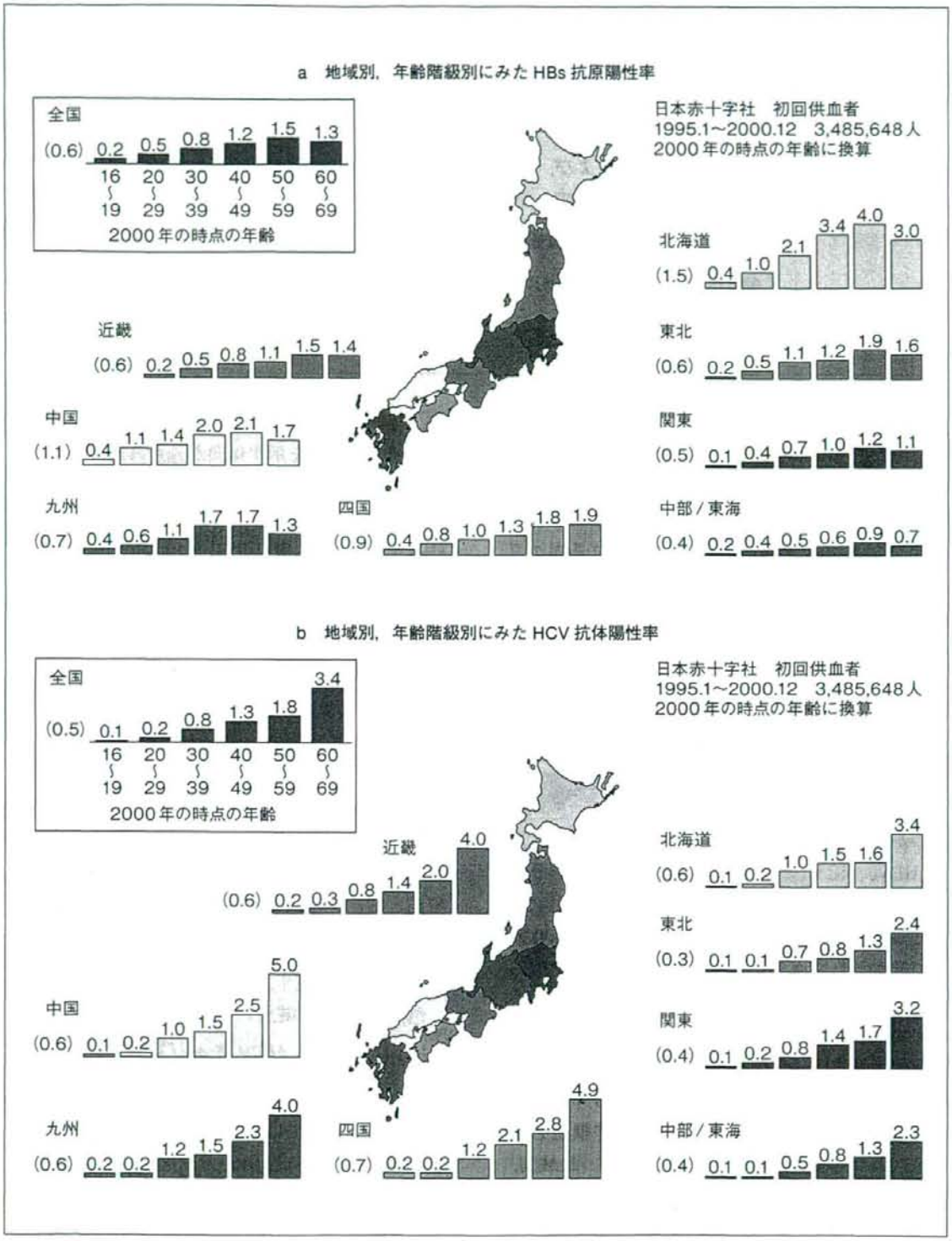

図4 地域別，年齢階級別にみたHBs抗原陽性率，HCV抗体陽性率
（厚生労働省：C型肝炎疫学研究班より）

- *2000年時点の人口約9,332万人(15歳から69歳)中,*
  「本人が自覚しないままの状態で社会に潜在している」
  キャリア数の推計値は,
  - *HBVキャリア数：96.8万人(80.7～112.9万人)*
  - *HCVキャリア数：88.5万人(72.5～104.5万人)*

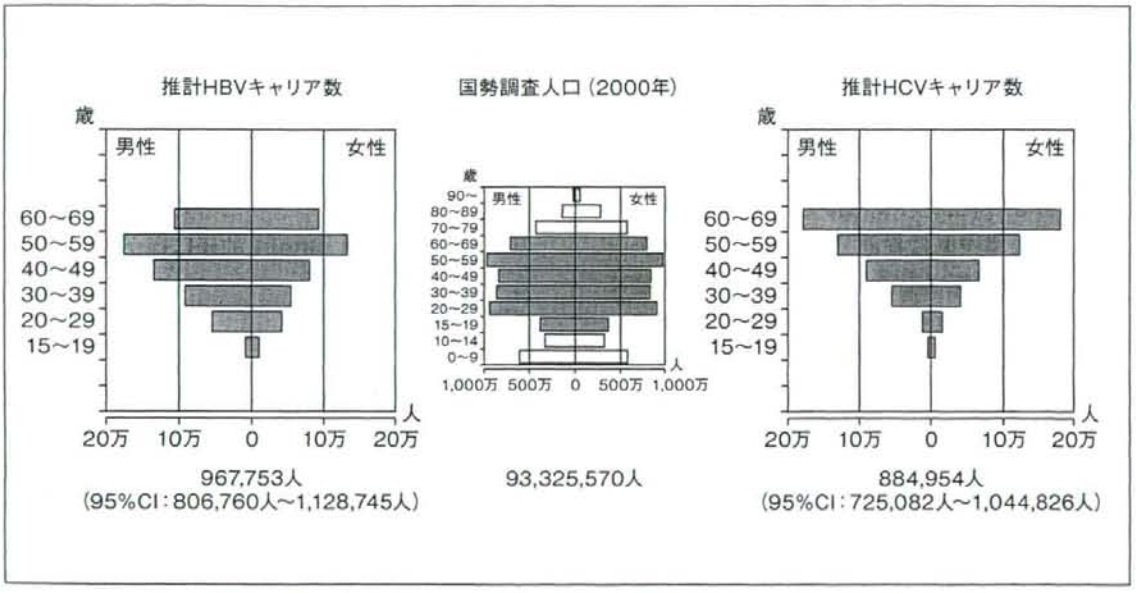

図5　わが国の人口ピラミッドと各年齢階級別にみた推計HBVキャリア数，HCVキャリア数
—15～69歳の年齢層における推計値—

似値を得ることはやや困難と考える.

一方，後者については，前項で示した日赤血液センターにおける献血者からみた年齢階級別のHBs抗原陽性率，HCV抗体陽性率と，HCV抗体陽性者の中に占めるHCVキャリア率(HCV抗体陽性者の70%)および，国勢調査資料から得られる年齢階級別人口から算出が可能[4]である．以上のことから，後者，すなわち「自覚症状がないまま社会に潜在している」HBVキャリア数，HCVキャリア数について推計したもの[4]を，2000年の時点におけるわが国の人口ピラミッドと併せて**図5**に示す.

2000年時点の15歳から69歳の人口約9,332万人の中に，本人が自覚しないままの状態で社会に潜在しているHBVキャリア数は96.8万人(80.7～112.9万人：95% 信頼区間)，HCVキャリア数は88.5万人(72.5～104.5万人：95% 信頼区間)と推計された．なお，この中には病・医院

● *肝発癌のリスクが高いと考えられる年齢層(40～69歳)において,「本人が自覚しないままの状態で社会に潜在している」キャリアの推計値は,71.4万人(HBV),75.9万人(HCV).*

へ通院もしくは入院しているウイルス肝炎の患者は含まれていないこと,また,献血時の問診により肝炎ウイルスに感染しているリスクのある人などはあらかじめ除外されることから,ここに推計したキャリア数は実態よりもやや低く見積もる可能性がある.しかし,約350万人にのぼる大規模調査の感染率を基に推計したキャリア数をbaseに,「通院・入院患者数」,「70歳以上の年齢層におけるキャリア数」などを加算することで,わが国のキャリア数全体を概算することができる.

2002年度から開始された肝炎ウイルス検診の対象年齢であり,肝発癌のリスクが高いと考えられる40歳から69歳の年齢層に限って,キャリア数を算出すると,当該人口約5,100万人中にHBVキャリアは71.4万人,HCVキャリアは75.9万人となった.これは,推計したキャリア数全体のそれぞれ,73.8%,85.8%と高率を占め,この年齢層にキャリアが偏在している[5]ことがわかる.また,図表には示していないが,推計キャリア数を地域別にみると,人口密度の高い関東地区,近畿地区,九州地区に多いことが明らかとなっている[5].

人口10万人あたりの肝癌死亡数は西日本地域で高い値を示したが,2006年当該死因による死亡実数33,662人のうち,大阪3,014人,東京2,851人,福岡2,024人,兵庫1,857人,神奈川1,782人,埼玉1,479人であり,前述の地域と重なることがわかる.

一方,広島県赤十字血液センターにおいて献血を契機に見出されたHCVキャリアに対する初診時の肝臓専門医による臨床診断の成績から,約52%が慢性肝炎と診断され,かつそのうち半数は初診後ただちに治療を開始したこと[6]からも,検診などを契機にみつかった自覚症状がないキャリアに対する診断・治療を的確に行う必要があると考えられる.

おわりに

わが国では,HBV母子感染予防対策事業による若年者集団(22歳以下)におけるHBVキャリア率の低下や,輸血用血液の安全性の向上などに加え,過去10年余にわたる広汎な血清疫学的調査[7]の結果から,特別な場合[8,9]を除き,新たなHBVキャリア,HCVキャリアの発生が低率である[10,11]ことが明らかとなっている.

これらの疫学的根拠と肝炎,肝癌治療の進歩を背景に,肝癌死亡の減少をめざしたキャリア対策の一環として,2002年から40歳以上の地域住民を対象とした肝炎ウイルス検査が開始されている.厚生労働省担当課の集計では,5年間にHBVキャリア100,983人,HCVキャリア99,950人が見出されている(受診者はそれぞれ約870万人,約863万人)が,肝炎ウイルス検診受診率,検診により見出されたキャリアの病・医院受診率は必ずしも十分ではない状況であることなどから,2008年度以降,国は医療補助も含めて「新し

い肝炎総合対策」を推進している状況にある．

今後は，地域・年齢ごとに潜在・偏在するキャリアの分布を念頭において，肝炎ウイルス検診の実施体制も含めた受診率の向上，キャリアの拾い上げから適切な治療に至る組織的な対応策を地域単位で確立し，実施に移すことが求められている．

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肝炎シリーズ [3]

透析医療施設における HCV 感染対策

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◆1．はじめに

これまでの調査から，近年の我が国の一般集団における C 型肝炎ウイルス（HCV）の新規感染は，ごく稀に起こるに過ぎないことが明らかになっている[1)2)]．しかし，透析医療施設など日常的に観血的処置を行っている医療現場における HCV 感染のリスクは，国際的にみても高い値を示すことが知られており[3)]，感染予防対策を立てることが必要とされている．

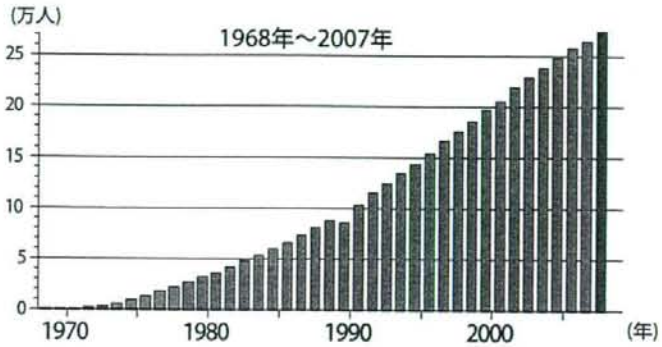

図 1　わが国の透析患者数の推移（文献[6)]より作図）

HCV 感染と B 型肝炎ウイルス（HBV）感染は，血液を介する感染であること，ウイルスの持続感染状態（キャリア）を持つこと，肝発がんとの関連が認められることなどの共通点が認められるが，C 型肝炎ウイルスは RNA 型，B 型肝炎ウイルスは DNA 型のウイルス構造を呈し，その感染病態は大きく異なる．

HCV に感染した場合，その 70％前後がキャリア化することから，閉鎖された集団において感染のリスク行為が繰り返された場合，感染源としての HCV キャリアが累積し最終的には，その集団内に HCV の感染爆発がおこることが，田辺らの調査成績[4)]から明らかとなっている．

本稿では，HCV キャリア率が高い環境下で観血的処置を繰り返すことから，感染発生のリスクが高いことが知られている透析医療施設における実態を把握することを目的とした前向き調査の成績[5)]と，感染予防対策のあり方，およびパイロットスタディの結果について紹介してみたい．

◆2. 慢性透析患者数の推移

日本透析医学会の統計調査委員会から出されている資料[6)]によると，慢性透析療法を受けている患者数はこの 20 年間，年々 1 万人余ずつ増え続けており，2007 年末の時点における患者数は 27 万 5119 人に達していることが明らかとなっている（図 1）．また，透析導入に至った原因疾患は 1998 年を境に糖尿病性腎症が第一位となり，2007 年には全体の 43.4％を占めるに至っていることが明らかとなっている（慢性糸球体腎炎 24.0％）．

◆3. 血液透析患者集団における HCV キャリア率

著者らは，透析医療施設における HCV 感染の実態を把握し，その感染予防対策を確立することを目的として 3 年余にわたる前向き調査を行った．1999 年 11 月から 3 ヵ月ごと，計 14 回にわたっ