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# Hepatitis B Virus DNA-negative Dane Particles Lack Core Protein but Contain a 22-kDa Precore Protein without C-terminal Arginine-rich Domain*

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**DNA-negative Dane particles have been observed in hepatitis B virus (HBV)-infected sera. The capsids of the empty particles are thought to be composed of core protein but have not been studied in detail. In the present study, the protein composition of the particles was examined using new enzyme immunoassays for the HBV core antigen (HBcAg) and for the HBV precore/core proteins (core-related antigens, HBcrAg). HBcrAg were abundant in fractions slightly less dense than HBcAg and HBV DNA. Three times more Dane-like particles were observed in the HBcrAg-rich fraction than in the HBV DNA-rich fraction by electron microscopy. Western blots and mass spectrometry identified the HBcrAg as a 22-kDa precore protein (p22cr) containing the uncleaved signal peptide and lacking the arginine-rich domain that is involved in binding the RNA pregenome or the DNA genome. In sera from 30 HBV-infected patients, HBcAg represented only a median 10.5% of the precore/core proteins in enveloped particles. These data suggest that most of the Dane particles lack viral DNA and core capsid but contain p22cr. This study provides a model for the formation of the DNA-negative Dane particles. The precore proteins, which lack the arginine-rich nucleotide-binding domain, form viral RNA/DNA-negative capsid-like particles and are enveloped and released as empty particles.**

Hepatitis B virus (HBV)[1] infects more than 300 million people and is a major cause of liver diseases. The HBV belongs to the Hepadnavirus family and is a small (42 nm) enveloped DNA virus, which possesses a 27-nm icosahedral nucleocapsid composed of core protein and a 3.2-kb partially double-stranded, circular genome (1). Although the term "Dane particles" refers to the 42-nm HBV particles (2) and is often used in reference to the complete HBV particles, electron microscopic studies have suggested that the DNA-negative "empty" Dane particles are predominant in sera (3–6). The capsids of the empty particles are thought to be composed of core protein but have not been studied in detail.

The HBV genome encodes two core-related open reading frames, precore and core genes (Fig. 1). These are expressed because of two in-frame ATG initiation codons located at the 5′ end of the genes. The first ATG encodes a 25-kDa protein (p25) containing the 29-amino acid (aa) precore sequence fused to the N terminus of the HBV core antigen (HBcAg). The p25 is directed toward the secretory pathway by a 19-aa signal sequence that is cleaved during translocation into the lumen of the endoplasmic reticulum (ER), producing a 22-kDa protein. Subsequent proteolytic cleavages within the arginine-rich C-terminal region (34 aa) generate a 17-kDa protein that is secreted as hepatitis B e antigen (HBeAg) (7–10). A heterogeneous population of these precore derivatives has been observed in the sera of patients and is serologically defined as HBeAg (9, 11, 12). Conversely, the second ATG specifies the 21.5-kDa HBcAg, which assembles into dimers that form the virus capsid (7, 9, 13–15). HBcAg is a 183-residue protein with two domains, the assembly domain that forms the capsid and the C-terminal arginine-rich domain that is responsible for RNA packaging (Fig. 1). The assembly domain, lacking the C-terminal domain, is sufficient for self-assembly into capsid particles. The arginine-rich C-terminal domain is involved in binding to the HBV RNA pregenome or the HBV DNA genome but is dispensable for HBV capsid assembly in *Escherichia coli* (16–19) and insect cells (20). The capsid is enclosed within an envelope containing the viral glycoprotein surface antigen (HBsAg) and released to the circulation as Dane particles.

We previously developed enzyme immunoassays (EIAs) for HBcAg (21) and HBV core-related antigens (HBcrAg) (22, 23). Serum specimens were pretreated with SDS to release and denature antigens and to inactivate antibodies. The HBcAg assay specifically measures core protein (21), and the HBcrAg assay measures precore/core proteins, including core protein and HBeAg (22, 23).

The present study investigated precore/core proteins in HBV-infected human sera using the new assays. The results suggest that most of the Dane particles were DNA-negative and were composed of a 22-kDa precore protein containing the uncleaved signal peptide and lacking the C-terminal arginine-rich domain. We present a new model for the formation of HBV DNA-negative particles.

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[1] The abbreviations used are: HBV, hepatitis B virus; HBcrAg, HBV core-related antigens; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; EIA, enzyme immunoassay; aa, amino acid; ER, endoplasmic reticulum; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; HBeAb, hepatitis B e antibody; rHBcAg, recombinant HBcAg; rHBeAg, recombinant HBeAg; LC, liquid chromatography; MS/MS, tandem mass spectrometry.

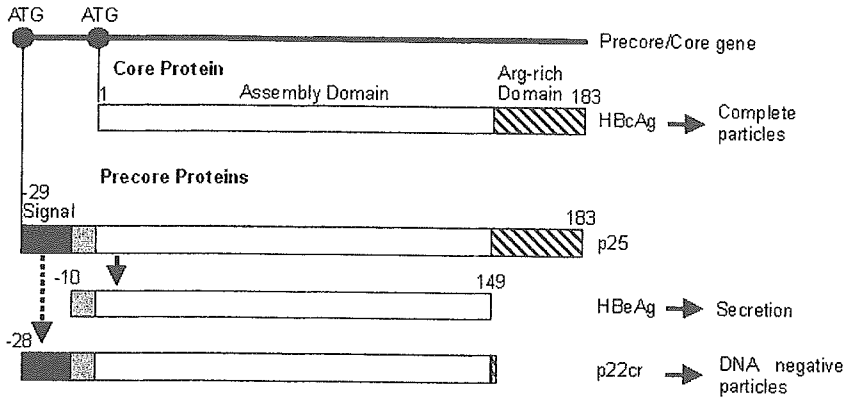


FIG. 1. **Schematics of precore/core genes and their products.** At its 5′ end, the precore/core gene contains two closely spaced ATGs (*black dots*) enclosing the precore region, which encodes a 29-aa precore sequence. Translation of the core mRNA results in the production of the cytoplasmic core protein, which assembles into icosahedral capsids enclosing the RNA pregenome, and then the nucleocapsids are enveloped and released as complete particles. Precore protein p25 is directed to the ER by a 19-aa-long signal sequence (*black boxes*) located at its N terminus. This signal sequence is removed during translocation into the ER, and then the C-terminal 34-aa-long arginine-rich domain (*hatched boxes*) is eliminated. Mature HBeAg is then secreted. p22cr is a novel precore protein identified with HBV DNA-negative particles.

## EXPERIMENTAL PROCEDURES

*Serum/Plasma Samples*—Hepatitis B plasma panels were purchased from Boston Biomedica, Inc. (BBI, West Bridgewater, MA), or ProMedDx (Norton, MA). Clinical sera were collected between 1997 and 2001 at the Shinshu University Hospital (Matsumoto, Japan) from patients with persistent HBV infection. Thirteen of these serum samples containing ≥0.05 ng/ml HBcAg were immunoprecipitated to examine HBcAg/HBcrAg ratios. Of the 30 total serum samples (from 23 males and 7 females), 22 were HBeAg-positive, and 7 were HBeAb-positive. The remaining sample was positive for both HBeAg and HBeAb. None of the 30 patients was treated with anti-viral agents such as interferon or lamivudine. All sera were stored at −30 °C or below until testing. The study design conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions involved in this study. A written informed consent was obtained from each patient.

*Recombinant HBV Core-related Antigens*—Recombinant HBcAg (rHBcAg, aa 1–183) and HBeAg (rHBeAg, aa −10–149) were expressed and purified as described (21, 22). The concentration of these antigens was determined using the BCA protein assay kit (Pierce) and bovine serum albumin standards according to the manufacturer's instructions.

*Monoclonal Antibodies and EIAs for HBcAg or HBcrAg*—Anti-HBcAg and anti-HBcrAg monoclonal antibodies were established as reported previously (21, 22). The HBcAg-specific monoclonal antibody, HB50, recognizes SPRRR repeats in the arginine-rich domain of HBcAg (21), whereas the anti-HBcrAg monoclonal antibody, HB91, recognizes aa 1–19 of HBcAg and thus reacts to denatured HBcAg, HBeAg, and other precore/core proteins (22).

HBcAg and HBcrAg were measured by EIA as described previously (21–23). The assays contain a sample pretreatment step that inactivates antibodies and dissociates antigens in samples. The assays can thus detect antigens within the viral envelope or complexed with antibodies in addition to free antigens.

*HBV Markers and HBV DNA Measurement*—HBeAg and HBsAg were measured by radioimmunoassay or by chemiluminescent immunoassay (Abbott, Tokyo), respectively. HBV DNA was detected by PCR using the Amplicor HBV monitor test (Roche Applied Science). Samples showing values over the detection range were remeasured after dilution to obtain quantitative results.

*Sucrose Density Gradient Ultracentrifugation*—Aliquots (1.7 ml) of 10, 20, 30, 40, 50, and 60% (w/w) sucrose in a solution containing 10 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA (pH 7.5) were carefully layered in a 12-ml Ultracentrifuge tube and left at room temperature for 6 h. HBeAg-positive plasma (0.1–1.0 ml) was layered on this sucrose gradient, and ultracentrifugation was performed at 200,000 × *g* for 15 h at 4 °C in a Beckman Sw40Ti rotor. Fractions were collected from the top to the bottom of the gradient. The density of each fraction was calculated from the weight and volume. Each fraction was diluted 10-fold and tested for HBcAg and HBcrAg as well as for HBsAg, HBeAg, and HBV DNA.

*Immunoprecipitation*—Immunoprecipitation was carried out using magnetic beads coated with polyclonal anti-HBsAg from the "HBV-Direct Mag kit" (JSR Corp., Tokyo) (24). A 200-μl aliquot of sample was mixed with 50 μl of reaction buffer from the kit and 50 μl of a magnetic bead suspension. The mixture was incubated for 30 min at room temperature with gentle agitation and then magnetically separated. HBcAg and HBcrAg in supernatant and precipitate were measured by EIA. Because some samples contain a large amount of HBsAg, which exceeds the capacity of anti-HBsAg beads, if the precipitated HBcAg ratio was less than 90%, the sample was diluted 10- or 100-fold and then reimmunoprecipitated.

*Electron Microscopy*—A 500-μl aliquot of HBV-positive plasma was subjected to ultracentrifugation on linear 10–50% (w/w) sucrose density gradients. The high density HBcrAg peak fractions (corresponding to Fig. 3*A*, fractions 23 and 24) and HBcAg peak fractions (corresponding to Fig. 3*A*, fractions 25 and 26) were separated by the second ultracentrifugation through linear 35–50% (w/w) sucrose density gradients. The fractions were fixed by adding paraformaldehyde solution to a final concentration of 4%. A 4-μl aliquot of each fraction was diluted in 90 μl of distilled water in 5-mm diameter polyallomer centrifugation tubes (Beckman Instruments), and copper grids filmed with Formvar membranes and treated additionally with poly-L-lysine were placed on the bottom of the tubes in the solution. Ultracentrifugation (200,000 × *g*, 4 °C, 2 h) was performed in a Beckman TLS-55 swinging bucket rotor to concentrate the virus particles and allow them to attach to the Formvar membranes on the copper grids. Afterward, the attached virus particles were negatively stained with 4% uranyl acetate and observed at an accelerating voltage of 80 kV in an electron microscope (H-7500, Hitachi, Tokyo). Fifteen electron micrographs of the virus particles from each fraction were taken randomly at a magnification of ×80,000. The number of virus particles in the 3.76 $\mu m^2$ area was then counted on each electron micrograph. The diameters of the virus particles in each fraction were also measured.

*Western Blot Analysis*—Samples were subjected to SDS-PAGE through a 15–25% polyacrylamide gel under reducing conditions. Proteins in the gel were electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore) at 15 V for 45 min. The membrane was blocked and probed using alkaline phosphatase-conjugated HB50 (for HBcAg) or HB91 (for HBcrAg) monoclonal antibody at room temperature for 1 h. The membrane was washed and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (KPL, Gaithersburg, MD) for 15 min (for HBcrAg) or 90 min (for HBcAg), respectively.

*N-terminal Amino Acid Sequence Analysis*—A 6-ml aliquot of HBV-positive plasma was subjected to ultracentrifugation on linear 10–60% (w/w) sucrose density gradients, and subsequently the high density HBcrAg peak fractions (Fig. 3*A*, fractions 23 and 24) were separated by gel filtration through Superose 6 HR (Amersham Biosciences). Void fractions were collected and ultracentrifuged at 200,000 × *g* for 15 h at 4 °C using a Beckman SW 50.1 rotor. The precipitate was separated by SDS-PAGE and electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore) at 15 V for 45 min. Proteins on the membrane were stained using Coomassie Brilliant Blue-R250. The N-terminal amino acid sequence of the 22-kDa band was analyzed using the Procise 494 cLC protein sequencing system at the Apro Life Science Institute, Inc. (Tokushima, Japan).

*Mass Spectrometry Analysis*—The 22-kDa protein was purified as described above. The 22-kDa band was cut from the SDS-polyacryl-

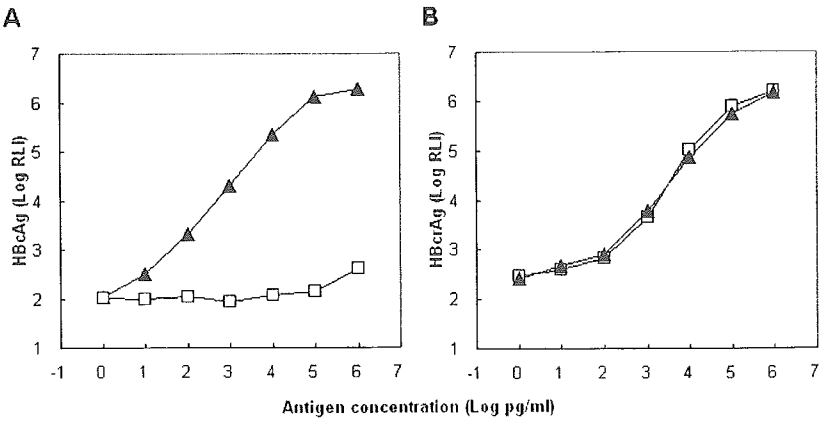


FIG. 2. **Reactivity of the HBcAg assay and the HBcrAg assay.** Recombinant HBcAg (▲) and HBeAg (□) were diluted and tested for the HBcAg assay (*A*) and the HBcrAg assay (*B*). The assay reactivity is shown as log relative luminescence intensity (*RLI*).

amide gel and digested in-gel by trypsin at 35 °C for 20 h. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) of the digested sample was performed on a Voyager-DE STR (Applied Biosystems) in positive ion reflection mode. External mass calibration was performed using four points bracketing the mass range of interest. Results were analyzed using the NCBI nonredundant data base (molecular mass range 15–30 kDa) by the MS-Fit 3.1.1 ProteinProspector 3.2.1 program (University of California), taking into account probable post-translational modifications. LC-MS/MS was performed using a Q-Tof2 (Micromass, Manchester, UK) quadrupole time-of-flight electrospray ionization mass spectrometer in nanoflow LC ionization mode. The analyses were performed at the Apro Life Science Institute, Inc.

*Statistical Analyses*—The virus particle numbers on each electron micrograph were statistically compared by Welch's *t* test. The diameters of the virus particles were statistically compared by Student's *t* test. Paired *t* tests were used to analyze differences between log concentrations of HBcrAg and those of HBcAg. Differences were considered significant at $p < 0.05$.

## RESULTS

*Specificity of HBcAg and HBcrAg EIAs*—The specificity of the HBcAg and HBcrAg assays was confirmed by using rHBcAg and rHBeAg. The HBcAg assay specifically reacted to rHBcAg but not to rHBeAg (Fig. 2*A*). The HBcrAg assay reacted equally to rHBcAg and rHBeAg (Fig. 2*B*).

*Density Distribution of HBV Precore/Core Proteins*—HBV DNA-positive plasma (ProMedDx 9990776, HBsAg-positive, HBeAg-positive, HBV DNA 9.1 log copies/ml) was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient. Fractions were tested for HBcAg, HBcrAg, HBsAg, HBeAg, and HBV DNA (Fig. 3*A*). HBcAg appeared in the high density fractions and peaked in the same fraction (fraction 25) as HBV DNA. HBsAg was distributed in fractions of lower density, and HBeAg was dispersed widely in fractions of much lower density. HBcrAg peaked in fraction 24, slightly lower in density than the HBV DNA and HBcAg peaks in addition to a peak corresponding to HBeAg at much lower density. The concentration of HBcrAg in fraction 24 was 13-fold higher than the concentration of HBcAg in fraction 25. The high density HBcrAg peak was therefore predominantly composed of precore proteins other than core protein.

High density HBcrAg fractions (Fig. 3*A*, fractions 23–26) were reanalyzed under gentler (30–50%) sucrose density gradient sedimentation (Fig. 3*B*). HBcrAg concentration peaked in lower density fractions than HBcAg and HBV DNA, indicating that high density HBcrAg clearly differs from HBcAg. HBsAg concentration exhibited a shoulder at the HBcrAg peak fraction.

*Immunoprecipitation by Anti-HBsAg*—Sucrose density fractions (Fig. 3*A*) were immunoprecipitated by the anti-envelope protein HBsAg. Most of the HBcAg (97.5, 97.8, 96.2, and 95.1% from fractions 23–26) was precipitated by anti-HBsAg. Although more than 94% (94.5, 94.1, and 94.3% from fractions 7, 10, 13) of low density HBcrAg was observed in the supernatant, more than 96% (96.2, 96.8, 96.9, and 96.5% from fractions 23–26) of high density HBcrAg was in the precipitate. These data suggest that similar to the core protein, the high density HBcrAg exists in enveloped particles.

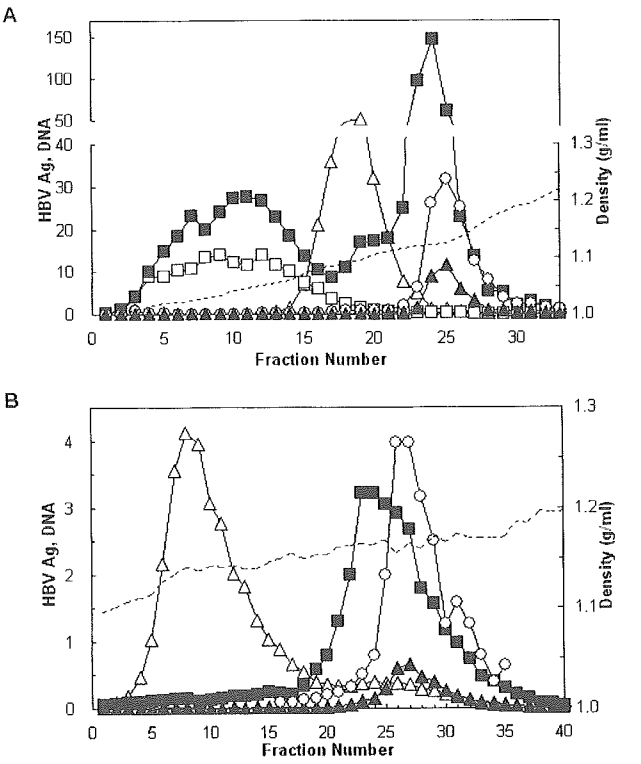


FIG. 3. **Sucrose gradient analysis of HBV-positive plasma.** *A*, ProMedDx HBV plasma 9990776 was subjected to ultracentrifugation using a 10–60% (w/w) sucrose density gradient. *B*, fractions 23–26 were reanalyzed by ultracentrifugation in a 30–50% sucrose density gradient. Density of each fraction is shown as a *broken line*. Fractions were diluted 10-fold and tested for HBeAg (□) (×10 signal/cutoff), HBsAg (△) ($\times 10^2$ IU/ml) in *A* and (IU/ml) in *B*), HBcrAg (■) (ng/ml), HBcAg (▲) (ng/ml), and HBV DNA (○) ($10^6$ copies/ml).

*Stability of HBcrAg Particles*—The HBV core forms very stable capsid particles resistant to denaturing pH, temperature, or detergents (25). Particle fractions of HBV-positive plasma were treated with or without 3% Nonidet P-40 detergent at 37 °C for 30 min and then subjected to gel filtration through Superose 6 HR (exclusion limit = $4 \times 10^7$ Da). Fractions were tested for HBcrAg and HBcAg. Regardless of Nonidet P-40 treatment, HBcrAg and HBcAg appeared in the void fractions (Fig. 4), indicating that HBcrAg formed high molecular mass (> $10^7$ Da) particles resistant to 3% Nonidet P-40

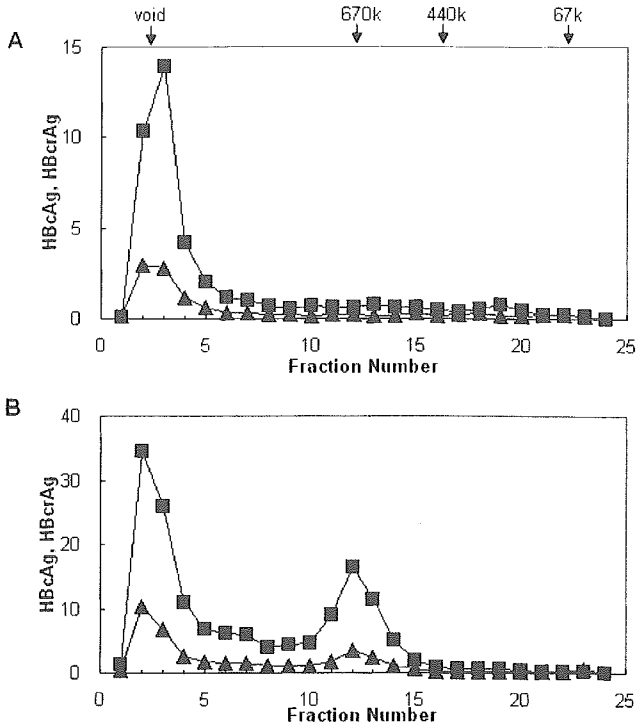


FIG. 4. **Gel filtration analysis of the particle fractions.** The particle fractions of a density gradient were treated without (*A*) or with 3% Nonidet P-40 detergent (*B*) and then subjected to gel filtration through Superose 6 HR. The elution from 6 to 18 ml were fractionated into 24 fractions and tested for HBcrAg (■) ($\times 10^2$ pg/ml) and HBcAg (▲) ($\times 10$ pg/ml).

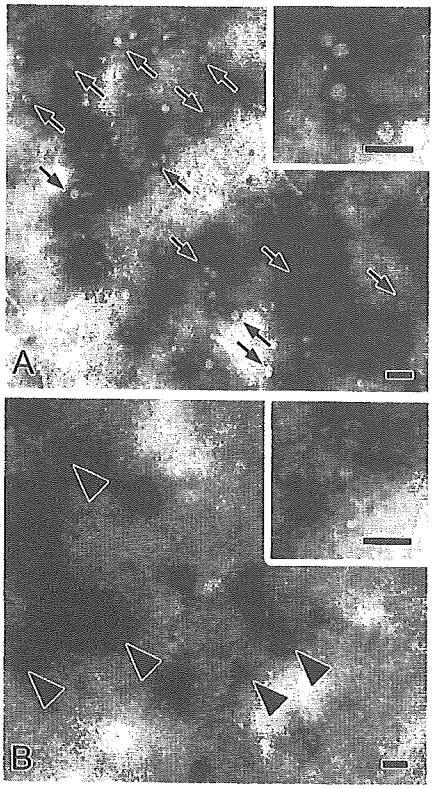


FIG. 5. **Electron micrographs of virus particles in the two density gradient fractions of HBV-positive plasma.** The particles in equal volumes of each fraction (Table I) were collected on copper grids by ultracentrifugation and negatively stained and observed under an electron microscope. Dane-like particles can be seen on the electron micrographs of both *fraction A* (*upper panel*, *arrows*, and *inset*) and *fraction B* (*lower panel*, *arrowheads*, and *inset*). *Bars*: 100 nm.

treatment, as did the HBcAg.

*Electron Microscopy*—HBcAg and HBcrAg in plasma 9990776 were separated by sequential sucrose density ultracentrifugation. The resultant HBcrAg-rich fraction (fraction A) contained 6.06-fold more HBcrAg than the HBcAg-rich fraction (fraction B) but contained only 3 and 38% of the HBV DNA and HBcAg, respectively, found in fraction B (Table I). Virus particles in the two fractions were concentrated and attached to the copper grids by ultracentrifugation and then negatively stained and observed under the electron microscope. Although virus particles appearing similar to Dane particles were observed in fraction B, more such Dane-like particles were seen in fraction A (Fig. 5), which contained HBV DNA at only 3% of that in fraction B. Fraction A contained 17.9 ± 11.6/3.76 $\mu m^2$ Dane-like particles, which was significantly more than in fraction B (5.6 ± 3.8/3.76 $\mu m^2$) ($n = 15$, $p < 0.001$) (Table I). The Dane-like particles in fractions A and B were not morphologically distinguishable (Fig. 5) but were quite similar to those reported previously (2–4, 6). The mean diameters of the measured particles were 41.5 ± 2.2 nm in fraction A and 42.0 ± 2.2 nm in fraction B (Table I). The mean diameters were not significantly different from one another ($n = 60$, $p = 0.27$) and were similar to the sizes reported previously (2).

*Identification of Particle HBcrAg as a 22-kDa Precore Protein (p22cr) Lacking the C-terminal Domain*—HBV DNA-positive plasma (BBI PHM935A-14) was subjected to a 10–60% sucrose density gradient and fractionated into 15 fractions. The fractions were then analyzed by Western blotting using monoclonal antibodies for HBcAg and HBcrAg (Fig. 6*A*). HBcAg was detected only in fraction 8 and the original plasma. Conversely, four bands were detected by anti-HBcrAg in plasma. HBeAg and two additional proteins, which were considered HBeAg precursors, were detected in low density fractions by anti-HBcrAg. A 22-kDa protein, which was termed p22cr, was also detected in fraction 8. To confirm whether p22cr was identical to HBcAg, the p22cr band was compared with the neighboring HBcAg band (Fig. 6*B*). The p22cr protein exhibited slightly higher molecular weight than HBcAg. A fainter HBcAg band was also detected by anti-HBcrAg. Because p22cr did not react with the HB50 anti-HBcAg antibody, SPRRR sequences (positioned at aa 155–174 as three repeats) were presumed absent. Furthermore, p22cr maintained its 22-kDa molecular mass without the *N*-glycosylation consensus site. These data suggest that p22cr contains a complete or nearly complete precore region, including the signal sequence.

The p22cr protein was purified, and the N-terminal amino acid sequence was analyzed. p22cr showed no significant amino acid signal (data not shown), suggesting that the N terminus of p22cr might be blocked.

We then applied mass spectrum analysis. Data from MALDI-TOF MS were analyzed by MS-Fit search using the NCBI non-redundant data base. The search selected 117 of 87,559 entries for the molecular mass range 15–30 kDa. The top 20 matches were all HBV core or precore proteins. Six of 50 input peptide masses matched five precore/core peptides (Table II) that spanned 40% (86 of 212 aa) of the sequence. The N-terminal precore tryptic peptide (peptide 1, aa −28 to aa −9) was found to be N-terminally acetylated and was, therefore, not directly accessible to Edman sequencing. p22cr lacked the first N-terminal methionine of the precore protein. Another peptide, peptide 5, was identified as a precore/core peptide comprising aa 128–150. LC-MS/MS analysis was also applied. Two peptide fractions corresponding to peptides 2 and 5 of Table II were recognized as HBV precore/core proteins. Thus, the p22cr protein was confirmed to be a precore protein from N-terminally

TABLE I

*HBcAg, HBcrAg, HBV DNA and Dane-like particles in fractions A and B*

HBcAg and HBcrAg in plasma were separated by sequential sucrose density ultracentrifugation. The HBV-DNA, HBcAg, and HBcrAg concentrations in the resultant HBcrAg-rich fraction (fraction A) and HBcAg-rich fraction (fraction B) are shown. The numbers of virus particles in the 3.76 $\mu m^2$ area were counted on each of 15 electron micrographs (Fig. 5).

| | HBV DNA | HBcAg | HBcrAg | Dane-like particles | |
|---|---|---|---|---|---|
| | | | | Number | Diameter |
| | *$\times 10^7$ copies/ml* | *ng/ml* | *ng/ml* | *in 3.76 $\mu m^2$* | *nm* |
| Fraction A | 13 | 81 | 2,823 | 17.9 ± 11.6[a] | 41.5 ± 2.2[b] |
| Fraction B | 398 | 210 | 466 | 5.6 ± 3.8[a] | 42.0 ± 2.2[b] |
| Ratios (A/B) | 0.03 | 0.38 | 6.06 | 3.20 | |

[a] Data are presented as mean ± S.D. $n = 15$; $p < 0.001$.
[b] Data are presented as mean ± S.D. $n = 60$; $p = 0.27$.

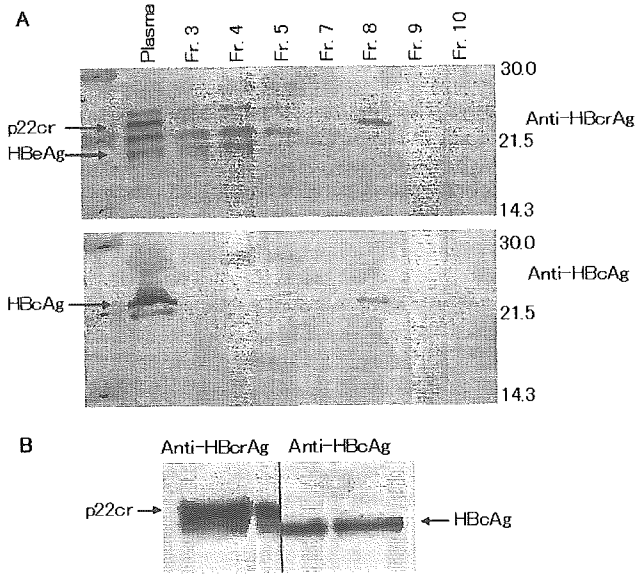


FIG. 6. **Western blot of HBcrAg.** HBV-positive plasma (BBI PHM935A-14) was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient and fractionated into 15 fractions. *A*, plasma and its sucrose density gradient fractions were analyzed by Western blotting using anti-HBcAg (HB50) or anti-HBcrAg (HB91) monoclonal antibodies. *B*, a membrane blot containing three *lanes* of *fraction 8* was cut in half and probed by anti-HBcAg or anti-HBcrAg.

acetylated aa −28 to at least aa 150.

*HBcAg and HBcrAg Levels in HBsAg-positive Particles from Chronic Hepatitis B Sera*—The levels of precore/core proteins were investigated in HBV particles of chronic hepatitis B sera. Sera were immunoprecipitated by anti-HBsAg, and then levels of HBcAg and HBcrAg in the supernatant and precipitate were measured. More than 91% of the HBcAg was detected in precipitate fractions. HBcrAg in precipitate fractions included p22cr and HBcAg. In the precipitate fractions, HBcAg concentration ranged from 0.08 to 165 ng/ml, whereas HBcrAg ranged from 0.59 to 1,079 ng/ml (Fig. 7*A*). Log concentrations of HBcrAg were significantly higher than those of HBcAg ($p < 0.001$). HBcrAg predominated over HBcAg in precipitates from both HBeAg-positive and -negative sera. HBcAg represented only 3.1–37.4% (median 10.5%) of HBcrAg (Fig. 7*B*), indicating that the remaining p22cr was the dominant precore/core protein in HBsAg-positive particles. Similar results were also obtained from high density fractions of the sucrose gradient in six tested samples.

## DISCUSSION

In the present study, we demonstrated that HBV DNA-negative Dane particles are dominant in serum and are composed of a precore protein p22cr, which contains an uncleaved signal sequence and lacks a C-terminal arginine-rich domain. Early electron microscopic and radiolabeling studies have suggested that less than 10% of Dane particles include full cores with viral DNA (3–6). However, the particle formation mechanisms have not been thoroughly examined. Core protein lacking the arginine-rich C-terminal domain can still assemble into capsid particles but fails to bind nucleic acids (16). Our findings present a new model for the formation of DNA-negative particles. The precore proteins, which lack the nucleotide-binding domain, form viral DNA-negative capsid-like particles, and the particles are enveloped and released to blood circulation.

Our new assays for HBcAg and HBcrAg enabled us to study precore/core proteins in HBV particles. The assays include sample pretreatment with SDS, which releases core protein from the particles, inactivates antibodies, and denatures antigens. Thus the HBcAg assay is able to detect the core protein in virion (21), and the HBcrAg assay is able to detect free HBeAg, HBeAg-antibody complex, and precore/core proteins in particles (22, 23). Unexpectedly, the HBcrAg assay detected abundant high density protein in addition to HBeAg and HBcAg (Figs. 3 and 6). The protein formed Nonidet P-40-resistant particles (Fig. 4) that did not contain HBV DNA but were enveloped by HBsAg. The protein was detected together with HBV DNA-negative particles that were morphologically identical to the complete virion (Fig. 5). The unknown precore/core protein proved to be a 22-kDa precore protein species (p22cr) containing the uncleaved signal peptide (Table II) and lacking the C-terminal arginine-rich domain (Fig. 6). The HBcrAg particles appear at a slightly lower density than HBcAg or HBV DNA (Fig. 3), which is also consistent with the observation that HBcrAg particles lack high density DNA components. Collectively, these data strongly suggest that p22cr forms the core of HBV DNA-negative Dane particles.

Our findings indicate that p22cr particles are more abundant than HBcAg capsid in sera (Figs. 3, 5–7, and Table I). In chronic hepatitis B sera, HBcAg comprised only 10.5% of HBcrAg (containing p22cr and HBcAg) in HBsAg-positive particles (Fig. 7). In addition, electron microscopic study indicated that Dane-like particles were more abundant in the HBcrAg-rich fraction than in the HBcAg/HBV DNA-rich fraction (Fig. 5 and Table I). This coincides with the previously reported abundance of empty particles (3–6). Empty and complete Dane particles were differently stained with uranyl acetate (3, 4, 6), but we could not distinguish Dane particles containing HBV DNA from those not containing HBV DNA. This might be due to differences in fixation and/or the negative staining procedure. We used paraformaldehyde for fixation to avoid biohazards.

The present study demonstrated that p22cr is a precore protein from aa −28 to at least aa 150 (Table II). The assembly domains (residues 1 to 149) self-assemble into capsids (16–19). In addition, precore protein containing the assembly domain could form capsid-like particles (26–28), whereas precore proteins are secreted as soluble HBeAg (7–12, 29, 30). A precore protein similar to p22cr, but containing the first methionine, has been isolated as soluble HBeAg from pooled sera of HBV

TABLE II
*MALDI-TOF MS analysis of p22cr*

The 22-kDa protein band was digested in-gel and analyzed by MALDI-TOF MS. The results were analyzed using the NCBI non-redundant data base, taking into account probable post-translational modifications. Five precore/core peptides matched to six of 50 input peptide masses are shown.

| Peptide | *m/z* observed | $[M+H]^+$ matched | Δ | Peptide sequence | Modifications | Amino acids | |
|---|---|---|---|---|---|---|---|
| | | | | | | Start | End |
| | | | *ppm* | | | | |
| 1 | 2233.1438 | 2233.1183 | 11.4048 | QLFHLCLIISCSCPTVQASK | N-terminally acetylated | −28 | −9 |
| 2 | 1237.6388 | 1237.6428 | −3.2413 | DLLDTASALYR | | 29 | 39 |
| 3 | 1913.9167 | 1913.8928 | 12.4530 | EALESPEHCSPHHTALR | | 40 | 56 |
| 3 | 1984.9153 | 1984.9299 | −7.1477 | EALESPEHCSPHHTALR | Acrylamide-modified Cys | 40 | 56 |
| 4 | 1552.7798 | 1552.8045 | −15.8855 | DLVVSYVNTNMGLK | | 83 | 96 |
| 5 | 2490.3663 | 2490.3720 | −2.2840 | TPPAYRPPNAPILSTLPETTVVR | | 128 | 150 |

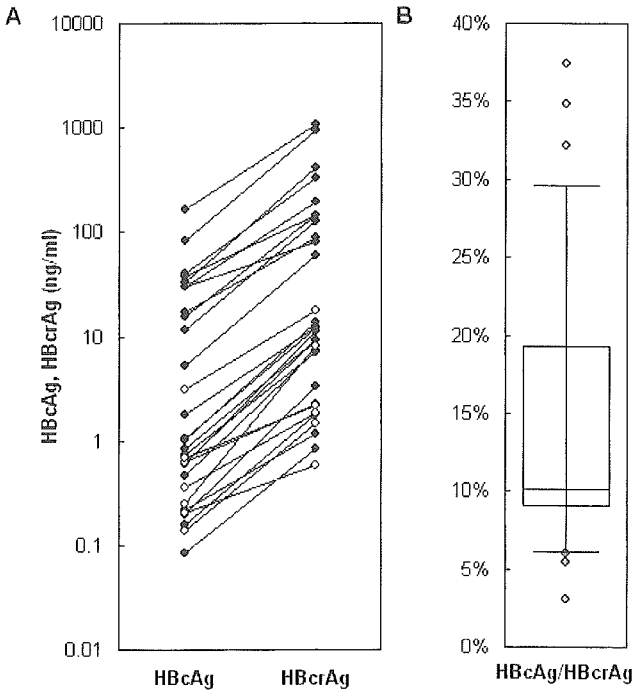


FIG. 7. **HBcAg and HBcrAg levels in HBsAg-positive particles of chronic hepatitis B serum.** HBsAg-positive particles were immunoprecipitated by anti-HBsAg-coated magnetic beads from 30 samples of HBV-infected sera. Precipitated proteins were eluted by SDS solution. Levels of HBcrAg and HBcAg in precipitate were measured by EIA. *A*, data are presented as HBcAg and HBcrAg concentrations per ml of serum. ●, HBeAg-positive; ○, HBeAg-negative sample. *B*, HBcAg percentage per HBcrAg in the precipitate. The *box plots* show the 10th, 25th, 50th, 75th, and 90th percentiles, and *diamonds* denote the outliers.

carriers (30). This could represent the soluble form of p22cr, which was secreted without processing of the signal peptide. Our findings indicate that the majority of p22cr exists in enveloped particles (Figs. 6 and 7).

Mass spectrum study indicated that the protein band contained precore peptide (Table II). We believe that this peptide was not derived from minor precore protein contamination of the major core protein because: (*a*) core protein concentrations in the original plasma were much lower than concentrations of precore/core proteins; (*b*) the purified sample appeared as a single band on SDS-PAGE; (*c*) the 22-kDa protein band could not be sequenced by Edman degradation, indicating that the N-terminal end of the peptide was blocked; and (*d*) the antibody targeting the C-terminal SPRRR repeats did not react with the 22-kDa p22cr (Fig. 6), suggesting that p22cr possesses the nearly complete N-terminal precore sequence.

Although the median HBcAg to HBcrAg (HBcAg + p22cr) ratio of HBsAg-positive particles was 10.5%, the actual ratios ranged widely from 3.1 to 37.4% (Fig. 7*B*). Because precore protein expression is abolished by precore nonsense mutation (31), the precore mutation must influence the HBcAg/HBcrAg ratios. In addition, the particle HBcAg/HBcrAg ratios would depend on the amount of precore proteins that are secreted as HBeAg or form p22cr particles. The ratios of particle-forming p22cr to soluble HBeAg in serum ranged from ~10:1 to 1:100.[2]

The manner in which precore protein containing the signal peptide forms particles remains unclear, but inefficient translocation of the precore protein might lead to particle formation in the cytosol. As with most secreted proteins, translocation of the precore protein across the ER membrane is mediated by signal recognition particles (8). However, translocation of the precore proteins is inefficient (8, 32, 33). In *Xenopus* oocytes, precore protein (p25, aa −29 to +183) was produced but not translocated into the ER lumen without processing (33). If translated precore proteins were to evade translocation to the ER, disulfide bridges would not form in the reducing environment of the cytosol. An intramolecular disulfide bridge between Cys-7 and Cys-61 determines the structure of the HBeAg (34, 35). HBe protein without Cys-7 also assembles into particles (29, 34–36). Conversely, Cys residues are not essential for the assembly of viral core particles (37). We therefore hypothesize that precore proteins remaining in the cytosol, which do not form disulfide bridges between Cys-7 and Cys-61, cannot assume the HBeAg conformation but can assemble into capsid-like particles.

The mechanisms for cleaving the C-terminal domain are unclear. Maassen *et al.* (38) reported that an N-terminal fusion core protein (with foreign sequences comprising 14 aa) assembles into capsid-like particles, but the fusion is sensitive to proteolytic attack within the arginine-rich C terminus. The uncleaved precore region (aa −28 to −1) might thus promote cleavage of the C-terminal domain.

Based on numerous *in vitro* or animal studies (14–19, 27, 29, 35, 38), the HBV capsid is believed to be a construct of core protein alone. However, nonsecreted precore protein and core protein can assemble to form hybrid nucleocapsids (28). The p22cr displayed a shoulder in virion fractions from density gradients (Fig. 3*B*, fraction 26–27), and the concentration of p22cr protein greatly exceeded that of HBcAg. The nucleocapsid of complete HBV particles could therefore contain p22cr.

Although the functions of the DNA-negative particles are largely unknown, the particles have been suggested to play a role in the persistence of HBV infection (3, 5, 6). p22cr in the particles may be a disturbing antigen for the host reactions. Overexpression of the precore gene results in inhibition of HBV replication in culture cells or transgenic mice (28, 39). The p22cr might be a molecule that inhibits HBV replication in

[2] T. Kimura, C. Ohue, A. Rokuhara, A. Matsumoto, E. Tanaka, K. Kiyosawa, and N. Maki, unpublished data.

human hepatocytes during natural infection. Furthermore, the number of particles containing p22cr or the antibodies specific for p22cr could be clinical markers for hepatitis B.

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HEPATOLOGY

# Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection

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**Abstract**

***Background and Aims***: Hepatitis B virus (HBV) core-related antigen (HBcrAg) and HBV core antigen (HBcAg) assays were developed for the measurement of serum HBV load. The aim of this study was to assess the clinical utility of these assays in Chinese patients with chronic genotype B and C HBV infection.

***Methods***: One hundred and ninety-three chronic hepatitis B patients were enrolled. Serum HBcrAg and HBcAg were measured by chemiluminescence enzyme immunoassay, and HBV-DNA was measured by using a sensitive polymerase chain reaction assay. The data were analyzed in patients with HBV genotype B (HBV/B) and genotype C (HBV/C). The HBcrAg/HBcAg ratio was calculated and compared between patients with and without hepatitis B e antigen (HBeAg).

***Results***: The concentrations of HBcrAg and HBcAg showed significant positive correlation with the HBV-DNA concentration in both HBV/B ($r = 0.79$, $P < 0.001$, and $r = 0.77$, $P < 0.001$, respectively) and HBV/C ($r = 0.87$, $P < 0.001$, and $r = 0.90$, $P < 0.001$, respectively). The cut-off for a positive HBcAg corresponded to approximately 4.5 log copies/mL, and that for a positive HBcrAg result corresponded to 3–4 log copies/mL. The HBcrAg/HBcAg ratio was higher in patients with HBeAg than in those without HBeAg.

***Conclusions***: The HBcrAg assay and HBcAg assay are clinically useful in viral quantitation of HBV/B and HBV/C. A combination of these assays would be a valuable tool for analyzing the clinical status of HBV infection.



**Key words**: hepatitis B e antigens (HBeAg), hepatitis B antigens, hepatitis B core antigens (HBcAg), hepatitis B virus, viral proteins.

## INTRODUCTION

Infection with hepatitis B virus (HBV) remains one of the major human infectious diseases and involves approximately 350 million people.[1] In a significant proportion of cases, infection progresses to cirrhosis and liver failure as well as hepatocellular carcinoma (HCC).[2] As therapeutic advances have emerged, detailed information is required to assess HBV replication in individual patients in clinical management.

Recently, two sensitive chemiluminescence enzyme immunoassays (CLEIA) specific for HBV were developed in our laboratory.[3,4] One is an HBV core-related antigen (HBcrAg) assay that measures the serum levels of hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg) simultaneously using monoclonal antibodies, and the other is an assay that measures the serum level of HBcAg. Although assessments of clinical performance relating to the HBcAg and HBcrAg assays have already been reported in Japanese patients,[3–5] an

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evaluation of these two antigen assays was not performed in patients with HBV genotype B. The aim of this study is to assess the clinical utility of the HBcAg and HBcrAg assays for measurement of HBV load in Chinese patients who are infected with genotype B or C.

## METHODS

### Patients

Patients attending the Second Hospital of Hebei Medical University, Shijiazhuang, in northern China, between June and August 2001, who had carried hepatitis B surface antigen (HBsAg) for at least 6 months, were enrolled for the study. Serum samples obtained from 193 patients (125 male and 68 female, median age 27 years, range 5–73 years) were examined. One hundred and eighty-two patients were diagnosed as chronic HBV carriers according to the consensus diagnostic criteria of HBV infection.[6] The remaining 11 patients had persistently normal alanine aminotransferase (ALT) levels, suggesting an inactive HBsAg carrier stage.[6] None of the 193 patients were treated with antiviral agents such as interferon or lamivudine. All were non-reactive for antibody to hepatitis C virus infection. All sera were stored at −20°C until use. The study design conformed to the 1995 Declaration of Helsinki, and was approved by ethics committees of our institutions. Informed consent was obtained from each patient.

### HBcAg CLEIA and HBcrAg CLEIA

Concentrations of HBcAg and HBcrAg were measured in serum using the CLEIA reported previously.[3,4] Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecyl sulfate. After incubation at 70°C for 30 min, 50 μL pretreated serum was added to wells coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies were added to the wells and incubated for 1 h at room temperature. After washing, substrate solution was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBcAg or HBcrAg concentration was read by comparison with a standard curve. Recombinant HBcAg (rHBcAg: amino acids 1–183 of precore/core gene product) and recombinant ProHBeAg (rProHBeAg: amino acids −10 to 183) were expressed in *Escherichia coli* and purified to single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Recombinant HBcAg and rProHBeAg were used as the standard for the HBcAg assay and the HBcrAg assay, respectively. The HBcrAg immunoreactivity for rProHBeAg at 10 fg/mL was defined as 1 U/mL[3] The cut-off for a positive HBcAg result was 4.0 pg/mL and that for HBcrAg was $1.0 \times 10^3$ U/mL (=immunoreactivity of rProHBeAg at 10 pg/mL), which were determined based on the mean +4 SD values of healthy control sera ($n = 160$ or 108) and sera of hepatitis C patients ($n = 55$ or 59).[3,4]

The HBcrAg/HBcAg immunoreactivity ratio was calculated in order to assess the relative amounts of HBeAg and HBcAg in sera. The immunoreactivity of HBcrAg (pg/mL) was divided by that of HBcAg (pg/mL) in each sample.

### Conventional HBV markers and genotyping of HBV

Using commercially available enzyme immunoassay kits, HBsAg, HBeAg, and anti-HBe were measured (Dinabbott, Tokyo, Japan). The levels of HBV-DNA in the serum samples were measured using an Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA) with a detection range between $4 \times 10^2$ and $4 \times 10^7$ copies/mL. Samples with an HBV-DNA level greater than $10^8$ copies/mL were measured after dilution in HBV-negative serum. Nucleic acids were extracted from 100 μL of sera using a Smitest Ex R&D kit (Genome Science Laboratories, Tokyo, Japan). HBV genotype was determined using restriction fragment length polymorphism.[7]

### Statistical analysis

The Mann–Whitney *U*-test was used for analysis of the quantitative data, and Fisher's exact test was used analysis of the qualitative data. The Spearman rank correlation was also employed where appropriate. Statistical analyses were done using the StatView software package (version 5.0; SAS Institute, Cary, NC, USA). A *P*-value of less than 0.05 was considered to be statistically significant.

## RESULTS

### Genotypic distribution

Among the 193 patients studied, 169 (87.6%) patients were infected with HBV of genotype C (HBV/C), 21 (10.9%) patients were infected with HBV/B, and three (1.5%) were infected with HBV/A. The clinical backgrounds of the patients who were infected with HBV/B and HBV/C are compared in Table 1. There were no statistical differences in clinical backgrounds, serum HBV-DNA levels, serum concentrations of HBcAg, or serum concentrations of HBcrAg between the patients infected with HBV/B and HBV/C.

### Correlation between HBcAg/HBcrAg and HBV-DNA concentrations

The correlation between the concentrations of HBcAg and HBV-DNA, and that of the concentrations of

**Table 1** Background characteristics of patients infected with hepatitis B virus (HBV) of genotype B and genotype C

| Features | Genotype B ($n = 21$) | Genotype C ($n = 169$) | $P$-value |
|---|---|---|---|
| Age (years)† | 22 (9–65) | 27 (5–73) | NS |
| No. males‡ | 12 (57.1%) | 111 (65.7%) | NS |
| HBeAg positivity‡ | 16 (76.2%) | 102 (60.4%) | NS |
| ALT (U/L)† | 50 (21–105) | 47 (10–2100) | NS |
| HBV-DNA (log copies/mL)†§ | 8.7 (4.4–9.4) | 7.5 (3.0–9.4) | NS |
| HBcAg (log U/mL)† | 6.3 (2.2–7.4) | 5.7 (1.9–7.5) | NS |
| HBcrAg (log U/mL)† | 8.3 (2.9–8.9) | 8.0 (2.5–9.0) | NS |

ALT, alanine aminotransferase; HBcAg, HBV core antigen; HBcrAg, hepatitis B virus core-related antigen; HBeAg, hepatitis B e antigen; NS, not significant. †Data are expressed as median (range). ‡Data are expressed as positive number (%). §HBV-DNA was measured by using an Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA).

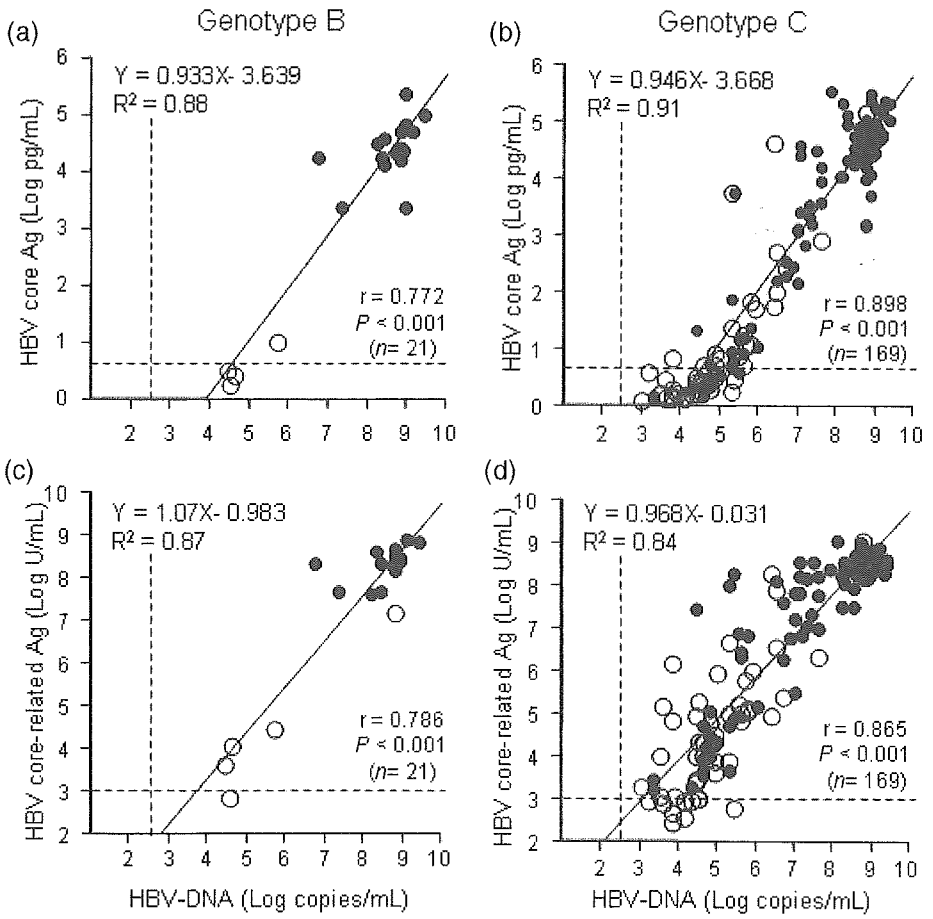


**Figure 1** Degree of correlation between the concentrations of hepatitis B virus (HBV) core antigen (HBcAg) and HBV-DNA, and those of hepatitis B virus core-related antigen (HBcrAg) and HBV-DNA. Correlations between the concentrations of HBcAg and HBV-DNA in the sera from patients infected with (a) HBV genotype B (HBV/B) and (b) HBV genotype C (HBV/C). Correlation between the concentrations of HBcrAg and HBV-DNA in the sera from patients infected with (c) HBV/B and (d) HBV/C, respectively. (●), Data from HBeAg-positive sera; (○), data from hepatitis B e antigen (HBeAg)-negative sera. HBV-DNA levels were determined by using the Amplicor HBV Monitor test (Roche Molecular Systems, Branchburg, NJ, USA). (- - -), Lower cut-off of the assays.

HBcrAg and HBV-DNA are shown in Figure 1. The serum concentrations of HBcAg and HBV-DNA correlated significantly in the patient group infected with HBV/B ($r = 0.772$, $P < 0.001$), as well as in the patient group infected with HBV/C ($r = 0.898$, $P < 0.001$). The serum concentrations of HBcrAg and HBV-DNA also correlated significantly in the patient group infected with HBV/B ($r = 0.786$, $P < 0.001$), as well as in the patient group infected with HBV/C ($r = 0.865$, $P < 0.001$). The cut-off for a positive HBcAg result was 4 pg/mL, which corresponded to approximately 4.5 log copies/mL (Fig. 1). The cut-off for a positive HBcrAg result corresponded to 3–4 log copies/mL (Fig. 1).

## HBcrAG/HBcAg ratio

The HBcrAg/HBcAg immunoreactivity ratio was calculated in each patient and was compared between the patients with and without HBeAg (Fig. 2). The data are represented in log scale. The median value of the

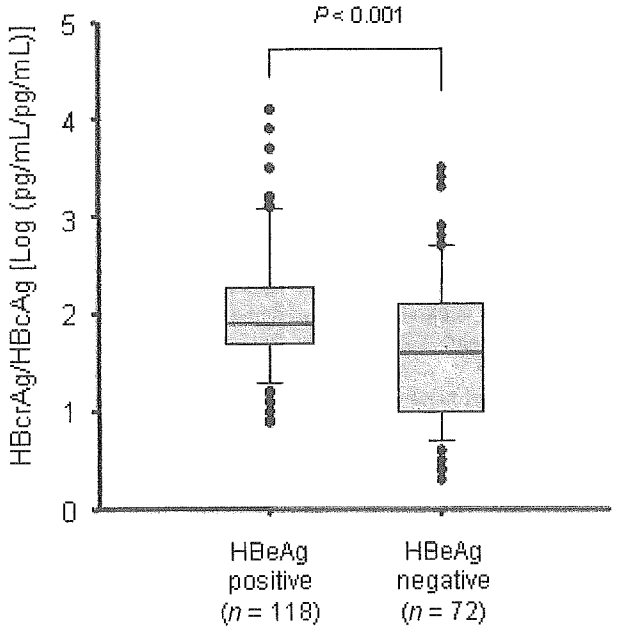


**Figure 2** Hepatitis B virus core-related antigen/HBV core antigen ratios in relation to hepatitis B e antigen (HBeAg) status. Data are represented by a box-plot showing the 25th percentile, median, and 75th percentile as vertical box. Tick marks above and below the box indicate the 90th and 10th percentiles (log representation). (●), Outlier data points.

HBcrAg/HBcAg immunoreactivity ratio was significantly higher in patients with HBeAg (median 1.9, range 0.9–4.1) than in patients without HBeAg (median 1.6, range 0.3–3.5; $P < 0.001$).

## DISCUSSION

In this report, an attempt was made to assess the clinical utility of the HBcAg and HBcrAg assays for the measurement of HBV load in the sera from Chinese patients who were infected with HBV/B or HBV/C. In a previous study, a good quality estimation of the accuracy of the HBcrAg assay in HBV/B-infected patients could not be obtained because of the small number of patients who were infected with HBV/B.[5] Twenty-one patients with HBV/B were enrolled in the present study. As a result, a significant positive correlation was observed between the serum concentrations of HBcAg and HBV-DNA, as well as between HBcrAg and HBV-DNA in both HBV/B- and HBV/C-infected Chinese patients. The HBcrAg assay has a high level of sensitivity, which was comparable with the real-time detection polymerase chain reaction.[5] The cut-off for a positive HBcAg result corresponded to a range of 4–5 log copies/mL. Because an HBV level less than 4 log copies/mL indicates inactive liver disease,[8,9] and an HBV level greater than 5 log copies/mL is associated with active liver disease,[10,11] the HBcAg assay could be valuable to postulate chronic active hepatitis B.

If all Dane particles contain one copy of HBV-DNA and 240 molecules of HBcAg, 9.0 log copies of HBV-DNA would correspond to 3.9 log pg (=$8.26 \times 10^3$ pg) of core protein. But in our experiment, approximately 4.5 log pg/mL of HBcAg was measured in sera containing 9.0 log copies/mL of HBV-DNA (Fig. 1), which is fourfold (0.6 logs) the calculated value. Although the HBV-DNA and HBcAg assays have some inaccuracies, this gap between 3.9 and 4.5 log pg/mL might indicate that the DNA-negative "empty" Dane particles were predominant in sera, as has been suggested by electron microscopy and radiolabeling studies.[12–14]

The HBcrAg assay detects HBcAg and HBeAg simultaneously, using monoclonal antibodies that recognize both denatured HBcAg and HBeAg, even in anti-HBe antibody-positive samples.[3] Current commercial HBeAg assays do not detect the HBeAg/anti-HBe complex, because the epitopes of HBeAg are masked by the anti-HBe antibody.[15] For capturing HBcAg, we used HB44, HB61, and HB114 immobilized monoclonal antibodies, which were the same as in the HBcrAg assay.[4] The HBcAg assay differs from the HBcrAg assay in the detection antibody, which recognizes core-specific SRRRR repeats in the C-terminal protamine-like nucleic acid binding domain, and is therefore specific for HBcAg. In the present report, the HBcrAg/HBcAg ratio was significantly higher in patients with HBeAg than in patients without HBeAg. Because the HBcrAg assay mainly reflects the levels of HBeAg and HBeAg/anti-HBe complex,[3] the HBcrAg/HBcAg ratio would represent the relative amounts of HBeAg and HBcAg. If this is true, this ratio could be used as a marker that indicates a balance of HBeAg production and HBV load at some points. As HBeAg states in sera largely depend on the HBeAg production from HBV, the mechanism of this result could be explained by the reduction of HBeAg in the sera, via mechanisms such as mutations in the precore and core promoter regions.[16–18] HBV viral load and the concentration of HBeAg vary widely in individual patients during the course of HBV infection. This variation and the immunological reaction of the host result in various pathological manifestations of HBV infection. It would therefore be more useful for diagnostic purposes to measure the HBcAg and HBcrAg levels simultaneously, instead of checking only the HBeAg state. Clearly, further analysis in longitudinal studies is required, and the mechanisms associated with these results remain to be explored.

In conclusion, we assessed the utility of the HBcAg and HBcrAg assays in Chinese patients with HBV/B and HBV/C. These results showed that these two HBV antigen assays are clinically useful in viral quantitation as well as HBV-DNA quantitation. Using a combination of these two assays could be more useful for analyzing clinical status in patients with HBV infection.

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**Clinical Studies**

# Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

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Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
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**Abstract:** *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

## Patients and methods

### Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

### Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing

over 7.0 log copy/ml HBV DNA were diluted 10- or 100-fold with normal human serum and re-tested to obtain the end titer.

Serum concentrations of HBVcrAg were measured using the CLEIA method reported previously (10, 11). Briefly, 100 μL serum was mixed with 50 μL pretreatment solution containing 15% sodium dodecylsulfate and 2% Tween 60. After incubation at 70 °C for 30 min, 50 μL pretreated serum was added to a well coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μL assay buffer. The mixture was incubated for 2 h at room temperature and the wells were then washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies against denatured HBc and HBe antigens (HB91 and HB110) were added to the well, and the mixture was incubated for 1 h at room temperature. After washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA) was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBVcrAg concentration was determined by comparison with a standard curve generated using recombinant pro-HBe antigen (amino acids, 10–183 of the precore/core gene product). The HBVcrAg concentration was expressed as units/ml (U/ml) and the immunoreactivity of recombinant pro-HBe antigen at 10 fg/ml was defined as 1 U/ml. In the present study, the cutoff value was tentatively set at 3.0 log U/ml. Sera containing over 7.0 log U/ml HBVcrAg were diluted 10- or 100-fold in normal human serum and re-tested to obtain the end titer.

### Statistical analysis

The Mann–Whitney *U*-test and Wilcoxon signed-ranks test were utilized to analyze quantitative data, and Fisher's exact test was used for qualitative data. A log-rank test was used to compare the occurrence of lamivudine resistance. Statistical analyses were performed using the SPSS 5.0 statistical software package (SPSS, Inc., Chicago, IL). A *P*-value of less than 0.05 was considered to be statistically significant.

## Results

Table 1 shows a comparison of the clinical and virological backgrounds of the 27 patients who showed lamivudine resistance and the 54 patients who did not. Median age, gender distribution and median follow-up period did not differ between the two groups, and the positive rate of HBe antigen was similar. Both HBV DNA and HBVcrAg levels at the beginning of lamivudine administration were similar between the two groups; however, both HBV DNA and HBVcrAg levels at 6 months after the start of lamivudine administration were significantly lower in the lamivudine resistance negative group than in the positive group. ALT level was normal at the beginning in eight (15%) of the 54 patients without lamivudine resistance and in two (7%) of the 27 patients with it ($P>0.2$).

Table 1. Comparison of the clinical and virological backgrounds of patients who showed lamivudine resistance and those who did not

| Characteristics | Appearance of lamivudine resistance: Negative ($n=54$) | Positive ($n=27$) | *P* |
|---|---|---|---|
| Age (years)* | 47.0 (24–79) | 50.6 (34–67) | 0.140† |
| Gender (male %) | 74% | 67% | >0.2‡ |
| Follow-up period (months)* | 16 (6–50) | 21 (9–43) | >0.2† |
| HBV genotype (A/B/C) | 2/2/50 | 0/1/26 | >0.2‡ |
| HBe antigen (positive %) | 59% | 70% | >0.2‡ |
| ALT (IU/ml)* | | | |
| Initial | 85 (22–713) | 95 (20–1140) | >0.2† |
| At 6 months | 27 (11–115) | 30 (15–92) | >0.2† |
| HBV DNA (log copy/ml)* | | | |
| Initial | 7.0 (3.5–9.1) | 7.3 (4.2–9.2) | >0.2† |
| At 6 months | <2.6 (<2.6–4.8) | 3.3 (<2.6–6.6) | <0.001† |
| HBVcrAg (log U/ml)* | | | |
| Initial | 6.2 (<3.0–8.8) | 7.3 (4.4–9.1) | 0.073† |
| At 6 months | 5.2 (<3.0–6.7) | 5.8 (4.7–8.4) | <0.001† |

HBe antigen, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; HBVcrAg, HBV core-related antigen. *Data are expressed as median (range). †Mann–Whitney *U* test. ‡$\chi^2$-test.

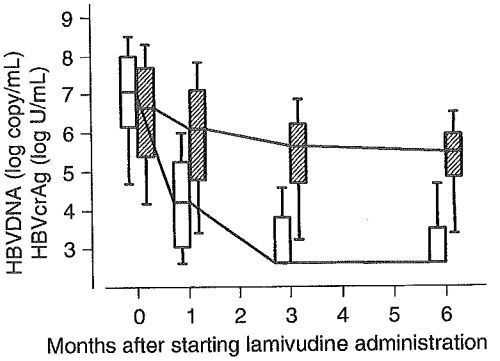


*Fig. 1.* Changes in the median levels of hepatitis B virus core-related antigen (HBVcrAg) and hepatitis B virus (HBV) DNA during lamivudine administration. The box plots show the 10th, 25th, 50th, 75th and 90th percentiles, with the open boxes indicating HBV DNA and shaded boxes indicating HBVcrAg. The median amount of decrease from the baseline in HBVcrAg levels was significantly smaller (Wilcoxon signed-ranks test) than that in HBV DNA level at 1 (2.80 log copy/ml vs. 0.27 log U/ml, $P<0.001$), 3 (3.60 log copy/ml vs. 0.83 log U/ml, $P<0.001$) and 6 months (3.90 log copy/ml vs. 1.15 log U/ml, $P<0.001$) after the initiation of lamivudine administration.

Figure 1 shows changes in HBV DNA and HBVcrAg levels during lamivudine treatment in all patients. The level of HBV DNA decreased rapidly and became undetectable at 3 months after treatment was initiated. On the other hand, although HBVcrAg levels decreased continuously, the median amount of decrease from the base-line was significantly lower than that in HBV DNA levels at 1, 3 and 6 months after starting lamivudine administration (Wilcoxon signed-ranks test, $P<0.001$ at all analyzed points in time).

Changes in HBV DNA and HBVcrAg levels during lamivudine administration are compared in Fig. 2 between the 27 patients who showed lamivudine resistance and the 54 patients who did not. Serum HBV DNA levels were found to decrease rapidly and become undetectable within 6 months in 45 (83%) of the 54 patients without lamivudine resistance. On the other hand, only 11 (41%) of the 27 patients with lamivudine resistance showed a similar rapid decrease, and the HBV DNA levels of the remaining patients stayed above the detection limit during the follow-up period. HBVcrAg levels decreased but did not reach levels lower than 4.7 log U/ml (5000 U/ml) in the 27 patients with lamivudine resistance. In 19 (35%) of the 54 patients without lamivudine resistance, on the other hand, the levels decreased to levels below 4.7 log U/ml within 6 months after the start of lamivudine administration. The level of HBVcrAg increased rapidly as did the level of HBV DNA when lamivudine resistance occurred.

The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months after the initiation of treatment than in the remaining 25 patients (Fig. 3). The cumulative occurrence of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. There was no occurrence of lamivudine resistance during the follow-up period in the 19 patients whose HBVcrAg levels were less than 4.6 log U/ml at 6 months after the initiation of lamivudine therapy (Fig. 3). On the other hand, lamivudine resistance occurred in 50% of the remaining patients within 2 years.

## Discussion

The HBVcrAg assay is a unique assay, which measures the amounts of e and core antigens

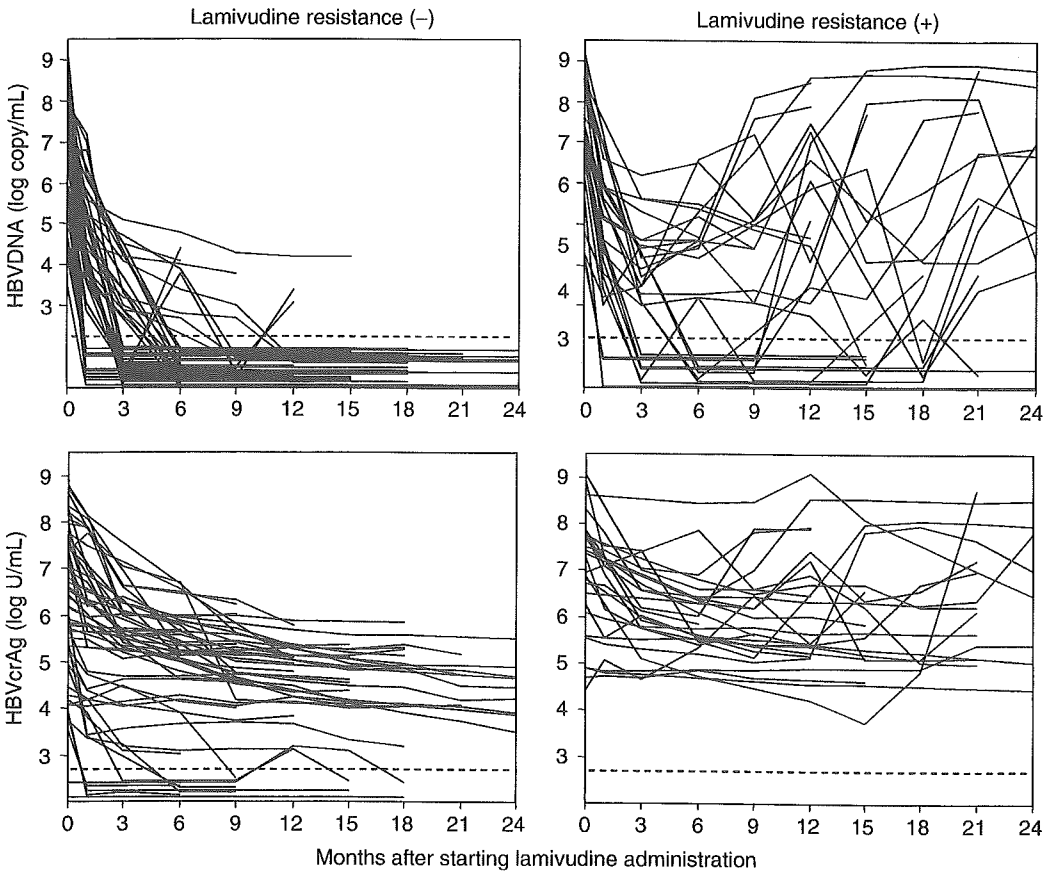


*Fig. 2.* Comparison of changes in serum hepatitis B virus (HBV) DNA and serum HBV core-related antigen (HBVcrAg) levels between patients who showed lamivudine resistance and those who did not. The broken lines indicate the detection limit of each assay.

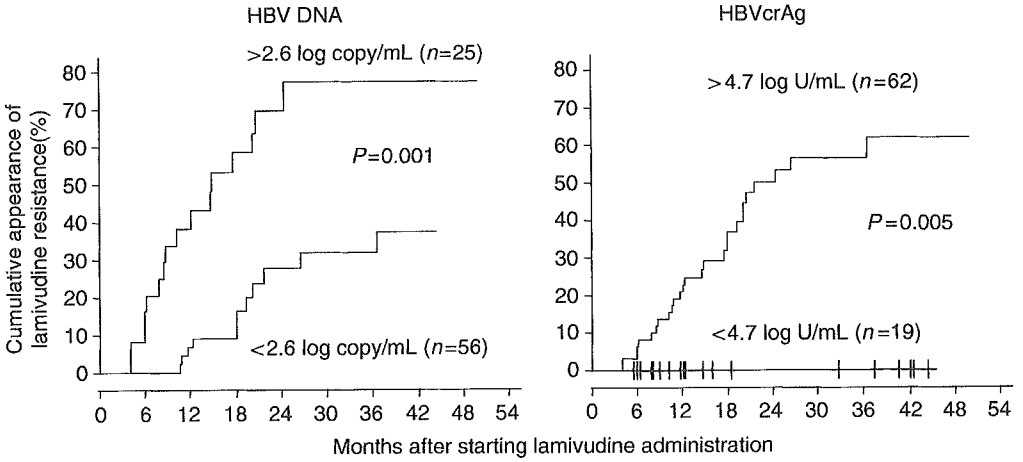


*Fig. 3.* Comparison of the cumulative occurrence of lamivudine resistance between patients who showed hepatitis B virus (HBV) DNA levels of less than the detection limit (2.6 log copy/ml) at 6 months after starting lamivudine administration and those who did not (left figure), and similarly between patients who showed HBV core-related antigen (HBVcrAg) levels of less than 4.7 log U/ml and those who did not (right figure).

coded by the core gene of the HBV genome with high sensitivity and a wide quantitative range. Serum HBVcrAg levels reflect the viral load in the natural course because these levels correlate linearly with those of HBV DNA (14, 15). On the other hand, the character of HBVcrAg is somewhat different from that of HBV DNA in patients undergoing anti-viral therapies such as lamivudine. That is, HBVcrAg levels decrease significantly more slowly than those of HBV DNA after the initiation of lamivudine administration.

HBV is an enveloped DNA virus containing a relaxed circular DNA genome, which is converted into a covalently closed circular DNA (cccDNA) episome in the nucleus of infected cells (18, 21–23). The cccDNA molecules serve as the transcriptional template for the production of viral RNAs that encode viral structural and non-structural proteins. Reverse transcription of the viral pregenomic RNA and second-strand DNA synthesis occur in the cytoplasm within viral capsids formed by the HBV core protein. Because lamivudine, a nucleoside analogue, inhibits reverse transcription of the pregenomic RNA, it directly suppresses the production of HBV virion. Thus, serum HBV DNA levels decrease rapidly after the initiation of lamivudine administration. On the other hand, the production of viral proteins is not suppressed by lamivudine because the production process does not include reverse transcription. Furthermore, it has been reported that the amount of cccDNA, which serves as a template for mRNA, decreases quite slowly after starting the administration of nucleoside analogues (24–26). Thus, it is reasonable that serum HBVcrAg levels decrease much more slowly than HBV DNA levels after the initiation of lamivudine therapy.

Significant markers that can predict the presence or absence of lamivudine resistance are clinically valuable because the emergence of this resistance and the subsequent recurrence of hepatitis are fundamental problems in lamivudine therapy. Serum markers that reflect the activity of HBV replication have been reported to be associated with the occurrence of lamivudine resistance (11, 12, 27, 28). However, neither the pretreatment existence of HBe antigen nor pretreatment levels of HBV DNA or HBVcrAg were found to be significant markers in the present study. These results may reflect a weak association between the pretreatment activity of HBV replication and the occurrence of lamivudine resistance (13, 29). Changes in HBV DNA and HBVcrAg levels after starting lamivudine administration clearly differed between patients with and without lamivudine resistance. Thus, HBV DNA and HBVcrAg levels at 6 months after starting lamivudine administration were analyzed to determine whether these levels might serve as predictive markers; both were found to be significantly lower in patients without lamivudine resistance at the tested point in time. Furthermore, patients who showed higher levels of HBV DNA and HBVcrAg at 6 months after the initiation of treatment were significantly more likely to develop lamivudine resistance than those who showed lower levels.

We believe that the measurement of HBV DNA levels is useful to identify patients who are at high risk for lamivudine resistance because as many as 70% of patients who were positive for HBV DNA at 6 months after starting lamivudine

administration developed lamivudine resistance within 2 years. However, a negative result of HBV DNA at 6 months does not necessarily guarantee the absence of lamivudine resistance because nearly 30% of such patients developed resistance within 2 years. On the other hand, HBVcrAg levels of less than 4.7 log U/ml at 6 months are a useful indicator of patients who are unlikely to develop lamivudine resistance, because no such patients developed resistance during the follow-up period in the present study. Lower serum HBVcrAg levels may reflect lower levels of cccDNA in hepatocytes because the mRNAs of HBVcrAg are transcribed from the cccDNA (18, 22, 23). This possibility may explain our finding that patients whose HBVcrAg levels decreased sufficiently were unlikely to develop lamivudine resistance, because cccDNA provides the templates for viral and pregenomic messenger RNA (18, 22, 23), which may be a source of lamivudine-resistant strains.

In conclusion, our results suggest that measurement not only of HBV DNA but also of HBVcrAg is useful for predicting the occurrence of lamivudine resistance. HBV DNA measurement is valuable for identifying patients who are at high risk of developing this resistance and HBcrAg measurement is valuable for identifying those who are at low risk.

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# Patients With and Without Loss of Hepatitis B Virus DNA After Hepatitis B e Antigen Seroconversion Have Different Virological Characteristics

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The characteristic differences between patients with and without loss of hepatitis B virus (HBV) DNA after achieving hepatitis B e antigen seroconversion were analyzed by comparing changes in HBV DNA and HBV core-related antigen levels during a period from 3 years before to 3 years after the seroconversion. Of the 24 seroconverters, 6 (inactive replication group) showed continuous loss of HBV DNA in serum after the seroconversion and the remaining 18 did not lose HBV DNA (active replication group). The HBV DNA level was similar between the two groups, while the HBV core-related antigen level was significantly lower in the active replication group than in the inactive replication group before the seroconversion. The levels of both HBV DNA and HBV core-related antigen decreased remarkably around the time of seroconversion in the inactive replication group, while these levels did not change or decreased slightly in the active replication group. After the seroconversion, the HBV DNA level was significantly higher in the active replication group than in the inactive replication group, while the HBV core-related antigen level was similarly low between the two groups. Because the serum level of HBV core-related antigen mainly reflects that of HBe antigen, the low level of HBV core-related antigen seen after seroconversion in both groups might have contributed to the occurrence of seroconversion. The precore and core promoter mutations which cause diminished excretion of hepatitis B e antigen were significantly more frequent in the active replication group than in the inactive replication group. It was therefore considered that the seroconversion was caused mainly by a decrease in viral replication in the inactive replication group, and mainly by a decrease in HBe antigen production in the active replication group. ***J. Med. Virol. 78:68–73, 2006.*** © 2005 Wiley-Liss, Inc.



**KEY WORDS:** HBV DNA; seroconversion; HBV core-related antigen; precore mutation; core promoter mutation

## INTRODUCTION

A total of 350 million people worldwide are estimated to be carriers of hepatitis B virus (HBV) [Maynard, 1990; Maddrey, 2000]. HBV is important as a causative agent for liver diseases such as chronic hepatitis and hepatocellular carcinoma, especially in Asian countries [Lee, 1997]. In the natural history of chronic HBV infection, seroconversion from hepatitis B e (HBe) antigen to its antibody (anti-HBe) is usually accompanied by a decrease in HBV replication and remission of hepatitis [Realdi et al., 1980; Hoofnagle et al., 1981; Liaw et al., 1983]. Thus, HBe antigen seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who continue to have elevated HBV DNA levels in the serum and active liver disease after the seroconversion [Bonino et al., 1986; Hsu et al., 2002].

Although the detailed mechanisms of HBe antigen seroconversion have not been fully clarified, several mutations in the HBV genome have been reported to be associated with the phenomenon. When the precore (pre-C) and core genes in the HBV genome are

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