Journal of Gastroenterology and Hepatology (2005) **20**, 1726–1730 DOI: 10.1111/j.1440-1746.2005.04087.x

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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Accepted for publication 9 December 2004.

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 1.0×10^3 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 4×10^2 and 4×10^7 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).

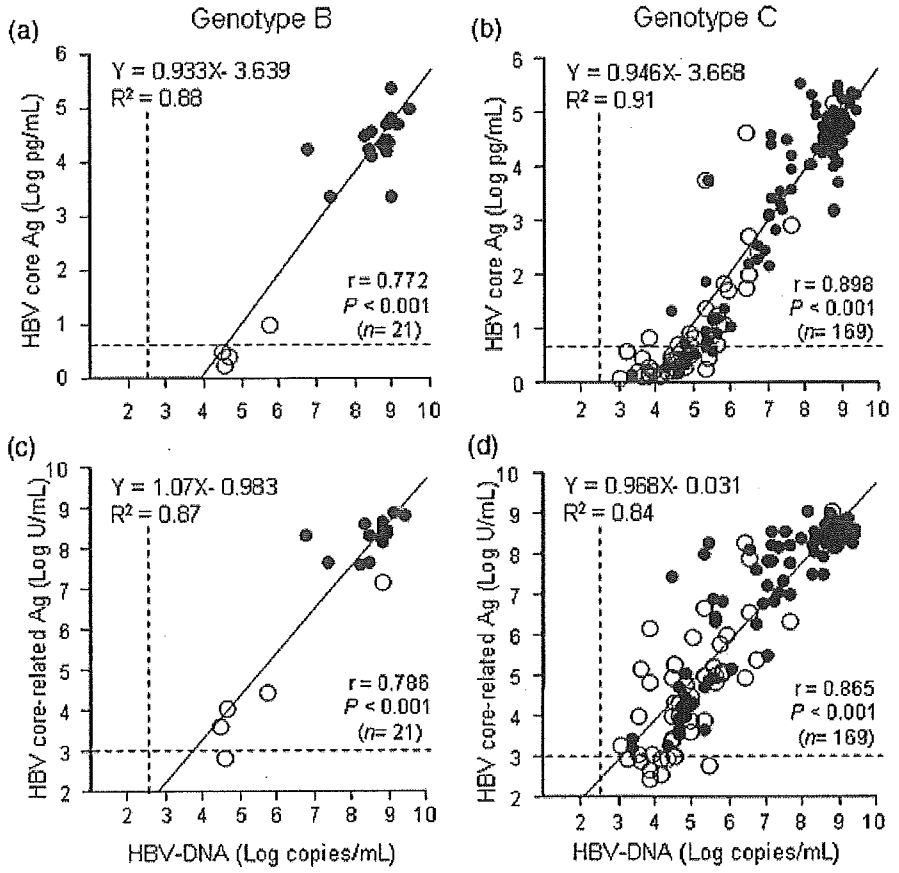

Figure 1 Degree of correlation between the concentrations of hepatitis B virus (HBV) core antigen (HBcAg) and HBV-DNA, and those of hepatitis B virus core-related antigen (HBcrAg) and HBV-DNA. Correlations between the concentrations of HBcAg and HBV-DNA in the sera from patients infected with (a) HBV 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

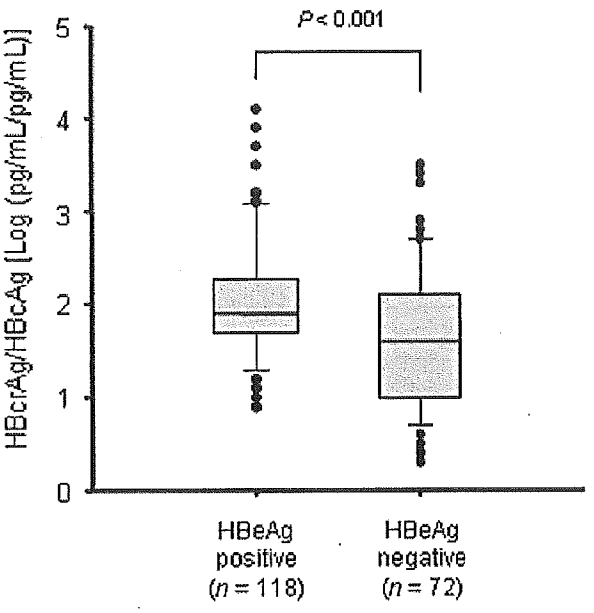

Figure 2 Hepatitis B virus core-related antigen/HBV core antigen ratios in relation to hepatitis B e antigen (HBeAg) status. Data are represented by a box-plot showing the 25th percentile, median, and 75th percentile as vertical box. Tick marks above and below the box indicate the 90th and 10th percentiles (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 (=8.26×10^3 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.

ACKNOWLEDGMENT

This study was supported in part by a research grant from the Japanese Ministry of Health, Labour and Welfare (no. 13670504).

REFERENCES

1 Lee WM. Hepatitis B virus infection. *N. Engl. J. Med.* 1997; **337**: 1733–45.
2 Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer* 1988; **61**: 1942–56.
3 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J. Clin. Microbiol.* 2002; **40**: 439–45.
4 Kimura T, Rokuhara A, Matsumoto A *et al.* New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV-DNA. *J. Clin. Microbiol.* 2003; **41**: 1901–6.
5 Rokuhara A, Tanaka E, Matsumoto A *et al.* Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen: a marker distinct from viral DNA for monitoring lamivudine treatment. *J. Viral Hepat.* 2003; **10**: 324–30.
6 Lok AS, McMahon BJ. Practice Guidelines Committee, American Association for the Study of Liver Diseases. Chronic hepatitis B. *Hepatology* 2001; **34**: 1225–41.
7 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett.* 1999; **450**: 66–71.
8 Lindh M, Horal P, Dhillon AP, Norkrans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histological activity in chronic hepatitis B. *J. Viral Hepat.* 2000; **7**: 258–67.
9 Manesis EK, Papatheodoridis GV, Hadziyannis SJ. Serum HBV-DNA levels in inactive hepatitis B virus carriers. *Gastroenterology* 2002; **122**: 2092–3.
10 Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000. Summary of a workshop. *Gastroenterology* 2001; **120**: 1828–53.
11 Martinot-Peignoux M, Boyer N, Colombat M *et al.* Serum hepatitis B virus DNA levels and liver histology in inactive HBsAg carriers. *J. Hepatol.* 2002; **36**: 543–6.
12 Alberti A, Diana S, Scullard GH, Eddleston WF, Williams R. Full and empty Dane particles in chronic hepatitis B antigen and presence of liver damage. *Gastroenterology* 1978; **75**: 869–74.
13 Gerin JL, Ford EC, Purcell RH. Biochemical characterization of Australia antigen: evidence for defective particles of hepatitis B virus. *Am. J. Pathol.* 1975; **81**: 651–68.
14 Sakamoto Y, Yamada G, Mizuno M *et al.* Full and empty particles of hepatitis B virus in hepatocytes from patients with HBsAg-positive chronic active hepatitis. *Lab. Invest.* 1983; **48**: 678–82.
15 Raimondo G, Recchia S, Lavarini C, Crivelli O, Rizzetto M. Dane particle-associated hepatitis B e antigen in patients with chronic hepatitis B virus infection and hepatitis B e antibody. *Hepatology* 1982; **2**: 449–54.
16 Carman WF, Jacyna MR, Hadziyannis S *et al.* Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; **2**: 588–91.
17 Okamoto H, Yotsumoto S, Akahane Y *et al.* Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J. Virol.* 1990; **64**: 1298–303.
18 Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J. Virol.* 1994; **68**: 8102–10.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 23, Issue of June 10, pp. 21713–21719, 2005
Printed in U.S.A.

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

Received for publication, February 10, 2005, and in revised form, March 31, 2005
Published, JBC Papers in Press, April 4, 2005, DOI 10.1074/jbc.M501564200

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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.

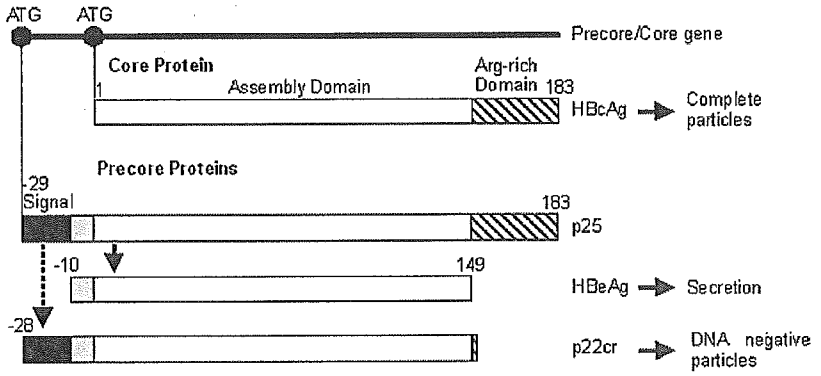

FIG. 1. **Schematics of precore/core genes and their products.** At its 5′ end, the precore/core gene contains two closely spaced ATGs (*black dots*) enclosing the precore region, which encodes a 29-aa precore sequence. Translation of the core mRNA results in the production of the cytoplasmic core protein, 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 μm^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-

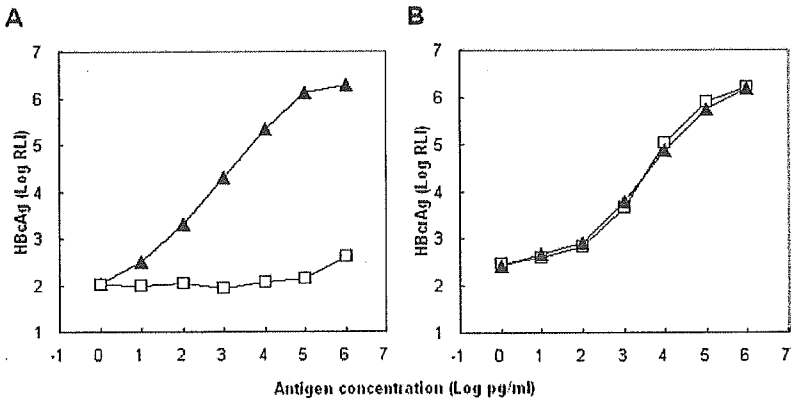

FIG. 2. **Reactivity of the HBcAg assay and the HBcrAg assay.** Recombinant HBcAg (▲) and HBeAg (□) were diluted and tested for the HBcAg assay (*A*) and the HBcrAg assay (*B*). The assay reactivity is shown as log relative luminescence intensity (*RLI*).

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.

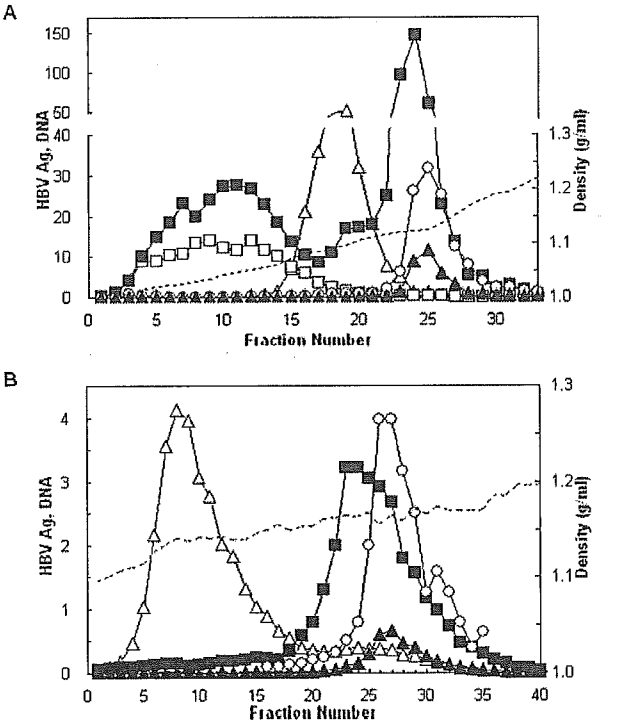

FIG. 3. **Sucrose gradient analysis of HBV-positive plasma.** *A*, ProMedDx HBV plasma 9990776 was subjected to ultracentrifugation using a 10–60% (w/w) sucrose density gradient. *B*, fractions 23–26 were reanalyzed by ultracentrifugation in a 30–50% sucrose density gradient. Density of each fraction 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 = 4×10^7 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

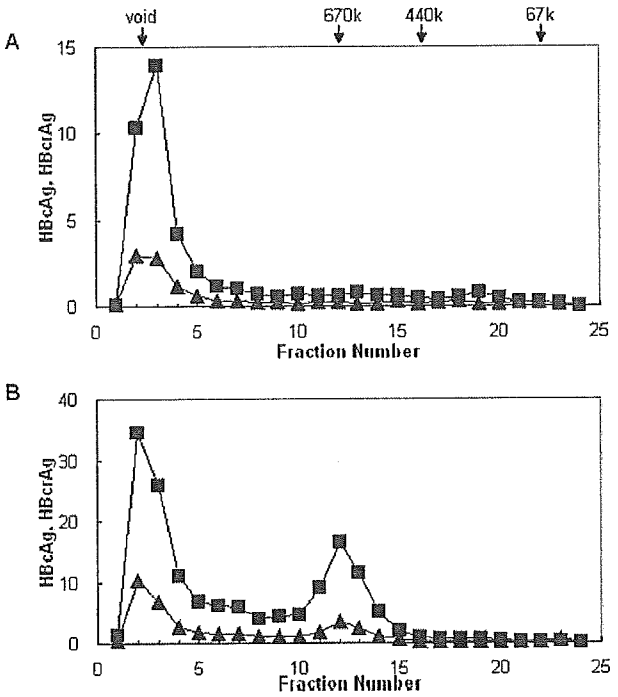

FIG. 4. **Gel filtration analysis of the particle fractions.** The particle fractions of a density gradient were treated without (*A*) or with 3% Nonidet P-40 detergent (*B*) and then subjected to gel filtration through Superose 6 HR. The elution from 6 to 18 ml were fractionated into 24 fractions and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

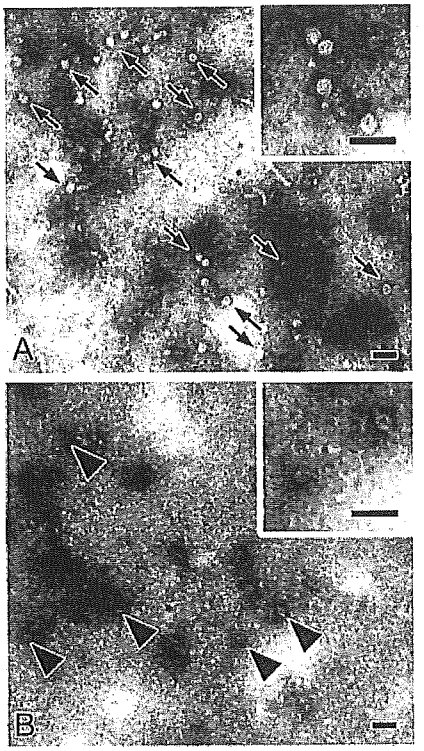

FIG. 5. **Electron micrographs of virus particles in the two density gradient fractions of HBV-positive plasma.** The particles in equal volumes of each fraction (Table I) were collected on copper grids by ultracentrifugation and negatively stained and observed under an electron microscope. Dane-like 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 μm^2 Dane-like particles, which was significantly more than in fraction B (5.6 ± 3.8/3.76 μm^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 μm^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 μm^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. $n = 60$; $p = 0.27$.

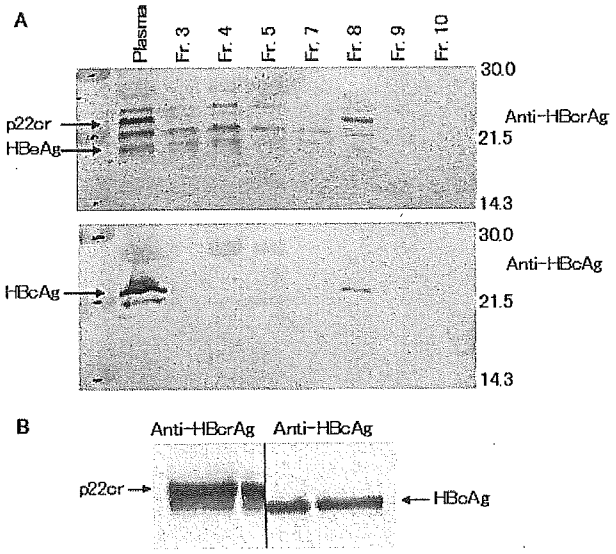

FIG. 6. **Western blot of HBcrAg.** HBV-positive plasma (BBI PHM935A-14) was subjected to ultracentrifugation through a 10–60% (w/w) sucrose density gradient and fractionated into 15 fractions. *A*, plasma and its sucrose density gradient fractions were analyzed by Western blotting using anti-HBcAg (HB50) 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

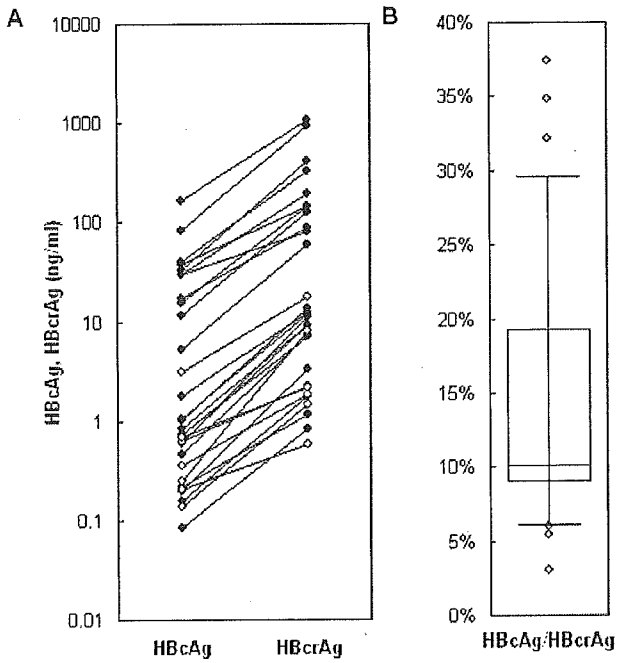

FIG. 7. **HBcAg and HBcrAg levels in HBsAg-positive particles of chronic hepatitis B serum.** HBsAg-positive particles were immunoprecipitated by anti-HBsAg-coated magnetic beads from 30 samples of HBV-infected sera. Precipitated proteins were eluted by SDS solution. Levels of HBcrAg and HBcAg in precipitate 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.

REFERENCES

1. Seeger, C., and Mason, W. S. (2000) *Microbiol. Mol. Biol. Rev.* **64,** 51–68
2. Dane, D. S., Cameron, C. H., and Briggs, M. (1970) *Lancet* **1,** 695–698
3. Gerin J. L., Ford, E. C., and Purcell, R. H. (1975) *Am. J. Pathol.* **81,** 651–668
4. Alberti, A., Diana, S., Scullard, G. H., Eddleston, W. F., and Williams, R. (1978) *Gastroenterology* **75,** 869–874
5. Takahashi, T., Kaga, K., Akahane, Y., Yamashita, T., Miyakawa, Y., and Mayumi, M. (1980) *J. Med. Microbiol.* **13,** 163–166
6. Sakamoto, Y., Yamada, G., Mizuno, M., Nishihara, T., Kinoyama, S., Kobayashi, T., Takahashi, T., and Nagashima, H. (1983) *Lab. Investig.* **48,** 678–682
7. Ou, J.-H., Laub, O., and Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1578–1582
8. Garcia, P. D., Ou, J.-H., Rutter, W. J., and Walter, P. (1988) *J. Cell Biol.* **106,** 1093–1104
9. Standring, D. N., Ou, J.-H., Masiarz, F. R., and Rutter, W. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8405–8409
10. Messageot, F., Salhi, S., Eon, P., and Rossignol, J.-M. (2003) *J. Biol. Chem.* **278,** 891–895
11. Takahashi, K., Machida, A., Funatsu, G., Nomura, M., Usuda, S., Aoyagi, S., Tachibana, K., Miyamoto, H., Imai, M., Nakamura, T., Miyakawa, Y., and Mayumi, M. (1983) *J. Immunol.* **130,** 2903–2907
12. Schlicht, H.-J. (1991) *J. Virol.* **65,** 3489–3495
13. Weimer, T., Salfeld, J., and Will, H. (1987) *J. Virol.* **61,** 3109–3113
14. Bottcher, B., Wynne, S. A., and Crowther, R. A. (1997) *Nature* **386,** 88–91
15. Conway, J. F., Cheng, N., Zlotnick, A., Wingfield, P. T., Stahl, S. J., and Steven, A. C. (1997) *Nature* **386,** 91–94
16. Gallina, A., Bonelli, F., Zentilin, L., Rindi, G., Muttini, M., and Milanesi, G. (1989) *J. Virol.* **63,** 4645–4652
17. Birnbaum, F., and Nassal, M. (1990) *J. Virol.* **64,** 3319–3330
18. Crowther, R. A., Kiselev, N. A., Bottcher, B., Berriman, J. A., Borisova, G. P., Ose, V., and Pumpens, P. (1994) *Cell* **77,** 943–950
19. Wingfield, P. T., Stahl, S. J., Williams, R. W., and Steven, A. C. (1995) *Biochemistry* **34,** 4919–4932
20. Beames, B., and Lanford, R. E. (1993) *Virology* **194,** 597–607
21. Kimura, T., Rokuhara, A., Matsumoto, A., Yagi, S., Tanaka, E., Kiyosawa, K., and Maki, N. (2003) *J. Clin. Microbiol.* **41,** 1901–1906
22. Kimura, T., Rokuhara, A., Sakamoto, Y., Yagi, S., Tanaka, E., Kiyosawa, K., and Maki, N. (2002) *J. Clin. Microbiol.* **40,** 439–445
23. Rokuhara, A., Tanaka, E., Matsumoto, A., Kimura, T., Yamaura, T., Orii, K., Sun, X., Yagi, S. Maki, N., and Kiyosawa, K. (2003) *J. Viral Hepat.* **10,** 324–330
24. Mukaide, M., Tanaka, Y., Katayose, S., Tano, H., Murata, M., Hikata, M., Fujise, K., Sakugawa, H., Suzuki, K., Zaunders, J., Nagasawa, Y., Toda, G., and Mizokami, M. (2003) *J. Gastroenterol. Hepatol.* **18,** 1264–1271
25. Newman, M., Suk, F.-M., Cajimat, M., Chua, P. K., and Shih, C. (2003) *J. Virol.* **77,** 12950–12960
26. Miyanohara, A., Imamura, T., Araki, M., Sugawara, K., Ohtomo, N., and Matsubara K. (1986) *J. Virol.* **59,** 176–180
27. Ou, J.-H., and Bell, K. D. (1990) *Virology* **174,** 185–191
28. Scaglioni, P. P., Melegari, M., and Wands, J. R. (1997) *J. Virol.* **71,** 345–353
29. Schlicht, H.-J., and Wasenauer, G. (1991) *J. Virol.* **65,** 6817–6825
30. Takahashi, K., Kishimoto, S., Ohori, K., Yoshizawa, H., Machida, A., Ohnuma, H., Tsuda, F., Munekata, E., Miyakawa, Y., and Mayumi, M. (1991) *J. Immunol.* **147,** 3156–3160
31. Lok, A. S., Akarca, U., and Greene, S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4077–4081
32. Ou, J.-H., Yeh, C.-T., and Yen, T. S. (1989) *J. Virol.* **63,** 5238–5243
33. Yang, S. Q., Walter, M., and Standring, D. N. (1992) *J. Virol.* **66,** 37–45
34. Nassal, M., and Rieger, A. (1993) *J. Virol.* **67,** 4307–4315
35. Schodel, F., Peterson, D., Zheng, J., Jones, J. E., Hughes, J. L., and Milich, D. R. (1993) *J. Biol. Chem.* **268,** 1332–1337
36. Wasenauer, G., Kock, J., and Shlicht, H.-J. (1992) *J. Virol.* **66,** 5338–5346
37. Zhou, S., and Standring, D. N. (1992) *J. Virol.* **66,** 5393–5398
38. Maassen, A., Rehfeldt, A., Kiessig, S., Ladhoff, A., Hohne, W. E., and Meisel, H. (1994) *Arch. Virol.* **135,** 131–142
39. Guidotti, L. G., Matzke, B., Pasquinelli, C., Shoenberger, J. M., Rogler, C. E., and Chisari, F. V. (1996) *J. Virol.* **70,** 7056–7061

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Hepatology Research 32 (2005) 173–184
HEPATOLOGY RESEARCH
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Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: A multicenter retrospective study of 2795 patients

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Received 6 December 2004; received in revised form 7 February 2005; accepted 9 February 2005

Abstract

A retrospective survey of Japanese patients histologically diagnosed with chronic hepatitis B was conducted to determine the effectiveness of lamivudine in preventing hepatocellular carcinoma (HCC). Of the 2795 patients who satisfied criteria for analysis after treatment from any of 30 medical institutions, 657 had received lamivudine and the remaining 2138 had not. A Cox regression model with liver biopsy as the starting point revealed seven factors related to HCC: lamivudine therapy, gender, family clustering of hepatitis B, age at liver biopsy, hepatic fibrosis stage, serum albumin level, and platelet count. In a matched case-controlled study, 377 patients in a lamivudine-treated group and 377 matched patients in a non-treated group were selected based on their propensity scores. The mean follow-up period was 2.7 years in the lamivudine group and 5.3 years in the control group. In the lamivudine group, HCC occurred in four patients (1.1%) with an annual incidence rate of 0.4%/(patient/year), whereas in the control group HCC occurred in 50 patients (13.3%) for a rate of 2.5%/(patient/year). A comparison of the cumulative HCC incidence between the two groups by the Kaplan–Meier method showed a significantly lower incidence of HCC in the lamivudine group ($p < 0.001$). These findings suggest that lamivudine effectively reduces the incidence of HCC in patients with chronic hepatitis B.

Keywords: Chronic hepatitis B; Hepatocellular carcinoma; Anti-viral treatment; Lamivudine

1. Introduction

An estimated 350 million people worldwide are chronically infected with the hepatitis B virus (HBV), most in southeast Asia [1,2]. In this region, infection occurs during infancy, including that through mother–child transmission. Infected persons with HBV are initially asymptomatic, and

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doi:10.1016/j.hepres.2005.02.006

active hepatitis emerges years later. In most patients, seroconversion from hepatitis Be antigen (HBeAg) to antibody to HBeAg (HBeAb) occurs spontaneously with age. At the same time, the virus levels decrease and hepatitis abates. Some patients, however, remain positive for HBeAg, and in those patients the hepatitis virus persists at high levels, resulting in the progression to hepatic cirrhosis, and the onset of hepatocellular carcinoma (HCC) in a high percentage of such patients [3–5]. The number of HBV carriers is decreasing in Japan and some other countries as a result of the prevention of mother–child transmission through the use of HBV vaccines and/or high-potency antibody to hepatitis B surface antigen (HBsAb) human immunoglobulin (HBIG) [6]. Even in these countries, however, only persons born after 1986 are protected by vaccination, and many chronic hepatitis B patients still need treatment. In the past, it was not easy to manage chronic hepatitis B using anti-viral agents such as interferon. In recent years, however, the development of lamivudine, a nucleoside analogue that inhibits reverse transcriptase, has drastically changed the treatment of hepatitis B [7–9]. By virtue of this inhibitory ability, lamivudine was developed as an anti-viral agent against human immuno-deficiency virus (HIV). It was later also found to be effective against HBV because HBV is a member of the Hepadnaviridae family, which utilizes reverse transcriptase in its replication process [10]. Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve liver histological findings in long-term treatment [11]. It is also useful when hepatitis B becomes severe due to acute exacerbation, as well as in the treatment of liver cirrhosis associated with symptoms of hepatic failure, such as ascites and edema [12–16]. However, a number of problems are associated with lamivudine therapy, such as relapse of hepatitis due to the appearance of YMDD mutant viruses and the difficulty of estimating the optimal time to discontinue the treatment [17,18]. In addition, until recently no adequate studies had been conducted to determine whether or not lamivudine inhibits the onset of hepatic cancer, even though it is known to slow the progression of histological changes in the liver. This lack of research is attributable partly to the need for long-term follow-up of a large number of patients and partly to the difficulty of conducting clinical trials. We conducted a multicenter study of a large number of registered patients to evaluate the effects of lamivudine on the course of hepatitis B and the onset of HCC. The data obtained were analyzed in a matched case-controlled study.

2. Materials and methods

2.1. Study design

The Inuyama Hepatitis Study Group designed this multicenter retrospective study to determine whether or not lamivudine is effective in preventing HCC. The subjects were Japanese patients with hepatitis B who were diagnosed with chronic liver disease by liver biopsy after 1980 and were followed up until March 2002. Each patient completed a questionnaire containing 16 items in four categories: background factors: date of birth, sex, family clustering of hepatitis B, and alcohol consumption during follow-up (80 g or more per day as ethanol); examination and test items: date of liver biopsy, grade and stage of histological findings of the liver, hepatitis Be antigen (HBeAg), antibody to HBeAg (HBeAb), albumin, asparate aminotransferase (AST), alanine aminotransferase (ALT), and platelet counts; clinical outcomes: the presence or absence of HCC during the follow-up period and the date of onset if present; lamivudine therapy: the presence or absence of lamivudine therapy during the follow-up period, and the date of initiation and duration of therapy if provided. The study was allowed by the review board of each participating institution. The names, ID numbers, and all other information that would directly identify individual patients were deleted to protect their privacy.

2.2. Patients

The present study included 3022 patients with chronic hepatitis B who underwent liver biopsy at any of 30 medical institutions after 1980. No patient had superinfection with hepatitis C virus and HIV. Two hundred and twenty-seven patients who had not answered the question about lamivudine treatment were excluded from the study. This left a total of 2795 patients for analysis. Among them, 657 patients had received lamivudine therapy and 2138 patients had not.

Histological findings of the liver were scored with respect to the grade of inflammation and stage of hepatic fibrosis according to the New Inuyama Histological Criteria [19] by a pathologist at each institution.

2.3. Lamivudine treatment

The lamivudine treatment group consisted of 657 patients who had received lamivudine therapy (100 mg/day). The median lamivudine treatment period was 18.9 months. Lamivudine therapy was continued until the end of the follow-up period in 45% of the patients.

2.4. Matched case-controlled study

In our analysis of the relationship between lamivudine therapy and hepatic carcinogenicity, the starting point was the day of liver biopsy. However, many patients in the lamivudine group (279 patients or 41.4%) initiated lamivudine therapy more than 2 years after liver biopsy, making them inappropriate subjects for the evaluation of the effects of lamivudine on hepatic carcinogenicity. For this reason, 377 patients who started lamivudine therapy within 2 years after liver biopsy were selected for analysis from the 657 patients in the lamivudine group. The interval from liver biopsy to lamivudine therapy was 5.8 ± 9.0 months, and the treatment

period was 23.1 ± 19.0 months (range 3–96 months). For the control group, seven factors were selected on the basis of the propensity scores from the 2138 patients who had not received lamivudine: age at the time of liver biopsy, gender, family clustering of hepatitis B, stage of hepatic fibrosis, serum albumin level, and platelet count. On that basis, 377 matching patients were selected for the control group [20].

2.5. Statistical analyses

A series of analyses was conducted using the day of liver biopsy as the starting point. Background factors at the time of liver biopsy were compared by the Student's *t*-test (numerical data) or the χ^2 test (categorical data), and differences were regarded as significant if $p < 0.05$ on both sides. Factors related to HCC were analyzed using a Cox regression model. The incidence of HCC was reported as an annual incidence rate (%/(patient/year)).

Because of the large differences in background factors between the lamivudine and control groups, the groups were matched for further analysis of HCC-related factors. For this analysis, all patients who had started lamivudine therapy within 2 years after liver biopsy were selected. The propensity score method was used to select patients from the control group [20]. Matching was done with respect to the HCC-related factors selected using the Cox regression model. After the matching, the incidence of HCC was shown by the Kaplan–Meier method and compared between the groups by the log-rank test. Differences were regarded as significant if $p < 0.05$ on both sides.

3. Results

3.1. Comparison of background factors

Table 1 demonstrates the comparison of background factors at the time of liver biopsy between the lamivudine and control groups. Significant differences were found in the mean age ($p < 0.001$), duration of follow-up ($p < 0.001$), history of IFN therapy ($p < 0.001$), inflammation of the liver ($p < 0.001$), HBeAg ($p < 0.001$), HBeAb ($p = 0.001$), serum albumin level ($p < 0.001$), AST level ($p = 0.011$), and platelet count ($p < 0.001$).

3.2. Evaluation of factors related to hepatic carcinogenicity by univariate analyses

HCC occurred in 31 of the 657 patients (4.7%) in the lamivudine group and in 239 of the 2138 patients (11.2%) in the control group. The mean follow-up periods after liver biopsy were 4.9 and 6.2 years in the lamivudine and control groups, respectively. Thus, the crude incidence of HCC determined was 1.0 and 1.8%/(patient/year) in the lamivudine and control groups, respectively.

Table 2 shows the incidences of HCC in the lamivudine and control groups in an analysis stratified with respect to background factors. In the lamivudine group, HCC did not occur in patients whose histological findings were grade 0 in inflammation and stage 0 in fibrosis, and significant intergroup differences were noted in this respect. No significant differences were observed other than in the histological findings.

3.3. Evaluation of factors related to hepatic carcinogenicity using a multivariate Cox regression model

Factors contributing to the incidence of HCC were analyzed using a Cox regression model (Table 3). The following variables were selected by the forward–backward stepwise selection method: lamivudine therapy (no therapy, $p = 0.002$), gender (male, $p < 0.001$), family history of hepatitis B (present, $p = 0.015$), age at the time of liver biopsy (older than 40 years, $p < 0.001$), stage of liver fibrosis (more than F2, $p < 0.001$), serum albumin level (less than 4.0 g/dL, $p = 0.001$), and platelet count (less than 150,000/μL, $p < 0.001$)). This analysis showed that lamivudine reduces the risk of HCC.

3.4. Evaluation of factors related to hepatic carcinogenicity by a six-factor matched case-controlled study

Matched case-control analyses were performed for six factors (sex, family history of hepatitis B, age at the time of liver biopsy, stage of liver fibrosis, serum albumin level, and platelet count). There were no significant differences in background factors between the groups, as shown in Table 4. The mean follow-up period in the control group (5.3 years) was about twice that in the lamivudine group (2.7 years). In the lamivudine group, HCC occurred in 4 of 377 patients (1.1%), with an annual incidence rate of 0.4%/(patient/year), compared to 50 of 377 patients (13.3%) and 2.5%/(patient/year), respectively, in the control group. A comparison of the cumulative HCC incidence between the two groups by the Kaplan–Meier method showed a significantly lower incidence in the lamivudine group ($p < 0.001$) (Fig. 1).

Next, the background factors were compared between patients with HCC and those without it in the lamivudine and control groups. In the lamivudine group (Table 5), the mean age was significantly higher in patients with HCC than in those without it (55.0 years versus 41.3 years, $p = 0.024$), but there were no significant differences in the other factors. In the control group (Table 6), the mean age was significantly higher in patients with HCC than in those without it (50.6 years versus 40.0 years, $p < 0.001$). Significant differences were also noted in the stage of liver fibrosis ($p < 0.001$), serum albumin level ($p < 0.001$), and platelet count ($p < 0.001$), suggesting that underlying liver disease was more advanced in patients who developed HCC.

Table 1
Comparison of background factors between lamivudine group and control group assessed at the time of liver biopsy

Parameter	Lamivudine group (n=657)	Control group (n=2138)	p-Value
Gender[a]			
Male	503 (76.6%)	1583 (74.0%)	0.194
Female	154 (23.4%)	555 (26.0%)	
Age (years)[b]	40.9 ± 11.0	37.3 ± 12.4	<0.001
Follow-up period (years)[b]	4.9 ± 4.4	6.2 ± 5.5	<0.001
Family clustering of hepatitis B[a]			
Yes	376 (57.2%)	1085 (50.7%)	0.011
No	242 (36.8%)	924 (43.2%)	
Unknown	39 (5.9%)	129 (6.0%)	
Drinking during the course of the study (>ethanol 80 g/day)			
Yes	69 (10.5%)	359 (16.8%)	<0.001
No	557 (84.8%)	1708 (79.9%)	
Unknown	31 (4.7%)	71 (3.3%)	
IFN therapy[a]			
Yes	269 (40.9%)	812 (38.0%)	<0.001
No	369 (56.2%)	1306 (61.1%)	
Unknown	19 (2.9%)	20 (0.9%)	
Liver histology			
Grade of inflammation[a]			
A0	15 (2.3%)	84 (3.9%)	<0.001
A1	194 (29.5%)	642 (30.0%)	
A2	283 (43.1%)	996 (46.6%)	
A3	142 (21.6%)	389 (18.2%)	
Unknown	23 (3.5%)	27 (1.3%)	
Stage of fibrosis[a]			
F0	12 (1.8%)	49 (2.3%)	0.491
F1	201 (30.6%)	721 (33.7%)	
F2	167 (25.4%)	524 (24.5%)	
F3	171 (26.0%)	491 (23.0%)	
F4	98 (14.9%)	331 (15.5%)	
Unknown	8 (1.2%)	22 (1.0%)	
HBeAg[a]			
+	355 (54.0%)	1272 (59.5%)	<0.001
–	280 (42.6%)	723 (33.8%)	
Unknown	22 (3.3%)	143 (6.7%)	
HBeAb[a]			
+	215 (32.7%)	642 (30.0%)	0.001
–	418 (63.6%)	1330 (62.2%)	
Unknown	24 (3.7%)	166 (7.8%)	
Albumin (g/dL)[b]	4.01 ± 0.49 (n=629)	4.14 ± 0.49 (n=1941)	<0.001
AST (IU/L)[b]	110.2 ± 131.8 (n=593)	94.5 ± 131.5 (n=2023)	0.011
ALT (IU/L)[b]	183.4 ± 211.1 (n=641)	163.5 ± 234.3 (n=2022)	0.056
Platelet count ($\times 1000/mm^3$)[b]	165.4 ± 54.9 (n=629)	176.9 ± 59.6 (n=1931)	<0.001

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.

4. Discussion

It is clear that this study has several limitations: it is not prospective, it is not randomized, there is no single regimen of lamivudine, and there is a lack of virological analysis (including that of the HBV genotype and that of YMDD mutations). It would be desirable to conduct a well-designed prospective study using controls. However, because lamivudine has been used in general practice under the insurance system in Japan, it is difficult to conduct a prospective and randomized control study of lamivudine therapy for chronic hepatitis B. In addition, it is ethically unacceptable to leave patients untreated for a long period of time in a control group, because lamivudine has been shown to abate hepatitis and improve histological findings of the liver [12–16].

Table 2
Comparison of the incidence of HCC in relation to each background factor between lamivudine group and control group

Parameter	Category	Group	Total number of patients (number)	No. of patients with HCC (number)	Average follow-up period (year)	Adjusted incidence of HCC (%/year)
Gender	Male	Lamivudine group	503	27	5.0	1.07
		Control group	1583	191	6.4	1.89
	Female	Lamivudine group	154	4	4.3	0.60
		Control group	555	48	5.6	1.54
Age (years)	<30	Lamivudine group	110	2	4.7	0.39
		Control group	642	8	5.9	0.21
	30≦ and <40	Lamivudine group	192	9	5.7	0.82
		Control group	646	52	6.8	1.18
	40≦ and <50	Lamivudine group	206	9	5.3	0.82
		Control group	491	75	6.7	2.28
	50≦	Lamivudine group	149	11	3.3	2.24
		Control group	359	104	5.3	5.47
Duration of lamivudine treatment (years)	<1	Lamivudine group	178	7	5.0	0.79
		Control group	–	–	–	–
	1≦ and <2	Lamivudine group	215	13	4.4	1.37
		Control group	–	–	–	–
	2≦ and <3	Lamivudine group	145	7	4.6	1.05
		Control group	–	–	–	–
	3≦	Lamivudine group	107	4	5.9	0.63
		Control group	–	–	–	–
Family clustering of hepatitis B	No	Lamivudine group	242	10	4.8	0.86
		Control group	924	100	6.4	1.69
	Yes	Lamivudine group	376	20	5.0	1.06
		Control group	1085	128	5.9	2.00
	Unknown	Lamivudine group	39	1	4.4	0.58
		Control group	129	11	8.2	1.04
Drinking during the course of the study (>ethanol 80 g/day)	No	Lamivudine group	557	23	4.8	0.86
		Control group	1708	158	5.8	1.59
	Yes	Lamivudine group	69	7	5.6	1.81
		Control group	359	76	7.8	2.71
	Unknown	Lamivudine group	31	1	3.8	0.85
		Control group	71	5	7.7	0.91
IFN therapy	No	Lamivudine group	369	19	4.2	1.23
		Control group	1306	167	6.0	2.13
	Yes	Lamivudine group	269	12	6.0	0.74
		Control group	812	70	6.5	1.33
	Unknown	Lamivudine group	19	0	2.6	0.00
		Control group	20	2	7.9	1.27
Liver histology Grade of inflammation	A0	Lamivudine group	15	0	9.3	0.00
		Control group	84	8	6.6	1.44
	A1	Lamivudine group	194	4	5.4	0.38
		Control group	642	59	6.4	1.44
	A2	Lamivudine group	283	15	4.9	1.08
		Control group	996	109	6.3	1.74
	A3	Lamivudine group	142	10	3.4	2.07
		Control group	389	52	5.5	2.43
	Unknown	Lamivudine group	23	2	6.1	1.43
		Control group	27	11	8.7	4.68

Table 2 (*Continued*)

Parameter	Category	Group	Total number of patients (number)	No. of patients with HCC (number)	Average follow-up period (year)	Adjusted incidence of HCC (%/year)
Stage of fibrosis	F0	Lamivudine group	12	0	7.2	0.00
		Control group	49	3	5.7	1.07
	F1	Lamivudine group	201	6	6.0	0.50
		Control group	721	29	6.7	0.60
	F2	Lamivudine group	167	8	4.7	1.02
		Control group	524	38	5.8	1.25
	F3	Lamivudine group	171	11	4.0	1.61
		Control group	491	61	6.0	2.07
	F4	Lamivudine group	98	6	3.6	1.70
		Control group	331	99	6.2	4.82
	Unknown	Lamivudine group	8	0	6.7	0.00
		Control group	22	9	8.3	4.93
HBeAg	−	Lamivudine group	280	10	4.2	0.85
		Control group	723	83	6.4	1.79
	+	Lamivudine group	355	19	5.3	1.01
		Control group	1272	134	6.0	1.76
	Unknown	Lamivudine group	22	2	6.2	1.47
		Control group	143	22	7.4	2.08
HBeAb	−	Lamivudine group	418	19	4.9	0.93
		Control group	1330	137	6.0	1.72
	+	Lamivudine group	215	10	4.7	0.99
		Control group	642	75	6.3	1.85
	Unknown	Lamivudine group	24	2	6.1	1.37
		Control group	166	27	7.4	2.20
Albumin (g/dL)	<4.0	Lamivudine group	257	19	4.5	1.64
		Control group	619	113	5.7	3.20
	4.0≦	Lamivudine group	372	9	4.9	0.49
		Control group	1322	90	6.1	1.12
AST (IU/L)	<50	Lamivudine group	187	7	5.7	0.66
		Control group	905	82	6.1	1.49
	50≦ and <100	Lamivudine group	200	14	4.7	1.49
		Control group	572	81	5.9	2.40
	100≦ and <200	Lamivudine group	142	7	5.1	0.97
		Control group	367	31	6.2	1.36
	200≦	Lamivudine group	64	2	4.4	0.71
		Control group	179	15	6.0	1.40
ALT (IU/L)	<50	Lamivudine group	117	5	4.7	0.91
		Control group	570	69	6.1	1.98
	50≦ and <100	Lamivudine group	155	7	4.9	0.92
		Control group	506	60	5.8	2.04
	100≦ and <150	Lamivudine group	109	9	4.7	1.76
		Control group	297	36	5.9	2.05
	150≦	Lamivudine group	260	9	4.8	0.72
		Control group	649	44	6.2	1.09
Platelet count ($\times 1000/mm^3$)	<150	Lamivudine group	254	18	3.8	1.86
		Control group	629	125	5.8	3.43
	150≦	Lamivudine group	375	11	5.3	0.55
		Control group	1302	67	6.1	0.84

Table 3
Estimation of effects of covariates following selection of regressor in Cox regression model

Category	Hazard ratio	95% Confidence interval (CI)	*p*-Value
Lamivudine therapy			
No	1		
Yes	0.49	0.31–0.77	0.002
Gender			
Male	1		
Female	0.42	0.28–0.62	<0.001
Family clustering of hepatitis B			
No	1		
Yes	1.44	1.08–1.94	0.015
Age at liver biopsy			
<40 y.o.	1		
≧40 y.o.	2.09	1.77–2.48	<0.001
Stage of liver fibrosis			
F0 or F1	1		
F2, F3, or F4	1.43	1.24–1.64	<0.001
Serum albumin level			
<4.0 g/dL	1		
≧4.0 g/dL	0.58	0.43–0.79	0.001
Platelet count			
<150 × 1000/μL	1		
≧150 × 1000/μL	0.53	0.38–0.73	<0.001

In the analysis of retrospective studies, great precautions are required in order to eliminate any bias between lamivudine-treated and non-treated groups. To minimize inter-group bias, we conducted with the cooperation of multiple medical institutions and a large number of patients ($n=2795$). The effect of lamivudine on HCC was ultimately analyzed in a matched case-controlled study. Because the time of liver biopsy was used as the starting point in our analysis, the analytical results were not expected to appropriately reflect lamivudine's effect if the therapy was started a long time after the biopsy. Therefore, from among the 657 patients who received lamivudine therapy, we selected 377 patients who started lamivudine therapy within 2 years after biopsy. For a control group, the same number of patients ($n=377$) without lamivudine therapy was selected from the 2138 subjects.

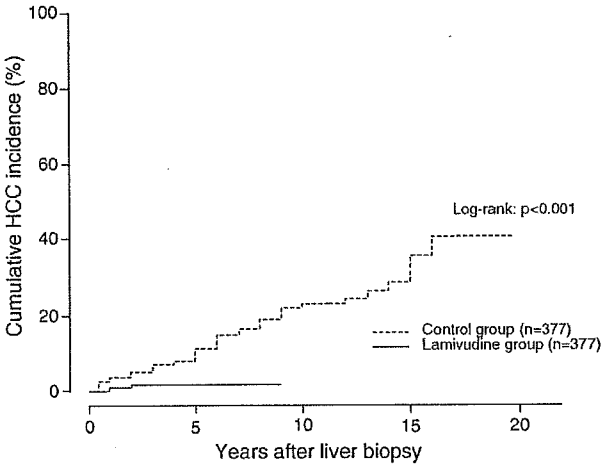

Fig. 1. Comparison of the cumulative HCC incidence between the lamivudine group (solid line) and the control group (broken line) by the Kaplan–Meier method in a case-matched control study. A significant difference was seen between the two groups ($p<0.001$, log-rank test).

The regimen was not the same in all patients who have been treated by lamivudine. It was transiently discontinued before being recommenced later in some patients, whereas it was uninterrupted throughout the follow-up period in the majority (63%) of subjects in the matched case-controlled study. The duration of lamivudine regimen was not taken into account in the design of our study. Some patients received lamivudine for relatively short periods to improve acute exacerbation of their clinical course in chronic hepatitis B. On the other hand, some patients received lamivudine for the long-term to suppress the development of HCC. In the analysis by a multivariate Cox regression model in all unmatched patients, lamivudine therapy was selected as one of the factors inhibiting the occurrence of HCC. In the matched case-controlled study, the annual occurrence rate of HCC was significantly lower (0.4%/(patient/year)) in the lamivudine group than in the control group (1.8%/(patient/year)), suggesting that lamivudine treatment is effective for inhibiting the occurrence of HCC.

Recently, Liaw et al. conducted a multicenter, centrally randomized, double-blind, placebo-controlled, parallel group study to evaluate the effects of lamivudine on the progression of chronic hepatitis B to hepatic cancer [21]. They randomized 651 patients with histologically confirmed (F3 and F4), compensated hepatic cirrhosis to receive either lamivudine or a placebo at a ratio of 2:1 and continued the treatment for up to 5 years. The study was terminated after a median treatment duration of 32.4 months (range 0–42) owing to a significant difference between the groups in the number of end points reached. The end points were reached by 7.8% of the patients receiving lamivudine and 17.7% of those receiving placebo (hazard ratio for disease progression, 0.45; $p=0.001$). The Child–Pugh score increased in 3.4% of the patients receiving lamivudine and in 8.8% of those receiving placebo (hazard ratio, 0.45; $p=0.02$), whereas HCC occurred in 3.9% of those in the lamivudine group and in 7.4% of those in the placebo group (hazard ratio, 0.49: $p=0.047$). The results of our analysis, which included patients with F0 through F2 hepatic fibrosis, were similar to those of Liaw et al. [21]. Thus, two studies demonstrated that the use of potent anti-viral agents such as lamivudine represents a major advance in the treatment of chronic hepatitis B and slows the progression of severe liver disease to liver cirrhosis as well as HCC.

Both hepatitis B and C are caused by persistent infection with hepatitis viruses, and both have a high probability of resulting in HCC. For this reason, these two diseases have a number of common traits, but some differences have been noted in their relationships with HCC. Among both

Table 4
Comparison of background factors between lamivudine group and control group assessed at the time of liver biopsy (matched case-controlled study)

Parameter	Lamivudine group (n=377)	Control group (n=377)	p-Value
Gender[a]			
Male	276 (73.2%)	273 (72.4%)	0.806
Female	101 (26.8%)	104 (27.6%)	
Age (years)[b]	41.5 ± 12.0	41.4 ± 12.2	0.950
Follow-up period (years)[b]	2.7 ± 2.1	5.3 ± 4.7	<0.001
Family clustering of hepatitis B[a]			
Yes	238 (63.1%)	242 (64.2%)	0.762
No	139 (36.9%)	135 (35.8%)	
Drinking during the course of the study (>ethanol 80 g/day)[a]			
Yes	38 (10.1%)	62 (16.4%)	0.007
No	333 (88.3%)	314 (83.3%)	
Unknown	6 (1.6%)	1 (0.3%)	
IFN therapy[a]			
Yes	129 (34.2%)	143 (37.9%)	0.046
No	236 (62.6%)	231 (61.3%)	
Unknown	12 (3.2%)	3 (0.8%)	
Liver histology			
Grade of inflammation[a]			
A0	6 (1.6%)	18 (4.8%)	0.001
A1	110 (29.2%)	101 (26.8%)	
A2	157 (41.6%)	186 (49.3%)	
A3	98 (26.0%)	72 (19.1%)	
Unknown	6 (1.6%)	0 (0.0%)	
Stage of fibrosis[a]			
F0	7 (1.9%)	6 (1.6%)	0.647
F1	103 (27.3%)	117 (31.0%)	
F2	95 (25.2%)	97 (25.7%)	
F3	107 (28.4%)	90 (23.9%)	
F4	65 (17.2%)	67 (17.8%)	
HBeAg[a]			
+	193 (51.2%)	220 (58.4%)	0.005
–	178 (47.2%)	141 (37.4%)	
Unknown	6 (1.6%)	16 (4.2%)	
HBeAb[a]			
+	126 (33.4%)	121 (32.1%)	0.030
–	245 (65.0%)	237 (62.9%)	
Unknown	6 (1.6%)	19 (5.0%)	
Albumin (g/dL)[b]	4.00 ± 0.51	4.00 ± 0.52	0.989
AST (IU/L)[b]	118.5 ± 155.4	95.5 ± 126.4	0.031
ALT (IU/L)[b]	191.7 ± 234.8	151.5 ± 180.5	0.009
Platelet count (×1000/mm^3)[b]	161.7 ± 52.7	164.3 ± 59.5	0.523

[a] Data are expressed as positive numbers (%).
[b] Data are expressed as means ± S.D.

hepatitis B patients and hepatitis C patients, HCC occurs mainly in those with advanced hepatic fibrosis, but the incidence of liver cirrhosis as a background of liver disease is lower in patients with B than in those with C. Furthermore, among hepatitis C patients HCC occurs mainly in those 60 years or older, while among hepatitis B patients it occurs mainly in those under 60 [22–24]. Studies on the cumulative incidence of HCC in hepatitis B patients showed that the HCC incidence increases linearly during the initial 12 years, plateaus, and then increases again in the 17th or 18th year [24,25]. In hepatitis C patients, on the other hand, the HCC incidence shows a continuous, linear increase [26,27]. Various findings obtained to date suggest that these clinical differences are related not only to differences in the hepatitis viral infection route and the timing of infection but also to differences in the mechanisms underlying cancer associated with hepatitis B and C. HCV is an RNA virus, and viral genes are not integrated into the host's genes, whereas HBV is a DNA virus with reverse-transcriptase activity. Thus, HBV genes are often integrated into the host's chromosomes