treatment characteristics that influenced the rate of decrease in HCV antibody titer. Additional studies are needed to elucidate the factors that affect the rate of decrease in the HCV antibody titer after eradication of HCV.

Previous studies have revealed that the antigenicities of HCV polypeptides differ according to genotype in some particular protein sequences, such as the core region or NS4 [32–34]. Typing techniques that detect genotype-specific antibodies have been developed as a means of serological typing [16] and are clinically useful for determination of HCV type [35, 36]. Serological type can be used for HCV typing when HCV is absent from serum [37] (for example, in patients with acute-phase self-limiting hepatitis C or patients with chronic hepatitis C in whom HCV was eradicated by antiviral therapy).

Maertens et al. [38] reported that antibodies to NS4 are usually cleared after resolution of HCV infection. In the present study, genotype-specific antibodies to NS4 were detected in many patients during follow-up but continued to decrease annually. In addition, a difference in the rate of decrease in titer between genotype-specific antibodies 1 and 2 was sometimes observed, and this caused a discrepancy between the serotype and genotype of the eradicated HCV in 2 patients. Thus, we found that serotype does not always correspond to eradicated HCV genotype in patients with SVR, and one should be careful to take this into account when genotype of eradicated HCV is analyzed.

In conclusion, HCV antibody titers decrease during the 10 years after eradication of HCV by IFN therapy. Because a decrease in HCV antibody represents, in part, the changes in immune status associated with HCV infection, this decrease in HCV antibody titer may be associated with changes caused by diseases related to HCV infection. These include, not only liver disease, but also extrahepatic disorders, such as mixed cryoglobulinemia, diabetes mellitus, lichen planus, and thyroid disease. Additional studies are needed to clarify the mechanisms of persistence and clearance of HCV antibody after the eradication of HCV and to investigate the association between the decrease in HCV antibody titers and patient immune status in patients with SVR. In addition, further studies are needed to examine the association between the decrease in HCV antibody titers and changes in extrahepatic manifestations associated with chronic HCV infection in patients with SVR.

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Hepatitis B virus genotype G is an extremely rare genotype in Japan

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Abstract

Background: Hepatitis B virus (HBV) has been classified into seven genotypes (A–G). HBV genotypes have a geographically characteristic distribution. Since HBV genotype G (HBV/G) was identified recently, little is known about the distribution of HBV/G in Japan. The aim of this study was to clarify this issue.
Patients and methods: Seven hundred and twenty-one serum samples obtained from patients with HBV in Japan were investigated. The patients included 149 asymptomatic carriers, 325 with chronic hepatitis, 129 with liver cirrhosis, and 118 with hepatocellular carcinoma. Six HBV genotypes (A–F) were determined by restriction fragment length polymorphim targeting to the S region of the HBV genome. Furthermore, HBV/G was investigated by polymerase chain reaction with hemi-nested primers derived from an HBV/G-specific nucleotide sequence.
Results: Of the 721 serum samples investigated, 12 subjects were classified as having HBV/A, 88 HBV/B, 610 HBV/C, 3 HBV/D, and 1 HBV/F. Seven subjects had a mixed infection with distinct genotypes, two with HBV/A and HBV/D, and five with HBV/B and HBV/C. HBV/G was not identified among the 721 samples.
Conclusion: HBV/G was not identified in a large cohort of patients with HBV, either single or dual infection. HBV/G seems to be an extremely rare genotype in Japan.

Keywords: Distribution; Genotypes; Hepatitis B virus; Japan; Polymerase chain reaction; Restriction fragment length polymorphism

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1. Introduction

Hepatitis B virus (HBV) infects approximately 350 million individuals worldwide and can cause a wide spectrum of liver disease [1]. HBV has been classified into seven genotypes based on an entire genome difference of more than 8% [2–4]. HBV genotypes have a geographically characteristic distribution [5]. HBV genotype A (HBV/A) and HBV/D are the most common genotypes worldwide, and account for the majority of cases in Europe and Africa. HBV/B and HBV/C are found in East Asia. HBV/E is confined to Africa, and HBV/F has been identified in indigenous populations of Central and South America. In 2000, a unique strain harboring a 36-base pair (bp) insertion into the core region was identified in France and was phylogenetically classified into the seventh genotype, G [4]. Thereafter, HBV/G was revealed to be distributed in San Francisco [6,7], Germany [8], Mexico [9], and Canada [10], and accounted for 1–5% in these areas. Although little is known about the virological and clinical characteristics of HBV/G, one of its unique characteristics is frequent coinfection with the other genotypes. In San Francisco, eight of the eight HBV/G patients were coinfected with HBV/A [6,7], and all of the HBV/G isolates from Canada were also coinfected with HBV/A, or HBV/A and HBV/C [10].

In Japan, HBV/C is the most common genotype, accounting for approximately 85% of all genotypes, and HBV/B follows with 12% [11–13]. However, little is known about the distribution of HBV/G in Japan. We have formerly investigated the 540 sera from patients with hepatitis B collected in and around Nagoya, and found that there were no HBV/G among them [14]. However, the serum samples in the study was obtained from a restricted area, a central part of Japan, therefore, further studies including serum samples collected from the other part of Japan had been required to conclude how often HBV/G distributed in Japan. Moreover, since HBV/G is frequently coinfected with the other genotypes, there is a possibility that HBV/G might exist as a minor population in the sera classified into the other six genotypes (A–F). At this time, to elucidate this issue, we conducted nationwide study of the distribution of HBV/G by analyzing sera obtained from patients with hepatitis B, including those whose genotypes were already known, using hemi-nested polymerase chain reaction (PCR) with HBV/G-specific primers. We also discussed the issues of HBV/G to date.

2. Materials and methods

2.1. Patients

Seven hundred and twenty-one serum samples were collected from patients with HBV in Japan. The patients resided in Hokkaido, Iwate, Yamagata, Niigata, Tokyo, Kanagawa, Nagano, Nagoya, Kyoto, Fukuoka, and Okinawa. The patients in this study were overlapped with some of the previous report [11]. They included 470 (65.1%) males and 251 (34.8%) females. The mean ± S.D. age was 43.6 ± 14.9 years (Table 1).

Table 1
Demographics of the 721 patients in this study

Sample	721
Gender (M:F)	470:251
Age (year)	43.6 ± 14.9
ALT (IU)	78.8 ± 115.8
ALP (IU)	240.8 ± 155.2
γ-GTP (IU)	52.2 ± 96.2
T. bil (mg/dl)	0.99 ± 1.60
HBeAg (%)	45.2
HBV DNA[a] (LGE/ml)	5.69 ± 1.84
Diagnosis	
Asymptomatic carrier	149
Chronic hepatitis	325
Liver cirrhosis	129
Hepatocellular carcinoma	118

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase; LGE, log genome equivalents; T. bil, total bilirubin; TMA, transcription-mediated amplification.

[a] Value was calculated using available data of transcription-mediated amplification of 255 subjects.

2.2. Detection of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) and HBV DNA level

HBsAg was detected by a particle-agglutination test using a commercial kit (Serodia: Fujirebio, Tokyo, Japan), and HBeAg was detected by ELISA using a commercial kit (Serodia: Kokusai-shiyaku, Tokyo, Japan), following the manufacturer's recommendations. Levels of HBV DNA were determined by the transcription-mediated amplification (TMA) method (Chugai Industry, Tokyo, Japan), and the results were expressed as log genome equivalents (LGE) per millilitre.

2.3. Determination of six HBV genotypes (A–F) by restriction fragment length polymorphism (RFLP)

DNA was extracted from 100 μl of serum samples using commercial kits (Smitest EX R&D: Genome Science, Fukushima, Japan) under manufacturer's recommendation. The extracted DNA was amplified in a 50-μl reaction mixture containing 0.5 μM of a sense primer MF1 (5′-YCC TGC TGG TGG CTC CAG TTC-3′: nt. 55–75), 0.5 μM of an antisense primer MR2 (5′-AAG CCA NAC ART GGG GGA AAG C-3′: nt. 730–709), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Co. Ltd., Tokyo, Japan), 0.2 mM each dNTPs, 3 mM $MgCl_2$, and 1× AmpliTaq Gold Buffer. The reactions were performed in a GeneAmp PCR system 9600 thermocycler. The sample was denatured at 96 °C for 9 min, and subjected to 40 cycles of PCR (95 °C for 1 min; 60 °C for 1 min; 72 °C for 1 min) followed by 72 °C for 5 min at final extension in a 96-well cycler (GeneAmp 9600; Perkin-Elmer, Norwalk, CT, USA). The amplified product

was subjected to the second round PCR with a sense primer MF2 (5′-GTC TAG ACT CGT GGT GGA CTT CTC TC-3′: nt. 246–271) and MR2 under the same condition as the first round PCR. The second round PCR product with the length of 485 bp was subjected to the digestion with five kinds of restriction enzymes. Genotype B could be distinguished by digestion with *Ear*I because of no recognition site of it was existed. Similarly, genotype C also could be distinguished by digestion with *Alw*I, as no recognition site of it was found within the amplified product. Only genotype E had a recognition site of *Nci*I, and only genotype F had no recognition site of *Hph*I. Finally, the distinction between genotypes A and D were done by digestion with *Nla*IV. Genotype A has a recognition site of *Nla*IV, result in the generation of fragments of 220 and 265 bp. While genotype D had two recognition site of *Nla*IV, result in generation of fragments of 34, 186, and 265 bp. Therefore, genotypes A and D were distinguished by if each of 220 and 186 bp were observed, respectively. The digested amplicon were run on 3% agarose gel stained with ethidium bromide and observed under UV light [15].

2.4. Identification of HBV/G

Nucleic acids extracted from serum were subjected to PCR with hemi-nested primers designed on the 36-bp insertion in the C gene of HBV/G genomes. In brief, the DNA was amplified by the first round of PCR for 40 cycles with HBHKF1 (sense: 5′-ACG GGG CGC ACC TCT CTT TAC-3′ [nt. 1519–1539]) and HBHKR2 that involved the 36-bp insertion characteristic of HBV/G (antisense: 5′-AGC CAA AAA GGC CAT ATG GCA-3′ [nt. 17–37 in the core gene of HBV/G]) in the presence of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The second round of PCR was performed for 40 cycles on the product of the first-round PCR with HBHKF2 (sense: 5′-GCA CTT CGT TTC ACC TCT GCA-3′ [nt. 1581–1601]) and HBHKR2. Then, the products were examined for fragments of 357 bp [15].

3. Results

3.1. Demographics, laboratory findings, and diagnosis of the patients

The mean value of alanine aminotransferase (ALT), alkaline phosphatase, gamma-glutamyl transpeptidase, and total bilirubin in the sera was 78.8 ± 115.8 IU, 240.8 ± 155.2 IU, 52.2 ± 96.2 IU, 0.99 ± 1.60 mg/dl, respectively (Table 1). Three hundred and twenty-six patients (45.2%) were positive for HBeAg. The mean value of HBV DNA measured by TMA was 5.69 ± 1.84 LGE per millilitre. One hundred and forty-nine patients (20.1%) were diagnosed as asymptomatic carriers, 325 (45.1%) with chronic hepatitis, 129 (17.9%) with liver cirrhosis, and 118 (16.4%) with hepatocellular carcinoma.

Table 2
Six genotypes (A–F) and HBV genotype G in 721 subjects from Japan

Genotype	No.	No. of HBV genotype G
A	12	0
A+D	2	0
B	88	0
B+C	5	0
C	610	0
D	3	0
F	1	0

3.2. HBV/G among 721 serum samples

Of the 721 serum samples investigated, 12 subjects were classified as having HBV/A, 88 HBV/B, 610 HBV/C, 3 HBV/D, and 1 HBV/F (Table 2). Seven subjects had a mixed infection with distinct genotypes, two with HBV/A and HBV/D, and five with HBV/B and HBV/C. HBV/G was not identified among the 721 samples.

4. Discussion

Several lines of evidence about the clinical significance of HBV genotypes have been accumulated in recent years. HBV/C causes more severe liver diseases than HBV/B by prolonging active hepatitis accompanying HBeAg production [16,17]. In a Western study, the rate of sustained remission after seroconversion was higher in genotype A than in genotype D hepatitis in patients who seroconverted to anti-HBe, and mortality related to liver disease was more frequent in genotype F than in genotype A or genotype D hepatitis [18]. Clinical data concerning HBV/G are very limited. One previous study analyzed 165 patients living in San Francisco and showed that the ALT level was higher in HBV/G than in HBV/C, and HBeAg was more prevalent in HBV/G than in HBV/C or HBV/D [7]. Further studies with a large sample size are warranted to confirm these findings.

Coinfection with distinct genotypes was seen also in other than HBV/G. In this study, coinfections with HBV/A and HBV/D as well as HBV/B and HBV/C were observed. In the previous study, analyzed 256 sera from the USA, Japan, Uzbekistan, Bangladesh, South Africa, and Cameroon, coinfection with distinct genotypes was identified in 28 subjects (10.9%) [19]. The occurrence of coinfection with distinct genotypes is important in virological aspects. It is reported that genomic recombination between distinct genotypes resulted in hybrid HBV strains, which causes distinct degree of liver diseases [20,21]. In such cases, genomic recombination never occurs without coinfection with distinct genotypes. However, clinical implication of coinfection with distinct genotypes per se still remains unanswered.

Ten years before the classification of HBV/G by Stuyver et al. [4], a unique strain with a 36-nucleotide insertion into the core region, which is known to a characteristic of HBV/G nowadays [22], was isolated from a homosexual man with hu-

man immunodeficiency virus infection [23]. Laboratory findings of his serum showed a few curious values. One was that HBeAg was detected in his serum in spite of a stop codon existing in the precore region of its genome, generally aborting the production of HBeAg at the stage of translation. Stuyver et al. also observed the same phenomenon, detection of HBeAg despite the stop codon in the precore region, and speculated that HBV/G might harbor another mechanism for producing HBeAg. Two years later, the mystery was solved by demonstration of coinfection with HBV/A in four of four sera with HBV/G [6]. It was explained that the HBeAg in the sera was produced by the coinfected precore wild type HBV/A. Furthermore, it was revealed that eight of the eight HBV/G patients from San Francisco were coinfected with HBV/A [7], and three of the three HBV/G patients were coinfected with HBV/A, or HBV/A and HBV/C in Canada [10]. These findings of the high frequency of coinfection of HBV/G with other genotypes give rise to another question, of whether HBV/G is competent to replicate by itself. An inoculation experiment in chimpanzees or an expression study in cultured cells would be required to answer this question.

The entire genome sequence of HBV/G has been reported from France [4,24], the USA [22], and Germany [8] so far. Interestingly, the sequence homology of these strains was surprisingly high. In one study in the USA, 10 HBV/G isolates, including 8 from San Francisco as well as 2 from France (FR1 [4] and B1-89 [24]), had a sequence homology of 99.3–99.8% among themselves [22]. Furthermore, another report from Germany showed that the HBV/G isolate (235/01) was nearly identical (sequence homology of the entire length was 99.7%) to both B1-89 and FR1 [8]. There are a few possible explanations for this finding. One possibility is that there are epidemiological links among French, German, and American HBV/G. A patient with HBV/G from Germany [8] and a homosexual male patient with HBV/G from San Francisco [23] were both positive for human immunodeficiency virus type-1. Thus, HBV/G might spread among a specific population, such as homosexual men or intravenous drug users. This would be also associated with the fact that HBV/G was not found among the patients in the current study, in which homosexual and intravenous drug were not included. The other possibilities are that HBV/G has a high genetic stability or was introduced into humans very recently. The mutation rate of HBV has been estimated to be 4.57×10^{-5} per site per year [25]. Thus, HBV/G might have an exceptionally low mutation rate under specific conditions, or the time since its introduction into humans might not have been long enough to gain a genetic diversity like that of the other six genotypes. To elucidate this issue, more HBV/G isolates from a wide variety of areas should be investigated.

In conclusion, HBV/G was investigated in a large cohort of patients with HBV from various areas in Japan, but no HBV/G isolate was identified, in either single or dual infection. The finding of the current nationwide study, the same as that of the previous study investigated the patients in a restricted area, indicates that HBV/G is extremely rare in Japan. Further studies with a large sample size from various areas in the world are required to further reveal the virological and clinical characteristics of HBV/G.

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Quantitative Detection of Hepatitis B Surface Antigen by Chemiluminescent Microparticle Immunoassay During Lamivudine Treatment of Chronic Hepatitis B Virus Carriers

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The usefulness of fully automated chemiluminescent microparticle immunoassay (Architect HBsAg QT) for monitoring serum levels of hepatitis B virus (HBV) during antiviral therapy remains unclear. Using this assay, hepatitis B surface antigen (HBsAg) was measured in 20 patients with chronic hepatitis B before and during lamivudine treatment. At the start of therapy, 12 patients had detectable hepatitis B e antigen (HBeAg) and 8 did not. The median serum HBV DNA level and HBsAg concentration (25th–75th centile) were 7.2 (6.1–7.8) log genome equivalents/ml and 3,932 (1,585–12,330) IU/ml, respectively. The HBsAg concentration was significantly higher in HBeAg positive than in HBeAg negative patients ($P=0.031$). There was a significant correlation between the HBsAg concentration and HBV DNA level ($r=0.490$, $P=0.027$). The HBsAg concentration negatively correlated with patient age ($r=-0.395$, $P=0.085$). After the start of lamivudine therapy, HBV DNA levels fell rapidly in all patients. Serum HBsAg concentrations also fell in most patients, but to a lesser extent. When drug-resistant variants emerged, serum HBsAg usually increased before biochemical breakthrough. Although HBV DNA was elevated persistently after the emergence of drug-resistant variants, the increase in HBsAg was transient. In some patients, the increase in HBsAg preceded the increase in HBV DNA. Monitoring of serum HBsAg concentrations with the use of Architect HBsAg QT, in addition to measurement of HBV DNA levels, is helpful for evaluating the response to lamivudine treatment and for the early detection of drug-resistant strains. ***J. Med. Virol. 75:235–239, 2005.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: HBsAg; HBV; HBV mutants; hepatitis B virus; YMDD variants; lamivudine therapy

INTRODUCTION

Hepatitis B virus (HBV) is the major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It is estimated that more than 350 million people worldwide are chronically infected with this virus [Lee, 1997]. Lamivudine, the first safe oral nucleoside analogue for the treatment of chronic hepatitis B, is now used widely. This compound suppresses effectively viral replication and prevents the progression of chronic liver disease [Lai et al., 1998; Dienstag et al., 1999; Lau et al., 2000]. However, prolonged treatment with lamivudine induces the emergence of variants with mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV polymerase gene. These mutants are resistant to lamivudine and can cause virological and/or biochemical breakthrough during therapy [Ling et al., 1996; Tipples et al., 1996]. It is therefore imperative that serum levels of hepatitis B e antigen (HBeAg) and HBV DNA are measured during lamivudine treatment to monitor the response to therapy and facilitate the early detection of drug-resistant strains [Kohmoto et al., 2003].

A simple and inexpensive method with a wide detection range is required for frequent measurement of serum HBV levels during antiviral treatment. Serum HBeAg, anti-HBe, and HBV DNA are currently the most important markers for assessing the response to lamivudine therapy. In contrast, HBsAg is used typically as a qualitative serological marker for diagnosing the presence of acute or chronic HBV infection. A fully

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automated chemiluminescent microparticle immunoassay (Architect HBsAg QT) for the quantitative detection of HBsAg has recently been introduced [Coleman et al., 1999; Deguchi et al., 2004]. Architect HBsAg QT can test many samples simultaneously in a short time and measure HBsAg concentrations over a wide detection range. However, the utility of this assay as a serological marker during lamivudine treatment has not yet been evaluated using clinical samples.

The aim of this study was to evaluate the Architect HBsAg QT as a method for monitoring viral loads during lamivudine treatment. Using this method, we measured HBsAg concentrations in patients with chronic HBV infection who received lamivudine. Changes in HBsAg concentrations were compared with those in HBV DNA before and after the emergence of YMDD variants.

MATERIALS AND METHODS

Patients

The subjects were 20 patients with chronic HBV infection (16 men and 4 women; mean age: 46 ± 12 years) who had received lamivudine between May 1995 and May 2003. The inclusion criteria were as follows: (1) persistent or fluctuating elevations of serum alanine aminotransferase (ALT) levels before therapy; (2) presence of HBsAg in serum; (3) presence of HBV DNA detectable by polymerase chain reaction (PCR); (4) absence of antibodies to hepatitis C virus and other likely causes of chronic liver disease; (5) no known use of corticosteroids, immunosuppressant drugs, or antiviral agents within 1 year before the start of therapy; and (6) no clinical signs of decompensated cirrhosis or hepatocellular carcinoma. Lamivudine was given orally at a dosage of 100 mg once daily for 12–18 months. Serum samples were obtained from the patients immediately before the start of therapy and at intervals of 1–2 months during therapy. Informed consent was obtained from all patients. Experimental procedures were in accordance with the Helsinki Declaration of 1975 (1983 revision) and were approved by the ethics committee of our hospital.

Routine Laboratory Tests

The following assays were done for all enrolled patients at the start of therapy: serum ALT activity, HBeAg, anti-HBe, and HBV DNA. Serum ALT was measured with an Autoanalyzer 7450 (Hitachi, Tokyo, Japan). HBeAg and anti-HBe were detected by radioimmunoassay. HBV DNA was measured by transcription-mediated amplification (TMA) with a hybridization protection assay (Chugai Diagnostics, Tokyo, Japan) as described elsewhere [Kamisango et al., 1999; Ide et al., 2001]. The detection range of this assay was between 3.7 and 8.7 log genome equivalents (LGE)/ml.

Quantitation of HBsAg

All serum samples were tested for HBsAg using the Architect HBsAg QT (Abbott Japan Corp., Tokyo) according to the manufacturer's protocol. Briefly, in the first step, the sample and anti-HBs-coated paramagnetic microparticles are combined. If HBsAg is present in the sample, it binds to the anti-HBs-coated microparticles. After washing, an acridinium-labeled anti-HBs conjugate is added. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured in relative light units. The amount of HBsAg in the sample is related directly to the relative light units detected by Architect HBsAg QT. Architect HBsAg QT is capable of measuring a wide range, from 0.05 to 250 IU/ml. Samples with an HBsAg level higher than 250 IU/ml require a 1:500 or greater dilution to bring them into the range of the calibration curve.

Detection of YMDD Mutant Strains

Mutations in the YMDD motif of the polymerase gene were examined by a line probe assay (INNO-LiPA HBV DR, Innogenetics NV, Belgium) [Stuyver et al., 2000] for patients who had a significant increase in HBV DNA during lamivudine treatment.

Statistical Analysis

Statistical analysis was performed with the use of the Statview SE + Graphics program, version 5.0 (SAS Institute, Cary, NC). The significance of correlations was evaluated by Spearman's rank analysis. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. A two-tailed *P* value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Baseline Characteristics of Patients

Among the 20 enrolled patients with chronic HBV infection, 12 had detectable HBeAg and 8 had undetectable HBeAg at the start of lamivudine therapy. The median serum ALT level, HBV DNA level, and HBsAg concentration (25th–75th centile) were 88 (59–220) IU/L, 7.2 (6.1–7.8) LGE/ml, and 3,932 (1,585–12,330) IU/ml, respectively.

Relations Between HBsAg Concentrations and Other Clinical Variables

The relations between concentrations of HBsAg and those of the other serological markers at the start of lamivudine therapy are shown in Figure 1. The HBsAg concentration was significantly higher in patients with detectable HBeAg than in patients without HBeAg ($P = 0.031$). When a value of 3.7 LGE/ml was assigned to HBV DNA levels below the lower detection limit of the TMA assay for calculation purposes, a significant correlation was obtained between measurements of HBsAg and of HBV DNA ($r = 0.490$, $P = 0.027$). The relation between the HBsAg concentrations and the ages of the patients at the start of therapy is shown in Figure 2. The HBsAg concentration negatively corre-

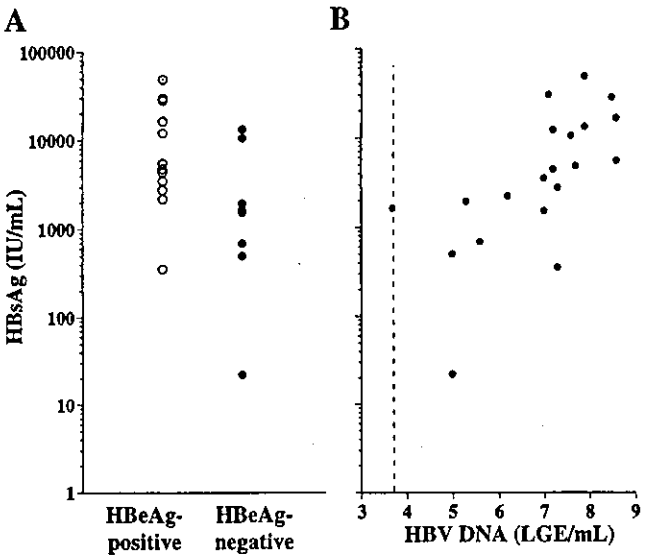

Fig. 1. A: Difference in hepatitis B surface antigen (HBsAg) concentrations between patients with and without detectable HBeAg at the start of lamivudine treatment. B: Correlation between serum HBsAg concentrations and serum hepatitis B virus (HBV) DNA levels measured at the start of therapy. The broken line indicates the detection limits of the assay.

lated with patient age, although the correlation was not statistically significant ($r = -0.395$, $P = 0.085$).

Changes in HBsAg and HBV DNA During Lamivudine Therapy

Changes in median HBsAg concentrations and median HBV DNA levels during the first 6 months of lamivudine therapy are shown in Figure 3. After lamivudine therapy commenced, HBV DNA levels fell rapidly in all patients. Serum HBsAg concentrations also fell in most patients, but the decrease in HBsAg was smaller and more gradual. HBsAg was detected during therapy in all patients.

Case Reports

YMDD mutant variants emerged in 12 patients between months 5 and 14 of therapy. The clinical courses of two patients with YMDD variants are shown in Figure 4. The first patient, a 45-year-old man, had received lamivudine since December 2001. At the start of therapy, HBeAg was positive. HBV DNA and HBsAg levels declined after the start of therapy. At month 8 of therapy, viral breakthrough associated with emergence of YMDD variants occurred. Increased serum HBV DNA and HBsAg levels were observed before biochemical breakthrough. After YMDD variants emerged, serum HBV DNA was persistently elevated, whereas the increase in HBsAg was transient.

The second patient, a 34-year-old man, participated in Japanese phase III clinical trials of lamivudine in 1995 and received 48 weeks of treatment. HBeAg was positive at the start of therapy. A liver biopsy specimen showed moderate inflammation and moderate fibrosis. After the

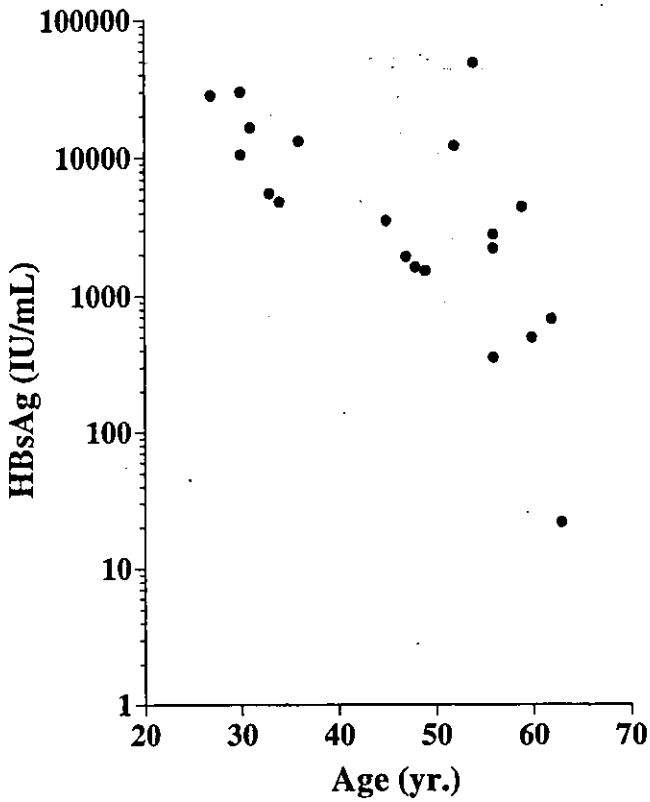

Fig. 2. Relation between HBsAg concentrations and age of patients at the start of lamivudine treatment.

start of therapy, there was a significant decline in HBV DNA, but the HBsAg level did not decrease. At month 7 of therapy, HBsAg was markedly elevated, with no significant increase in HBV DNA. About 4 months subsequently, a breakthrough in ALT activity associated with YMDD variants occurred.

DISCUSSION

Monitoring of HBeAg and HBV DNA levels in serum is important for assessing the response to antiviral treatment. However, because of the possible presence of HBeAg negative variants with mutations in the precore or basic core promoter region among some chronic HBV carriers, it is difficult to correlate the absence of HBeAg with the level of HBV replication. Several assays for serum HBV DNA are available commercially: the branched DNA signal amplification assay [Jen et al., 2001], TMA assay [Kamisango et al., 1999; Ide et al., 2001], and PCR-based assays [Gerken et al., 1998]. The branched DNA assay is based on the hybridization of HBV DNA to oligonucleotide probes. This assay is not sensitive enough to measure low serum HBV levels during antiviral therapy. TMA assay and PCR-based nucleic acid amplification tests have been developed to provide more sensitive, quantitative methods for the detection of HBV DNA. However, these methods involve cumbersome procedures and high costs, and generate divergent results.

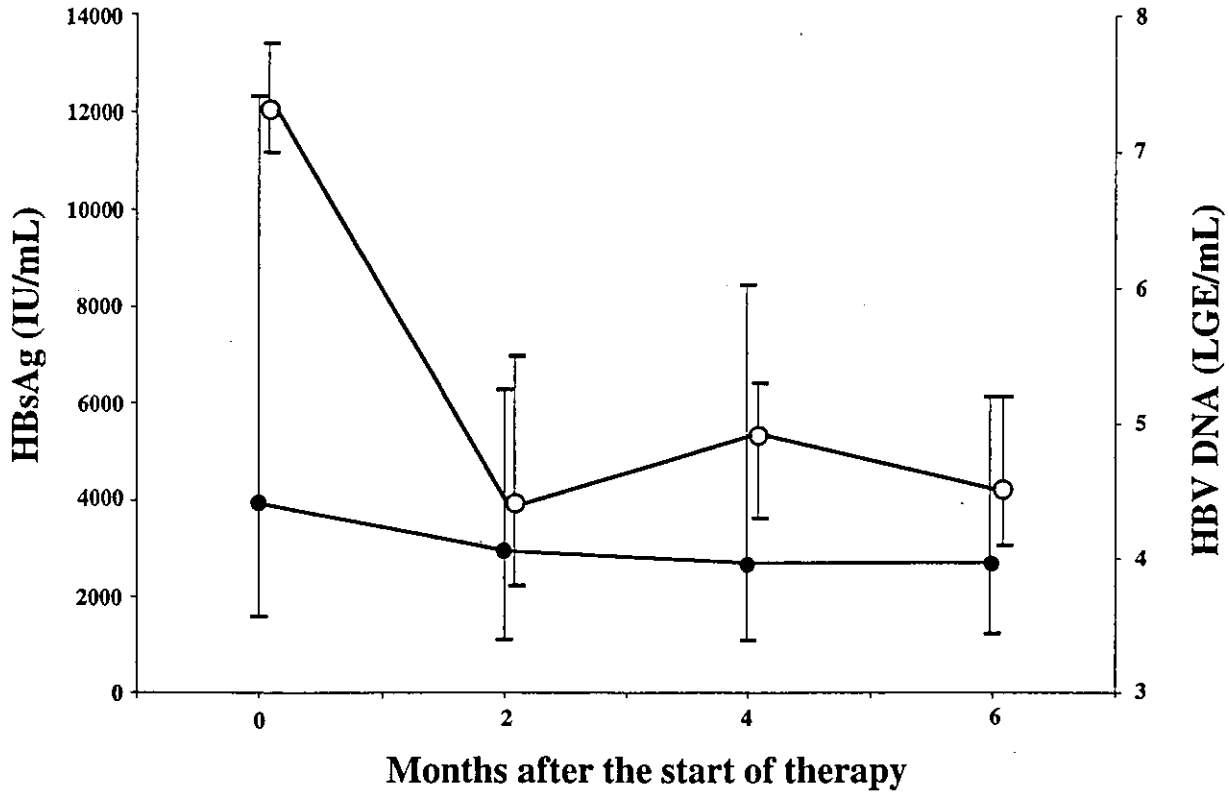

Fig. 3. Changes in HBsAg concentrations (●) and in HBV DNA levels (○) during the first 6 months of lamivudine therapy. Values are medians, with bars showing the 25th–75th centile.

In this study, the Architect HBsAg QT was used to monitor HBsAg concentrations during lamivudine treatment. This method measures HBsAg with high intra-assay precision and inter-assay reproducibility over a wide detection range. The lower limit of detection was 0.05 IU/ml, which is equivalent or superior to that of other commercially available immunoassays. Coleman et al. [1999] reported that several known HBV vaccine-escape variants with mutations in the "a" determinant within the hydrophilic region of HBsAg are detected by this assay. The Architect HBsAg QT is a simple, sensitive, specific, reproducible, and inexpensive method that produces results rapidly and accurately. The wide detection range of this method is

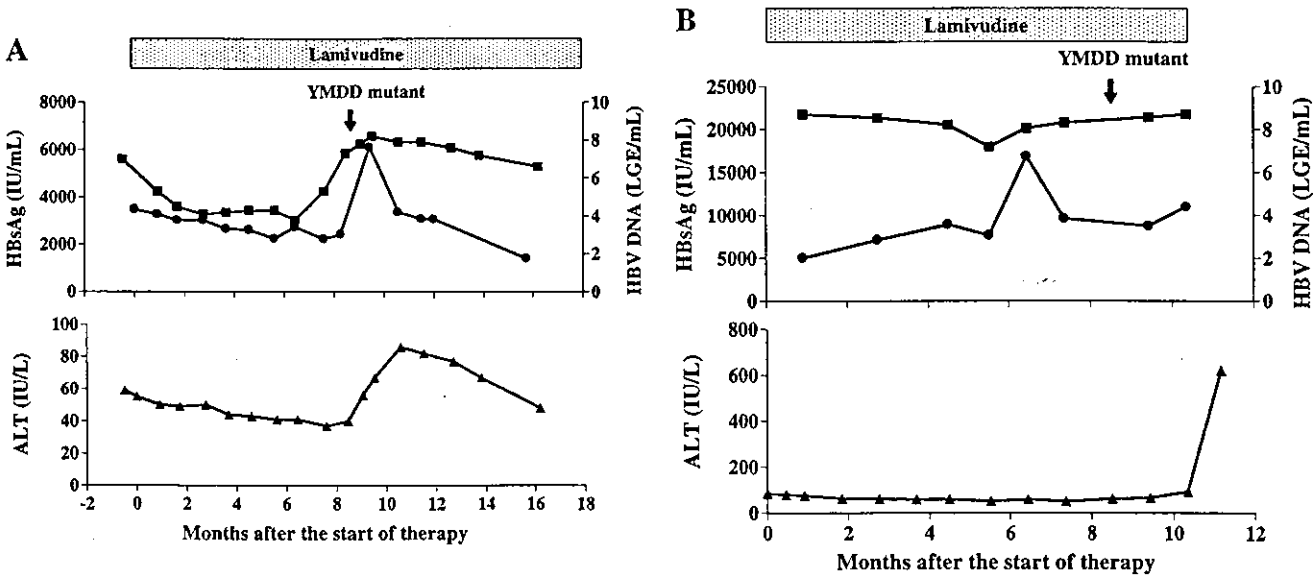

Fig. 4. Clinical courses of two patients (A), a 45-year-old man and (B), a 34-year-old man. ●, HBsAg concentration; ■, HBV DNA level. ▲, ALT activity. YMDD, tyrosine-methionine-aspartate-aspartate.

appropriate for assessment of response to antiviral therapy, and the low cost of the assay permits frequent testing of serum samples.

Many elderly patients have undetectable HBeAg levels and low titers of HBV DNA. In our study, the serum HBsAg concentration was lower in elderly patients than in younger patients. The age-related decrease in serum HBsAg levels may be due to low viral replication. Fan et al. [2001] reported that the frequency of deletion mutants in the pre-S region of HBV DNA is increased in patients with low viral replication and proposed that several pre-S deletion mutants cause a remarkable decrease in the synthesis and secretion of small surface antigens. The emergence of the pre-S deletion mutants may also be related to the decrease in HBsAg levels in the elderly.

A positive correlation was found between HBsAg and HBV DNA levels before lamivudine treatment. Early in the course of therapy, changes in HBsAg concentrations appeared to correlate with changes in HBV DNA levels. However, the decrease in HBsAg was much smaller than that in HBV DNA during lamivudine treatment. Kimura et al. [2003] reported that the decrease in hepatitis B core antigen (HBcAg) level as measured by a sensitive enzyme immunoassay was also smaller than that in the HBV DNA level during therapy. One possible explanation for these differences is that covalently closed circular DNA (cccDNA) may continue to remain in hepatocytes during lamivudine treatment. Lamivudine strongly inhibits HBV reverse transcriptase and HBV DNA production, but has little or no effect on cccDNA [Lee, 1997]. Consequently, viral proteins such as HBsAg or HBcAg might be persistently translated and released into the circulation, even during therapy.

When YMDD mutant variants emerged, an increase in serum HBsAg concentrations as well as in HBV DNA was usually observed before biochemical breakthrough. Although serum HBV DNA was persistently elevated after the emergence of YMDD variants, the increase in HBsAg was transient, similar to the elevation of ALT activity (Fig. 4A). We speculate that the release of HBsAg into serum from hepatocytes might be associated in part with the lysis of hepatocytes involved in the replication of YMDD variants and liver inflammation. Further studies are needed to test this hypothesis. In most patients, the increase in serum HBsAg was preceded by an increase in HBV DNA. In some, however, serum HBsAg increased a few months before the increase in HBV DNA (Fig. 4B). Monitoring of serum HBsAg concentrations in addition to HBV DNA levels may thus facilitate the early detection of drug-resistant mutant variants during lamivudine treatment.

In summary, the Architect HBsAg QT measures serum HBsAg concentrations easily and inexpensively. This assay may be useful for monitoring the response to antiviral treatment, which requires the frequent measurement of serological markers. Monitoring changes in HBsAg concentrations during lamivudine treatment may provide useful information on the activity of HBV and the emergence of drug-resistant variants, as well as changes in levels of HBeAg, anti-HBe, and HBV DNA. If possible, the response to lamivudine therapy should be monitored by assessing both serum HBsAg concentrations and serum HBV DNA levels.

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Hepatitis B virus DNA integration in hepatocellular carcinoma after interferon-induced disappearance of hepatitis C virus

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Hepatitis B virus DNA integration in hepatocellular carcinoma after interferon-induced disappearance of hepatitis C virus

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ABSTRACT

OBJECTIVES: Hepatocellular carcinoma (HCC) has been reported in patients in whom hepatitis C virus (HCV) was eliminated by interferon therapy. We examined the pathogenesis of HCC in patients with sustained viral response.

METHODS: Operable HCC developed in 7 of 342 patients cured of HCV infection by interferon monotherapy. No patient abused alcohol or had diabetes mellitus or obesity. Resected specimens of HCC were histologically evaluated. DNA extracted from HCC was examined by polymerase chain reaction to locate hepatitis B virus (HBV) DNA. HBV integration sites in human genome were identified by cassette-ligation-mediated PCR.

RESULTS: HBV DNA was not amplified in serum samples from any of the 7 patients with HCC and was found in liver in 4 patients. In the latter 4 patients, HBV DNA was integrated into the human genome of HCC. In 2 of these patients, covalently closed circular HBV was also detected. The patients with HBV DNA integration were free of HCV for more than 3 years. In 2 of the 3 patients without HBV DNA integration, the surrounding liver showed cirrhosis. The liver of HCC with HBV DNA integration had not progressed to cirrhosis. Three of the 4 tumors with HBV integration had one integration site each, located at chromosomes 11q12, 11q22-23, and 22q11, respectively. The other tumor had two integration sites, situated at chromosomes 11q13 and 14q32. At chromosome 11q12, HBV DNA was integrated into protein-coding genome, the function of which remains unclear.

CONCLUSION: Integrated HBV DNA may play a role in hepatocarcinogenesis after the clearance of HCV by interferon treatment.

INTRODUCTION

Interferon (IFN) has potent antiviral activity against hepatitis C virus (HCV). Previous studies have shown that IFN can reduce the incidence of hepatocellular carcinoma (HCC) in patients with HCV infection (1-3). After complete eradication of HCV by IFN therapy, HCC was thought to rarely occur (4). Recent studies have shown that HCC develops in 2.5% to 4.2% of such patients (5-7). These patients may have had advanced liver fibrosis at the time of HCV eradication, and subclinical tumors might have already existed in the liver at the end of IFN therapy (8). In some patients, however, HCC might develop from liver without fibrosis several years after the eradication of HCV by IFN. The etiology of such cases of HCC remains obscure. New regimens combining IFN with antiviral drugs can improve the rate of HCV clearance (9, 10). The risk of HCC might increase in patients with chronic hepatitis who have complete responses to IFN therapy. It is important to delineate the features of HCC occurring after elimination of HCV. Occult hepatitis B virus (HBV) infection is defined as the detection of HBV DNA in the serum or liver of patients without hepatitis B surface antigen (11). In patients with chronic hepatitis C, occult HBV coinfection may exacerbate liver disease (12). Occult HBV infection is present in a substantial proportion of patients with HCV infection and has a pro-oncogenic effect (13). In the present study, we examined resected liver specimens to evaluate the role of occult HBV infection in the development of cancer after the clearance of HCV by IFN treatment. We also describe the clinical course of such patients with HCC.

METHODS

Patients

At our department, 1286 patients with chronic hepatitis C without cirrhosis and without hepatitis B surface antigen (HBsAg) received IFN monotherapy for 24 weeks from 1992 through 2002. In 342 patients, serum HCV RNA disappeared, and alanine aminotransferase activity (ALT) was within the normal range for 6 months after the end of IFN therapy. We are now monitoring 144 of these patients every half year. HCC was diagnosed in 7 patients, 4 of whom were regularly monitored (cases 1, 4, 6 and 7). Seven patients underwent hepatectomy at our hospital. Their clinical characteristics are described in Table 1. No patient had alcohol abuse, drug usage, or diabetes mellitus. All patients had a body mass index of less than 25 kg/m^2. The surrounding liver tissue was pathologically classified according to the criteria proposed by Desmet et al (14).

Detection of HBV DNA in serum and liver

DNA was extracted from 100 µl of serum or 10 µg of liver tissue by means of proteinase K digestion followed by phenol/chloroform extraction, as described previously (15). HBV DNA in serum or in liver was amplified with specific primers for HBX, HBS, and HBC (sequences of the primers shown in Table 2). Amplification was done in a thermal cycler for 35 cycles: 95°C for 30 sec, 55°C for 60 sec, and 72°C for 60 sec in 40 µl of a reaction buffer containing 30 pmol of the two appropriate primers, four deoxynucleotides each at a concentration of 100 mM, PCR buffer, and 2.5 units of Gold Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). With 2 µl of the first PCR product, a second PCR was done. To examine covalently closed circular HBV (cccHBV) in liver, extracted DNA was amplified with primers P23, 24, 25, and p26 (Table 2). The amplification procedure and primers have been described previously (16).

Detection of integrated HBV DNA in human genome

We used cassette-ligation-mediated polymerase chain reaction (PCR) to detect HBV DNA integrated into the human genome as described previously (15). Briefly, 10 μg of DNA was digested with *Eco*RI, *Hin*dIII, or *Pst*I and ligated to double-stranded DNA cassettes with compatible ends. The cassette-ligated DNA fragments were used as a template for nested PCR with the cassette- and HBV-specific primers. One microliter of the DNA solution was amplified in 40 μl of a reaction buffer containing 10 pmol of the two appropriate primers, four deoxynucleotides each at a concentration of 100 mM, PCR buffer, and 2.5 units of LATaq polymerase. The amplifications were carried out in a thermal cycler for 33 cycles (45 sec at 94°C, 2 min at 55°C, 2 min at 72°C), followed by final extension for 10 min at 72°C. With 1 μl of the first PCR product, a second PCR was done. Table 2 shows the sequences of the primers used. The amplified cassette-ligated DNA fragments were subcloned and sequenced with a DNA sequencing system (377A, Applied Biosystems, Tokyo). To identify the integrated site of the host genome, we used the GenomeNet (***http://www.genome.ad.jp***) to compare the sequences adjacent to the integrated HBV DNA with the human sequence.

Statistical analysis

Ages, intervals, and tumor sizes in the two groups were compared by Student's *t* test.

RESULTS

Pathological findings of the resected liver

The 7 liver tumors were diagnosed as 4 poorly differentiated HCC and 3 moderately differentiated HCC (Table 3). The surrounding liver tissues were diagnosed as chronic hepatitis. The stage of liver fibrosis was VI in 2 specimens, II in 3 specimens, and I in 2 specimens. The activity grade was II in 4 specimens and I in 3 specimens. There was no evidence of fat deposits in any of the specimens.

HBV DNA in serum

HBV DNA was not detected in serum of any of the 7 patients with HCC.

HBV DNA in liver

We detected HBV DNA in 5 of the 7 HCC and 3 of 5 noncancerous liver samples (Figure 1). In detail, HBx was detected in 3 of the 7 tumors and 1 specimen of noncancerous liver tissue. HBc was detected in 4 tumors and 3 liver tissues. HBs was detected in 2 tumors and 3 liver tissues. Covalently closed circular HBV was detected in case 2 (both HCC and noncancerous liver) and in case 4 (only liver tissue).

HBV DNA integrated in human genome

Our results provide evidence that HBV DNA was integrated into human genome in 4 of the 7 patients with HCV infection in whom HCC developed after complete responses to interferon therapy (cases 2, 5, 6, and 7). HBV DNA was integrated into chromosome 11q23 in case 2, chromosome 22q11.23-12 in case 5, chromosome 11q12 in case 6, and chromosomes 11q13 and 14q32 in case 7. In case 6, HBV DNA was integrated into protein-coding sequences, hypothetical LOC387771 protein, the function of which remains unclear. HCC developed more than 3 years after clearance of HCV in the patients with HBV DNA integration (Table 4). The interval from HCV eradication to the diagnosis of cancer was significantly longer in HCC with HBV DNA integration than in

HCC without it. In HCC without HBV DNA integration, noncancerous liver tissue showed cirrhosis. In HCC with HBV DNA integration, the fibrosis stage of liver tissue was 1 or 2.

Clinical courses of the 7 patients

In 4 patients, in whom more than 3 years had elapsed since the clearance of HCV RNA, HBV DNA was integrated into the human genome of HCC. In 2 of the 3 patients without HBV DNA integration, the surrounding liver showed cirrhosis. In contrast, the surrounding liver of HCC with HBV DNA integration did not progress to cirrhosis. Four patients are alive as of this writing. Tumor recurrence has not been detected in 2 of these patients (Figure 2). The other 3 patients have died: 2 died of tumor progression and 1 of a myocardial infarction at operation. There was no correlation between clinical outcome after surgical treatment and HBV DNA integration.