TABLE I. Characteristics of Patients With or Without Occult HBV Infection

	HBV DNA (−)	HBV DNA (+)
Number	21	22
Mean age	35.2 ± 10.5	32.8 ± 13.5
Type of hemophilia (A/B)	17/4	17/5
Severity of hemophilia (mild/moderate/severe)	1/1/19	1/3/18
HBs-antibody (+/−)	14/7	13/9
HBc-antibody (+/−)	17/4	20/2
HBc-antibody titer#	129.4 (39.1–897.8)*	291.3 (80.4–914.5)*
HIV (+/−)	13/8	11/11
HIV RNA concentration (copies/μl)##	7.6 (0.2–35)	0.4 (0.2–0.5)
Under limit of quantitation sensitivity	7 (53.8%)	9 (81.8%)
CD4+ cell count##	449.9 ± 300.1	577.7 ± 272.6
HCV (+/−)	20/1	18/4
HCV RNA concentration (copies/μl)###	383.5 (17–810)	498.8 (85–830)
HCV genotype (1a/1b/2a/2b/3a/4a)###	3/3/3/2/6/3**	10/3/1/2/2/0**
Serum ALT levels (IU/L)###	57.1 (12–208)	71.8 (10–209)

HBV, hepatitis B virus; HBs, hepatitis B viral surface; HBc, hepatitis B viral core; HIV, human immunodeficiency virus; HCV, hepatitis C virus; ALT, alanine aminotransferase.
#Only in patients with positive HBc-antibody.
##Only in patients with HIV coinfection.
###Only in patients with HCV coinfection.
*$P = 0.0476$ by Mann–Whitney U test.
**$P = 0.0230$ for 1a or 1b versus other genotypes by Chi-square test.

the rates of positive HBs antibody and HBc antibody were similar between HBV DNA-positive and -negative patients. The rates of both HIV and HCV coinfection were similar regardless of occult HBV infection. In addition, when compared in combination with HBs and HBc antibody, or in combination with HIV and HCV, there was no difference in the rate of patients with HBV DNA (Table II). In patients with positive HBc antibody, however, the antibody titer was higher in HBV DNA-positive patients than in HBV DNA-negative patients ($P = 0.0476$, Mann–Whitney U test). In patients with HCV, HCV genotype 1 (1a or 1b) was significantly more prevalent in patients with HBV DNA than in patients without HBV DNA ($P = 0.0230$, Chi-square test).

In all five patients without HCV coinfection, serum alanine aminotransferase (ALT) levels were continuously normal regardless of occult infection with HBV. In patients with HCV infection, there was no significant difference in serum ALT level which was calculated as the average value of four to six analyses over 1 year, between HBV DNA-positive and -negative patients. When this comparison was restricted to patients with HBV detectable by PCR specific for the S-region only, serum ALT levels in HBV DNA-positive patients were significantly higher than those in HBV DNA-negative patients (patients with HBV, 120.3 ± 66.6 vs. patients without HBV, 57.1 ± 44.0; $P = 0.0162$, Mann–Whitney U test).

Genotype of Occult HBV

HBV genotyping was carried out based on the sequence of the S-region in seven patients in whom HBV DNA was detected by PCR specific for the S-region. Genotype C, which is the major genotype observed in the Japanese patients with chronic hepatitis B without hemophilia, was detected in all seven patients.

DISCUSSION

The clinical significance of occult HBV infection for patients with chronic hepatitis C has been described in recent reports [Cacciola et al., 1999; Sagnelli et al., 2001] and remains controversial [Kao et al., 2002]. These reports consider the influence of HBV occult infection on advanced liver disease [Cacciola et al., 1999; Sagnelli et al., 2001], development of hepatocellular carcinoma [Sheu et al., 1992; Paterlini et al., 1993], and reduced response to interferon [Zignego et al., 1997; Cacciola et al., 1999]. The importance of HBV occult infection has been reported in immunosuppressive patients, even in those without HCV coinfection [Xunrong et al., 2001]. In these patients, reactivation of HBV caused liver damage and sometimes resulted in liver failure.

Patients with hemophilia are at high risk of exposure to transfusion-transmissible virus such as HIV, HBV, HCV, and GBV-C. The high prevalence of infection with HIV [Tsuchie et al., 1985; Kroner et al., 1994], HCV

TABLE II. Rate of Hepatitis B Virus DNA Detection (%)

HBsAb(+) HBcAb(+)#	HBsAb(+) HBcAb(−)	HBsAb(−) HBcAb(+)	HBsAb(−) HBcAb(−)
13/25 (52.0)	0/2 (0)	7/12 (58.3)	2/4 (50.0)
HIV(+) HCV(+)##	HIV(+) HCV(−)	HIV(−) HCV(+)	HIV(−) HCV(−)
11/21 (52.4)	3/3 (100)	10/17 (58.8)	1/2 (50.0)

#HBsAb, hepatitis B viral surface antibody; HBcAb, hepatitis B viral core antibody.
##HIV, human immunodeficiency virus; HCV, hepatitis C virus.

[Makris et al., 1990; Troisi et al., 1993], and GBV-C [Hanley et al., 1998; Toyoda et al., 1998] has been reported in many studies. The status of serological markers on HBV infection has also been reported [Kumar et al., 1993; Goedert et al., 2002]. However, occult HBV infection in this population has not been examined. Because a large number of patients with hemophilia have HIV infection and changes in immune status in these patients can occur partly due to the disease itself and partly to the effect of HAART therapy, clarification of the status of occult HBV infection in these patients is important because of the potential for reactivation of occult HBV in association with changes in immune status.

HBV DNA was detected in serum in around one-half of the patients. The rate of detection was similar to that of HBV DNA detected in serum of the Japanese patients without hemophilia who have chronic HCV infection [Fukuda et al., 1999]. Neither the severity of hemophilia nor coinfection with HIV and HCV indicated the potential for occult HBV infection. In a previous study, Nunez et al. [2002] found no HIV-infected patients (most were intravenous drug users) in whom occult HBV infection could be confirmed. In contrast, we confirmed occult HBV infection in 11 HIV-infected patients. Only a high HBc antibody titer, which has already been reported to be an indicator of occult HBV infection [Nirei et al., 2000], and HCV genotype 1 (1a or 1b) in patients coinfected with HCV may indicate the high risk of occult HBV coinfection.

On the basis of our results, occult HBV infection appears to have no significant clinical impact when the infection is evaluated by the HBV detection for the C-region. On the contrary, occult HBV may increase serum ALT levels, which indicates severe liver damage, in patients with HCV infection when HBV DNA is positive by PCR for the S-region. Further study will be required to clarify the difference in clinical significance of HBV occult infection between PCR positive for the C-region and that positive for the S-region.

The HBV genotype detected in the Japanese patients with hemophilia was exclusively genotype C, which is the most common genotype in Japan. This shows the distinct characteristics of occult HBV infection in hemophilia patients in Japan, which are different from those of other transfusion-transmissible viruses in this population. The genotypes of viruses such as HCV or GBV-C in the Japanese hemophilia patients are foreign and not domestic genotypes [Kinoshita et al., 1993; Toyoda et al., 1998]. This is because, in this population, transmission of these viruses has been by imported blood products, as well as HIV transmission in this population [Tsuchie et al., 1985]. In contrast, only the domestic HBV genotype was found in the Japanese hemophilia patients. This, together with the lack of difference in the prevalence of occult HBV infection between hemophilia patients with HCV and HCV-infected patients who have not undergone repeated transfusions in Japan, suggests a route of transmission of this virus, different from that in cases of HIV, HCV, and GBV-C infection. The lack of correlation in the rate of coinfection between HIV or HCV and HBV also supports this suggestion. Screening for HBV in blood donors using HBs antigen as a marker started in 1973 in Japan. Some patients have a history of blood transfusion, which may have caused the occult HBV infection. Nosocomial infection in relation to the injection of blood products through the repeated use of needles, syringes, or other medical instruments, which could have occurred under medical conditions in Japan prior to the 1970s, might have also played a role.

In summary, among 43 Japanese patients with hemophilia, occult HBV infection was observed in about one-half of patients without detectable HBs antigen, a prevalence similar to that of the Japanese patients with chronic HCV infection. Occult HBV infection did not have significant clinical implications as a whole, although patients in whom HBV was detected with S-region-specific PCR showed higher ALT levels. The HBV genotype was exclusively a domestic type, suggesting a different route of transmission of HBV from that of HCV or HIV in this population. Further studies are required to determine occult HBV infection in the Japanese patients with hemophilia.

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Mutations of the Interferon Sensitivity-Determining Region (ISDR) Correlate With the Complexity of Hypervariable Region (HVR)-1 in the Japanese Variant of Hepatitis C Virus (HCV) Type 1b

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Hepatitis C virus (HCV) genotype 1b comprises mainly two subtypes in Japan, each named for its geographic prevalence (Japan-specific, J type; worldwide, W type). Because the newly identified subtypes have not been fully characterized, the present study directed this issue from virological viewpoints such as hypervariable region (HVR)-1 as well as interferon (IFN) sensitivity-determining region (ISDR). Fifty chronic hepatitis patients with HCV 1b (31 men and 19 women; mean age 50.5 years) were enrolled, and J/W type was determined according to envelope 1 (E1) sequence as described previously (23 J type and 27 W type). Correlations between age, number of HVR-1 clones, HVR-1 diversity, and ISDR mutations were analyzed in J and W type patients independently. In addition, the sequences of the three HCV regions obtained for the determination of the above genetic factors were studied phylogenetically. The number of HVR-1 clones was significantly higher for J type in comparison with W type ($P=0.044$). In the J type-infected patients, the ISDR mutation number was correlated inversely with HVR-1 clone number ($P=0.0001$, $r=-0.734$) and HVR-1 diversity ($P=0.0001$, $r=-0.722$). However, this correlation was not observed in the W type patients. W type patients showed a significant correlation between age and HVR-1 clone number ($P=0.015$, $r=0.462$). Phylogenetic study revealed that the nonstructural (NS) 5A sequence, which is obtained for ISDR type determination, can distinguish between J and W types. The inverse correlation in J type patients between ISDR mutations and HVR-1 complexity may explain the usefulness of the ISDR for prediction of IFN response only in Japanese patients. This suggests that the ISDR is not directly related to IFN responsiveness, but the degree of HVR-1 complexity may be more important. ***J. Med. Virol. 74:54–61, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: J type; W type; subtype measurement; E1; E2; NS5A

INTRODUCTION

Hepatitis C virus (HCV), a leading cause of chronic liver disease worldwide, is presently classified into six major types on the basis of nucleotide sequences [Poynard et al., 2003]. The geographic distributions of HCV genotypes vary; genotype 1b is found worldwide and is the predominant type in certain areas, such as Central Europe and Northern Asia including Japan. Phylogenetic comparisons of HCV 1b isolates in GenBank revealed that genotype 1b comprises three major subpopulations, each with a particular geographic prevalence [Nakano et al., 1999]. J type is found predominantly in Japan, NJ type is rarely found in Japan, and W type is distributed worldwide. HCV 1b isolates from Japanese hepatitis patients are primarily W type (approximately 60%) and J type (approximately 40%).

A portion of the amino acid sequence of the nonstructural (NS) 5A segment of HCV genotype 1b, termed the interferon sensitivity-determining region (ISDR), was initially reported to be correlated with responsiveness to interferon (IFN) therapy in Japanese population [Enomoto et al., 1995, 1996]. Subsequent studies in Europe failed to confirm this association [Khorsi et al., 1997; Zeuzem et al., 1997], and the usefulness of the ISDR as a predictor of IFN responsiveness became controversial. In our previous report, a comparison of predictive factors for IFN response between the J and W

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subtypes revealed different predictive value of the ISDR, which could explain the differences in usefulness of the ISDR system between the Japanese and European [Nakano et al., 1999].

In the present study, we investigated subtype-specific features, including the hypervariable region (HVR)-1 of HCV genotype 1b. HVR-1 is located at the *N*-terminus of the envelope 2 (E2) glycoprotein, which forms the virus surface antigen, and is thought to play an important role in viral escape of the host immune response [Taniguchi et al., 1993; Koizumi et al., 1995]. Previous investigators suggested that a less complexity of HVR-1 quasispecies is predictive for a preferable response to IFN therapy and is independent of other predictive factors such as viral load [Hino et al., 2000; Yeh et al., 2002]. We here found another important subtype-specific feature, which could further explain the J type-specific usefulness of the ISDR system. We also present an easier method for subtype determination with the use of the NS5A sequence, which is utilized in ISDR type determination.

MATERIALS AND METHODS

Patients

Over a 3-year period, 50 chronic hepatitis patients with HCV 1b visited our hospital for further evaluation of a positive HCV antibody in the sera at heath check screening. They were not treated at the first visit, and sera taken at the first visit were used in the present study. Subsequent examinations revealed that no patients had signs of advanced liver cirrhosis or hepatocellular carcinoma. All were negative for both anti-HBc and anti-human immunodeficiency virus in their sera. Informed consent was obtained from all patients. Some patients are currently being treated with IFN and ribavirin to eradicate HCV viremia, and they will continue treatment as long as they can tolerate the drugs' side effects.

It has been suggested that higher doses of IFN treatment of chronic hepatitis patients with HCV have a higher success rate for eradication of the virus [Vrolijk et al., 2003]. On the basis of this suggestion, we intended to maintain our patients on IFN therapy for as long as they could tolerate various side effects. Adjustments in dose to minimize side effects resulted in different IFN doses for each patient. Many of the treated patients tolerated the therapy well. Unfortunately, these variations in therapy protocols made it difficult to compare IFN responses among patients enrolled in the present study. Thus, the final IFN response of each patient was not included.

Methods

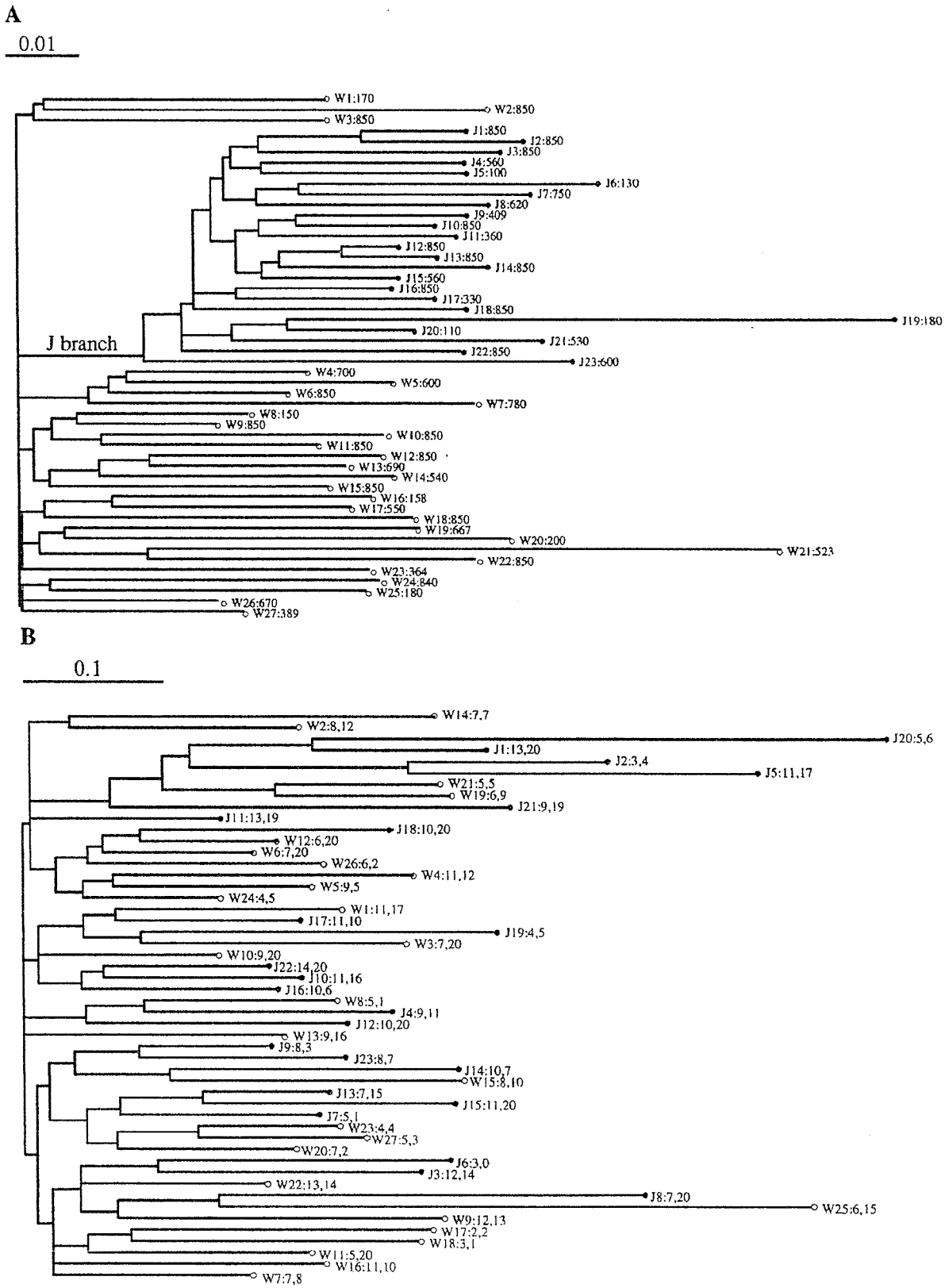
RT-PCR. Serum HCV RNA was detected and quantified with a commercial RT-PCR kit (Amplicor HCV; Roche Diagnostics, Branchburg, NJ) according to the manufacturer's instructions. HCV genotyping was carried out with a second-generation reverse hybridization line-probe assay (Inno-LiPA HCV II, Innogenetics, Ghent, Belgium) according to the manufacturer's instructions. After extraction of RNA from HCV 1b-positive patient sera, regions including partial envelope 1 (E1), HVR-1, and ISDR were amplified by nested RT-PCR with Smith's [Smith et al., 1997], Toyoda's [Toyoda et al., 1996], and Enomoto's [Enomoto et al., 1996] primer sets for the determination of J/W subtype, HVR-1 complexity, and ISDR mutations, respectively. PCR products were sequenced directly with an auto-sequencer, and sequences for nucleotides (nt) 982–1275, nt 1477–1551, and nt 6954–7280 were obtained (nt positions are according to the HCV-J strain [Enomoto et al., 1996]).

J/W subtype. Partial E1 sequences were used to calculate the subtype score as described below. Phylogenetic tree analysis of HCV 1b isolates in GenBank identified 8 nt residues that differed between the J and W subtypes [Nakano et al., 1999]. The subtype score was determined by counting the number of nt identical to those specific for each subtype, and the isolate was then classified accordingly. Twenty-three patients (J1–J23) carried J type HCV 1b, and 27 patients (W1–W27) carried W type HCV 1b. This method yielded results consistent with those of the phylogenetic tree analysis (Fig. 1A).

Complexity of HVR-1. The complexity of HVR-1 in quasispecies was determined with both the clone number determined by fluorescence single-stranded conformation polymorphism (SSCP) and nucleotide diversity determined from direct sequencing as described previously [Toyoda et al., 1996]. Briefly, HVR-1 clone number was calculated from obtained fluorescence SSCP data with the Fragment Manager software system (Pharmacia, Tokyo, Japan), and HVR-1 diversity was determined as described below. Mutated sites were identified with Alignment software (Fujitsu, Tokyo, Japan). Entire clones were analyzed directly, and sites with non-conserved nt on the HVR-1 were examined and quantified. The number of sites where nt were not conserved was calculated and considered to be an indication of diversity.

ISDR mutations. Mutations in the ISDR were examined according to Enomoto's method [Enomoto et al., 1996]. Briefly, ISDR sequences were translated into amino acid (aa) sequences. ISDR with aa sequences identical to the prototype HCV-J strain were considered to be wild type. For those ISDR with differences in sequences, the number of variant residues was counted.

Phylogenetic and statistical analyses. Phylogenetic analysis was performed with the neighbor-joining method with the Genetyx software package (Software Development Co., Tokyo, Japan). Statistical analysis for sex comparisons was performed by chi-square test without Yates correction. Other data are expressed as the mean with 95% confidence intervals, and the Student's *t*-test was used for comparisons. Correlations between values were tested with the Spearman rank correlation coefficient. *P*-values of less than 0.05 were considered to be statistically significant. StatView


A
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W1:170
W2:850
W3:850
J1:850
J2:850
J3:850
J4:560
J5:100
J6:130
J7:750
J8:620
J9:409
J10:850
J11:360
J12:850
J13:850
J14:850
J15:560
J16:850
J17:330
J18:850
J19:180
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J22:850
J23:600
J branch
W4:700
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W7:780
W8:150
W9:850
W10:850
W11:850
W12:850
W13:690
W14:540
W15:850
W16:158
W17:550
W18:850
W19:667
W20:200
W21:523
W22:850
W23:364
W24:840
W25:180
W26:670
W27:389
B
0.1
W14:7,7
W2:8,12
J20:5,6
J1:13,20
J2:3,4
J5:11,17
W21:5,5
W19:6,9
J21:9,19
J11:13,19
J18:10,20
W12:6,20
W6:7,20
W26:6,2
W4:11,12
W5:9,5
W24:4,5
W1:11,17
J17:11,10
J19:4,5
W3:7,20
W10:9,20
J22:14,20
J10:11,16
J16:10,6
W8:5,1
J4:9,11
J12:10,20
W13:9,16
J9:8,3
J23:8,7
J14:10,7
W15:8,10
J13:7,15
J15:11,20
J7:5,1
W23:4,4
W27:5,3
W20:7,2
J6:3,0
J3:12,14
W22:13,14
J8:7,20
W25:6,15
W9:12,13
W17:2,2
W18:3,1
W11:5,20
W16:11,10
W7:7,8

Fig. 1.

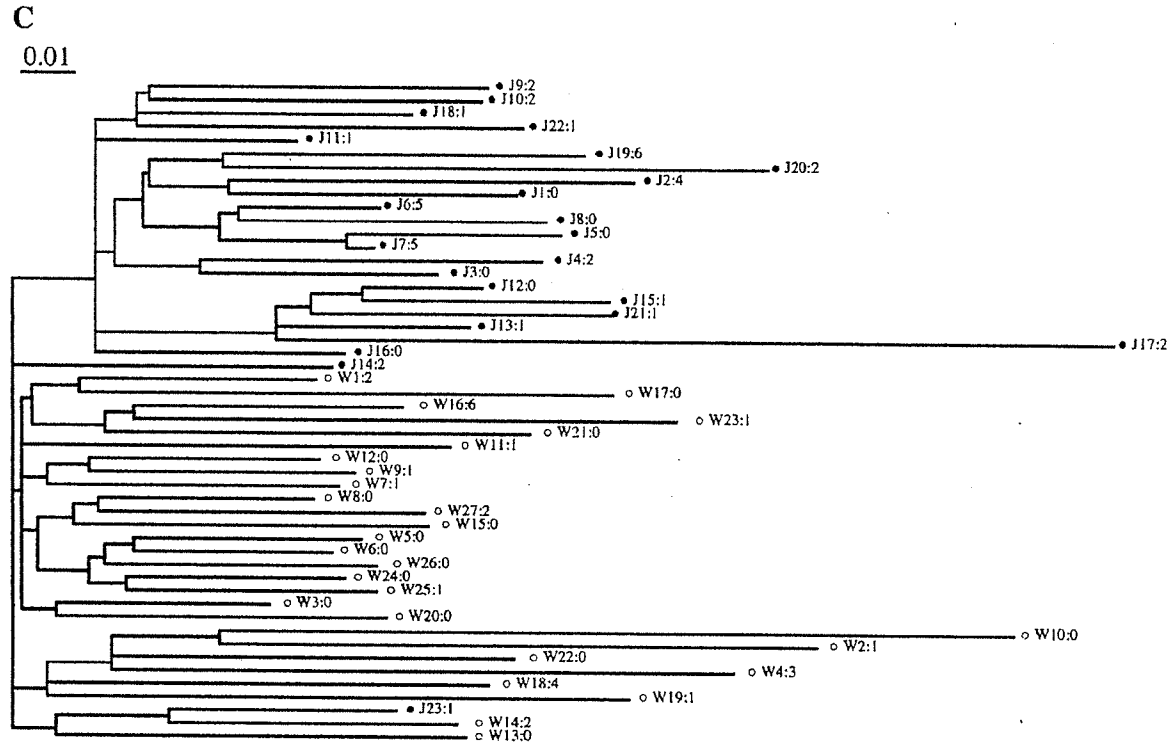

Fig. 1. **A:** Phylogenetic analysis and subtype determination. Classification of J and W types was performed as described in "Materials and Methods." Fifty chronic hepatitis patients with HCV genotype 1b were classified into 23 J type patients (closed circles; J1–J23) and 27 W type patients (open circles; W1–W27). The branch of clustered J type sequences is indicated as "J branch." The number following each patient number indicates serum viral load (KIU/ml). The horizontal bar indicates the number of nucleotides substitutions per site. **B:** Phylogenetic E2 analysis. The *N*-terminal portion of the E2 region containing HVR-1 from the same patients was analyzed. The numbers following each patient number indicate HVR-1 clone number and HVR-1 diversity. The horizontal bar indicates the number of nucleotides substitutions per site. **C:** Phylogenetic NS5A analysis. The NS5A region containing the ISDR from the same patients was analyzed. The number following each patient number indicates the number of ISDR mutations. The horizontal bar indicates the number of nucleotides substitutions per site.

5.0 software (SAS Institute, Inc., Cary, NC) was used for all calculations.

RESULTS

Patient Characteristics and Viral Factors (Table I)

There were no significant differences in sex or age between the patient groups. Viral loads were similar between the patient groups. The numbers of HVR-1 clones were significantly higher in J type-infected patients than in W type patients ($P = 0.044$). HVR-1 diversity and ISDR mutation numbers were slightly higher in J type patients, but this difference was not statistically significant.

Phylogenetic Subtype Analysis (Fig. 1A)

The scoring method for subtype classification yielded results consistent with those of the phylogenetic analysis (Fig. 1). Individual patient data, including viral load, HVR-1 clone number, HVR-1 diversity, and ISDR mutation number, are indicated.

TABLE I. Patient Profiles and Viral Factors

	Subjects n = 50	J type n = 23	W type n = 27	*P*-value
Sex (male/female)	31/19	14/9	17/10	0.879
Age (years old)	50.5 (47.1–53.9)	50.8 (46.1–55.5)	50.3 (45.0–55.5)	0.878
Viral load (KIU/ml)	608 (532–684)	597 (477–718)	617 (514–721)	0.794
HVR-1 clone number	7.94 (7.07–8.81)	8.87 (7.49–10.3)	7.15 (6.05–8.25)	0.044[a]
HVR-1 diversity	11.1 (9.09–13.0)	12.2 (9.09–15.3)	10.1 (7.46–12.8)	0.295
ISDR mutation number	1.30 (0.84–1.76)	1.70 (0.94–2.45)	0.96 (0.39–1.54)	0.110

Mean values with 95% confidence intervals in parentheses are indicated.
HVR, hypervariable region; ISDR, interferon sensitivity-determining region.
[a]$P < 0.05$.

TABLE II. Correlation Between Age, Viral Load, HVR-1, and ISDR

	Age	Viral load	HVR-1 clone number	HVR-1 diversity	ISDR mutation number
J type (n = 23)					
Age	—	$P = 0.931$	$P = 0.504$	$P = 0.612$	$P = 0.090$
Viral load	r = −0.019	—	$P = 0.132$	$P = 0.174$	$P = 0.116$
HVR-1 clone number	r = −0.147	r = 0.324	—	$P = 0.0001^b$	$P = 0.0001^b$
HVR-1 diversity	r = 0.112	r = 0.293	r = 0.710	—	$P = 0.0001^b$
ISDR mutation number	r = 0.362	r = −0.337	r = −0.734	r = −0.722	—
W type (n = 27)					
Age	—	$P = 0.739$	$P = 0.015^a$	$P = 0.125$	$P = 0.336$
Viral load	r = 0.067	—	$P = 0.789$	$P = 0.055$	$P = 0.141$
HVR-1 clone number	r = 0.462	r = 0.054	—	$P = 0.016^a$	$P = 0.364$
HVR-1 diversity	r = 0.303	r = 0.374	r = 0.460	—	$P = 0.586$
ISDR mutation number	r = 0.193	r = 0.291	r = 0.182	r = −0.110	—

P- and r values are indicated.
HVR, hypervariable region; ISDR, interferon sensitivity-determining region.
[a] $P < 0.05$.
[b] $P < 0.01$.

Correlation Between Age, Viral Load, HVR-1, and ISDR (Table II, Fig. 2)

Correlations between age and several viral features were analyzed independently for each subtype group. For both subtypes, there were significant correlations between HVR-1 clone numbers and HVR-1 diversity ($P = 0.0001$, r = 0.710 for J type; $P = 0.016$, r = 0.460 for W type), suggesting that both are reliable markers of HVR-1 complexity. In J type patients, the ISDR mutation numbers were inversely correlated with HVR-1 clone numbers ($P = 0.0001$, r = −0.734) and

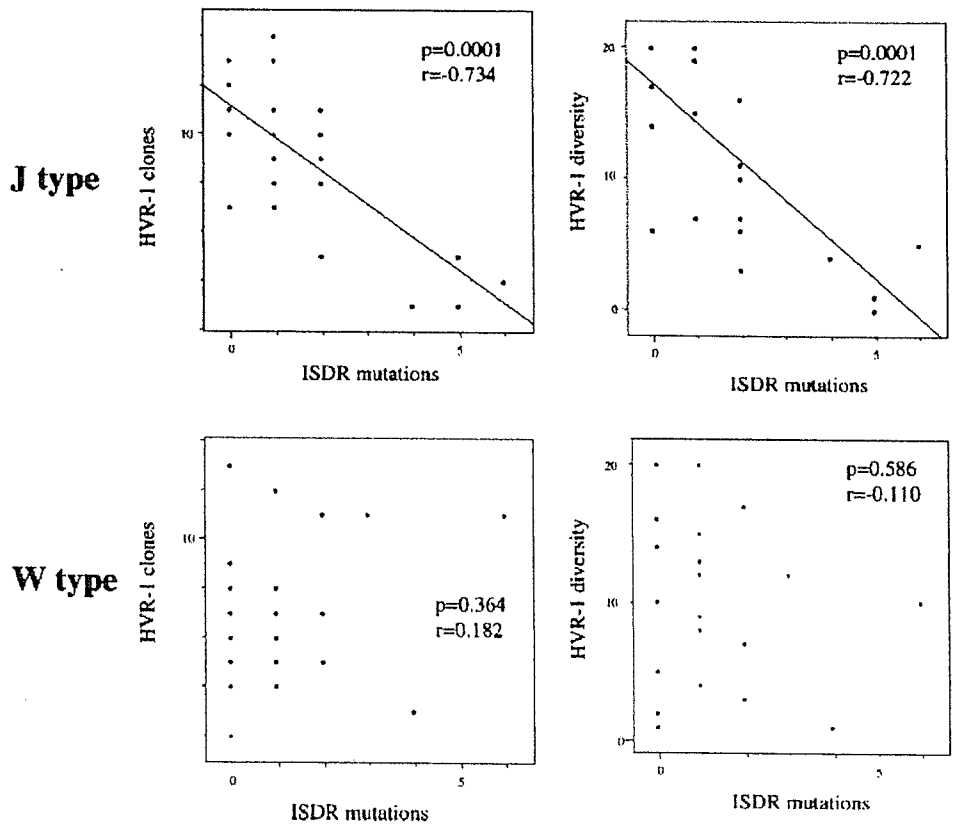

Fig. 2. Correlation between HVR-1 complexity and ISDR mutations. Scattergrams of HVR-1 clone number (left panels, vertical axes) and diversity (right panels, vertical axes), and ISDR mutation number (horizontal axes) for J type (upper panels) and W type (lower panels) HCV 1b are shown. Correlation values are also shown in each panel.

HVR-1 diversity ($P = 0.0001$, $r = -0.722$), whereas such correlations were not observed in W type patients. In W type patients, there was a significant correlation between age and HVR-1 clone numbers ($P = 0.015$, $r = 0.462$).

Phylogenetic Tree Analysis of E2 *N*-Terminal and NS5A Regions (Fig. 1B,C)

Phylogenetic tree analysis of the E2 *N*-terminal region (nt 1477–1551) revealed no association of phylogenetic position of each sample in the E1 region. No phylogenetic features in the distributions of the number of clones or diversity in quasispecies were detected. The phylogenetic distance between each sequence was relatively long compared to those in the other two regions. All J and W sequences were distributed diffusely, and no clustered branches were detected. In contrast, phylogenetic tree analysis of the NS5A region revealed that 22 of 23 J type patients were clustered on the same branch, whereas the clustered branch was relatively short in length compared to the one drawn with the E1 region. Thus, the NS5A region showed strong correlation with the E1 region with respect to J/W subtype distribution, suggesting that the NS5A region can identify J/W type effectively. No phylogenetic features of the NS5A region with respect to ISDR mutation numbers were detected.

J/W Type Determination by NS5A Sequence (Table III, Fig. 3)

To characterize the nt residues specific for each subtype with respect to the NS5A region, alignments of nt 6954–7280 were compared (Fig. 3). Six nt residues were relatively specific for an alternative subtype. The specific nt are summarized in Table II. Two of 6 nt residues led to substitutions in aa sequence (from Thr to Ser or Pro at aa 2278 and from Arg to Lys at aa 2279). These two residues are part of the PKR-binding domain (aa 2209–2281) but not the ISDR (aa 2209–2248). J/W subtype determination of new HCV1b-infected patients is possible by viewing the phylogenetic tree of the NS5A region when it is drawn with many subtype-identified sequences. Alternatively, investigation of the specific nt residues indicated in Table III may aid in subtype determination.

DISCUSSION

We reported previously that differences in the usefulness of ISDR sequences for prediction of IFN responses between patients from Japan and those from other countries led to the identification of three subtypes of HCV genotype 1b, which were named on the basis of their distribution [Nakano et al., 1999; predominantly two subtypes in Japan; Japan-specific J type and worldwide W type]. Our subsequent studies identified several clinical differences between J type- and W type-infected patients, even though both patient groups showed similar IFN response rates, serum viral loads, and numbers of ISDR mutations [Nakano et al., 2001a,b; Otagiri et al., 2002]. The IFN response in patients infected with J type HCV 1b was related to transfusion history, low viral load, and ISDR mutation, whereas that in W type-infected patients was associated with female sex and low viral load [Nakano et al., 2001a]. W type was also associated with histologically more active disease and faster development of cirrhosis, whereas J type showed a relatively low pathogenicity [Nakano et al., 2001b]. Such subtype-dependent characteristics may explain the controversial findings of clinical studies of HCV 1b in Japan and other countries [Nakano et al., 2001a,b].

Considering that the usefulness of the ISDR is restricted to J type-infected patients, J/W subtype determination should precede ISDR measurement in HCV 1b-infected patients in whom physicians intend to treat with IFN. Because subtype measurement requires a large amount of work, including sequencing of the E1 region and phylogenetic analysis, this procedure may not be appropriate for routine clinical use. In addition, unless the result is "J type," the ISDR system is irrelevant. In the present study, we devised a simple method to determine subtype and ISDR type with a sequence from the NS5A region. The present method will allow for ISDR use as a more powerful predictive tool of IFN sensitivity.

We compared the complexity of HVR-1 between J and W types. HVR-1 is part of the E2 glycoprotein of HCV and appears to be the target of neutralizing antibodies. HCV strains with HVR-1 mutations may escape antibody neutralization, and such escape from immune selection may allow for the viral persistence that accompanies the complexity of HVR-1 quasispecies [Taniguchi et al., 1993; Koizumi et al., 1995]. The age-dependent increases in HVR-1 clone number in W type-infected patients in the present study may be a reflection of this phenomenon. Because HVR-1 is likely involved in the interaction between host and virus, the complexity of HVR-1 may be associated with the clinical features of HCV infection [Farci et al., 2000]. In liver transplant recipients, the increased complexity of HVR-1 quasis-

TABLE III. Dominantly Distributed Nucleotide Residues

nt residues	6995	7161	7165	7205	7214	7229
J type	A	A	G	C	A	T
W type	G	T/C	A	T	T	C
Involved aa sequences	2222	2278	2279	2292	2295	2300
J type	Pro	Thr	Arg	Asp	Pro	Ser
W type	—	Ser/Pro	Lys	—	—	—

Dashes indicate amino acid residues identical to those of J type.

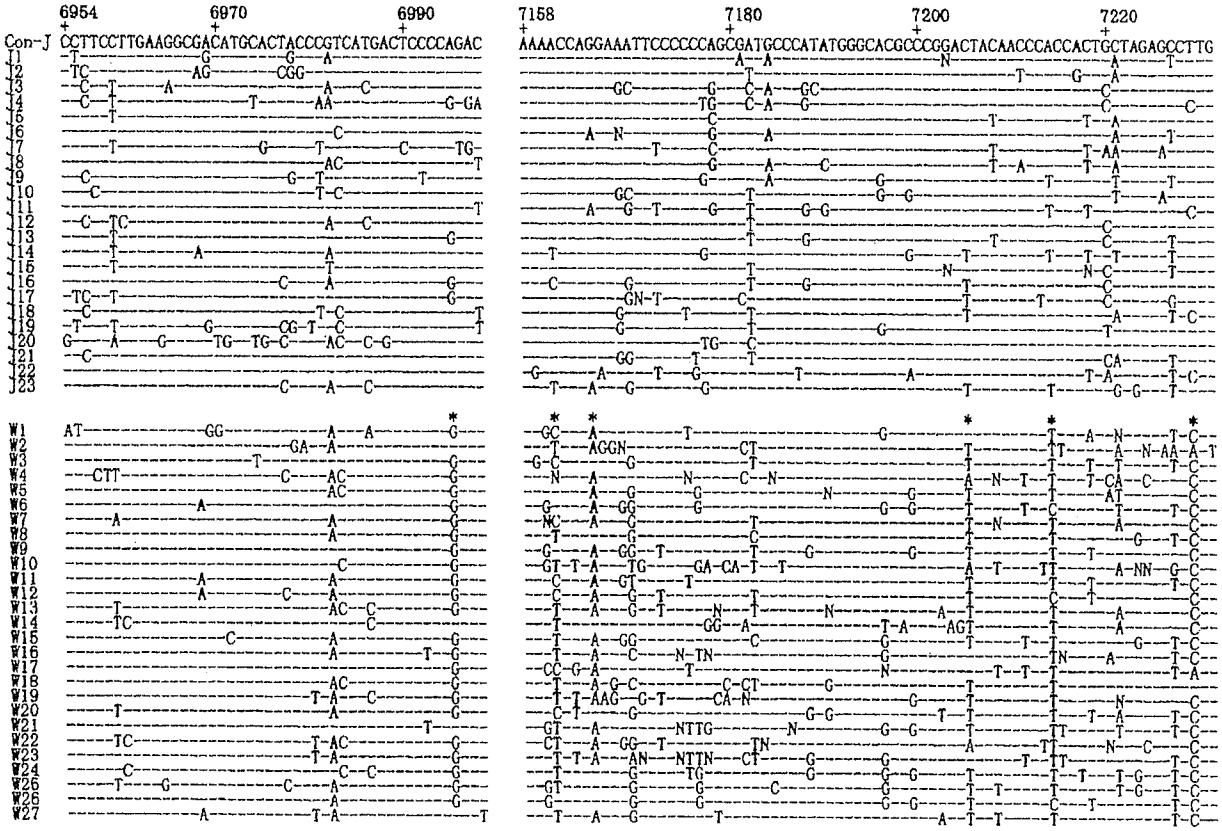

Fig. 3. Nucleotide sequence alignments of the NS5A region. The relatively conserved regions are removed. The top sequence, Con-J, is a consensus sequence deduced from J type patients J1–J23. Dashes indicate nt residues identical to those of the Con-J sequence. The six stars (*) indicate the nucleotide positions where the nucleotide distributions apparently differ between J and W type patients.

pecies is associated with less aggressive hepatitis [Lyra et al., 2002]. Longitudinal analysis of HCV-infected renal transplant recipients revealed that quick diversification of HVR-1 quasispecies is associated with reduced progression of liver fibrosis [Izopet et al., 2000]. Thus, the high complexity of HVR-1 quasispecies appears to be associated with low pathogenicity of the virus. One possible hypothesis for this association was given [Lyra et al., 2002]: that host immune pressure drives the genetic complexity of HVR-1, and the stronger immune pressure directed against HCV is, the more protective it is against liver disease. In the present study, HVR-1 quasispecies of J type-infected patients were more complex than those of W type-infected patients, which may explain our previous finding that J type HCV 1b is less pathogenic than W type one [Nakano et al., 2001b].

The ISDR system is useful in Japan because of the predominance of J type HCV 1b in infected Japanese patients, as described previously [Nakano et al., 1999]. Some investigators have hypothesized a possible mechanism for the association between ISDR mutations and IFN response [Noguchi et al., 2001]. They reported that the NS5A protein containing the wild-type ISDR sequence blocks IFN-induced RNA-dependent protein kinase activity, and that this may interfere with the cellular response to IFN. Mutations in the ISDR might affect this ability, resulting in better IFN response. However, this scenario does not explain why the correlation is restricted to patients infected with J type HCV 1b. In the present study, the number of ISDR mutations was inversely correlated with HVR-1 complexity in a J type-specific manner. This could explain the J type-specific correlation between ISDR mutations and IFN responses. It is known that the less complex the HVR-1 region, the higher the response to IFN [Hino et al., 2000; Yeh et al., 2002]. In J type-infected patients, the higher ISDR mutation rate, which is related to reduced complexity of HVR-1, should yield a more robust IFN response. In contrast, in W type-infected patients, the higher rate of ISDR mutation, which is not related to HVR-1 complexity, would not be predictive of IFN response. Our present findings suggest that ISDR mutations are not directly related to IFN responsiveness and that the degree of HVR-1 complexity is more important than the ISDR in determining IFN responsiveness.

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MAJOR ARTICLE

Changes in Hepatitis C Virus (HCV) Antibody Status in Patients with Chronic Hepatitis C after Eradication of HCV Infection by Interferon Therapy

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Background. Changes in hepatitis C virus (HCV) antibody status were followed for 10 years after the eradication of HCV by interferon (IFN) therapy in 30 patients with chronic hepatitis C who showed a sustained virological response.

Methods. HCV core antibody titer, third-generation HCV recombinant immunoblot assay (RIBA) grade (measuring the presence of antibodies for core, NS3, NS4, and NS5 antigens), and genotype-specific antibodies to the HCV NS4 region were measured annually with commercially available kits for these antibodies.

Results. For grade of HCV antibody determined by RIBA, the most significant decrease was observed with anti-NS5 antibody, followed by anti-NS4, anti-NS3, and anti-core antibodies, in that order. Tests for anti-NS5 and anti-NS4 antibodies had negative results in almost 50% of patients 10 years after eradication of HCV. In contrast, the results of tests for anti-core antibody were still markedly positive in most patients. However, anti-core antibody titer decreased continuously during the 10-year follow-up period. Antibodies to the NS4 region specific for HCV genotypes 1 and 2 also decreased during the follow-up period. Differences in the rate at which antibody titers decreased were observed between antibodies for genotypes 1 and 2; as a consequence, the serological type of HCV changed during the follow-up period in some patients.

Conclusions. HCV antibody titer appears to continue to decrease during the 10 years after eradication of HCV by IFN therapy.

Chronic infection with hepatitis C virus (HCV) is one of the most common infections worldwide. The number of patients with chronic hepatitis due to HCV infection is estimated at 170 million worldwide, 2.7 million in the United States, and 1.2 million in Japan. HCV infection is one of the important causes of hepatocellular carcinoma, and in addition, a relationship between HCV infection and disorders other than liver disease—such as mixed cryoglobulinemia, diabetes mellitus, and lichen planus—has been suggested [1–3].

Treatment with IFN has been used to induce the normalization of the serum alanine aminotransferase (ALT) level, with a disappearance of the HCV RNA in serum in some patients with chronic HCV infection. Patients with normal ALT levels and the absence of HCV RNA in serum >6 months after the end of IFN therapy are usually described as having a sustained virological response (SVR). In such patients, HCV RNA continues to be absent, and HCV is considered to be eradicated [4].

A few studies have focused on features of patients with SVR and have specifically focused on the incidence of hepatocellular carcinoma and the resolution of liver fibrosis [5–12]. However, no studies have examined changes in HCV antibody status in patients with SVR after eradication of HCV. In the present study, we prospectively followed-up and investigated serial changes in various HCV antibodies after eradication of HCV in patients with chronic hepatitis C who achieved SVR to IFN therapy.

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PATIENTS, MATERIALS, AND METHODS

Patients. A total of 751 patients with histologically and virologically proven chronic hepatitis C received IFN therapy at Ogaki Municipal Hospital (Ogaki, Japan) during 1989–2004. Of these patients, 288 showed SVR, which was defined as the continuation of normal serum ALT levels and the absence of HCV RNA in serum >1 year after the end of IFN therapy. At present, 201 of these patients with SVR continue to undergo regular follow-up and laboratory testing as outpatients every 3–6 months. Thirty patients with follow-up periods of >10 years were analyzed in the present study. At each follow-up visit, serum samples were obtained and stored at −80°C until analyzed.

Written informed consent was obtained from each patient at the time that serum samples were collected. The entire protocol was approved by the ethics committee of Ogaki Municipal Hospital and was carried out in compliance with the Helsinki Declaration.

Serum test for HCV RNA and genotyping of HCV. The presence of HCV RNA in serum obtained from each patient at 1, 3, 5, 8, and 10 years after the end of IFN therapy was determined by nested RT-PCR [13]. HCV genotype was determined by RT-PCR with genotype-specific primers [14].

Serological tests for anti-HCV core antibody titer, recombinant immunoblot assay (RIBA) grade, and genotype-specific antibodies. Anti-HCV core antibody specific for the c22-3 antigen was measured by radioimmunoassay with a commercially available kit (Ortho HCV Cire-Ab Irma Test; Mitsubishi Kagaku Iatron) according to the standard. Semiquantitative titer of antibody against HCV was measured by a third-generation RIBA [15] with use of the Chiron RIBA HCV Test 3.0 (Chiron) according to the manufacturer's instructions. This assay detects antibodies directed to both structural antigens (core antigen, c22 synthetic peptide) and nonstructural antigens (NS3 antigen, c33c recombinant protein; NS4 antigen, mixed 5.1.1 and c100 peptides; NS5 antigen, recombinant protein). In the assay, the intensities of colored bands on the nitrocellulose strip are proportional to amounts of bound antibody and are graded as negative, 1+, 2+, 3+, and 4+, according to the manufacturer's instructions. Sample reactivity to superoxide dismutase, to which all the HCV antigens were fused, was also assessed.

Genotype-specific antibodies against the HCV NS-4 region were measured by ELISA [16] with an Immucheck F-HCVGr assay (International Reagents) according to the manufacturer's instructions. Titers of genotype-specific antibodies C14-1 and C14-2 were measured. Samples with a cut-off index (COI) of >1.0 were judged as positive. Serological type 1 included samples with a C14-1/C14-2 antibody COI ratio >2 or samples positive for C14-1 antibody and negative for C14-2 antibody. Serological type 2 included samples with a C14-2/C14-1 antibody COI ratio >2 or samples negative for C14-1 antibody and positive for C14-2 antibody. The serological type was classified as 1 and 2 when the sample was positive for both C14-1 and C14-2 antibodies and the COI ratio of C14-1 to C14-2 was <2. The serological type was classified as undetermined when tests for both C14-1 and C14-2 antibodies had negative results.

RESULTS

Patient characteristics. The presence of HCV RNA in serum was confirmed before initiation of IFN therapy by nested RT-PCR in all 30 patients. The study group included 16 men and 14 women, and the mean age (±SD) was 50.5 ± 10.5 years at the start of IFN therapy. HCV genotypes, determined on the basis of Simmonds' nomenclature [17], were 1b (10 patients), 2a (12 patients), and 2b (3 patients). HCV genotype could not be determined or was mixed in the remaining 5 patients. Histological study of the liver biopsy specimens obtained within 3 months before the start of the IFN therapy revealed activity grades to be A1 (21 patients), A2 (7 patients), and A3 (2 patients). Grades of fibrosis were F0 (5 patients), F1 (16 patients), F2 (4 patients), and F3 (5 patients), determined on the basis of the classification by Desmet et al. [18]. Twenty-three patients received IFN-α, and the remaining 7 received IFN-β.

In all 30 patients, HCV RNA was not detected in serum samples obtained at 1, 3, 5, 8, and 10 years after the end of IFN therapy, and ALT levels in serum samples were less than the normal limit throughout the follow-up period. No patients showed immunosuppression before or during IFN therapy or during the follow-up period.

Changes in annual HCV RIBA grade and HCV core antibody titer after the eradication of HCV by IFN therapy. Annual changes in semiquantitative antibody titers for HCV core protein (c22) are shown in figure 1A. In most patients, the antibody titer for c22 was maintained at 4+ during the 10 years of follow-up. However, when we analyzed HCV core antibody (c22-3) annually, the titer decreased over the 10-year follow-up period (figure 2). In contrast to the titer of HCV core antibody as determined with use of RIBA, titers for HCV NS3 (c33c), NS4 (5.1.1 and c100), and NS5 antibodies decreased serially after eradication of HCV by IFN therapy. The decrease in antibody was most marked for antibodies specific for NS5, NS4, and NS3, in that order (figures 1B, 1C, and 1D).

We next compared the clinical characteristics of patients who had a rapid decrease of HCV antibodies after eradication of HCV with those of patients who did not have a rapid decrease. We found no difference in patient characteristics, including age, sex, pretreatment HCV RNA concentration, HCV genotype, ALT level, and liver histological findings.

Changes in HCV genotype-specific antibodies in NS4 region after the eradication of HCV by IFN therapy. Decreases in the titers of 2 kinds of HCV genotype-specific antibodies—

anti-HCV genotype 1 (1a or 1b) and anti-HCV genotype 2 (2a or 2b)—to HCV NS4 region were measured annually (figure 3). Titers of both antibodies decreased continuously after the eradication of HCV, and the rate of decrease was similar between the 2 antibodies. However, the rate of decrease of these 2 antibodies was sometimes different between genotype 1– and genotype 2–specific antibodies. This difference caused a discrepancy between the original HCV genotype and the serological data, and it resulted in the incorrect determination of the genotype of the eradicated HCV on the basis of serological typing in 2 of 30 patients (table 1).

DISCUSSION

There are several reports of changes in HCV antibody titers or RIBA grade in patients with acute hepatitis C after spontaneous eradication of HCV during the acute phase of the illness in a case series with a few patients [19–21]. Other reports have documented changes in HCV antibody status during and after the end of IFN therapy [22–26] and changes after spontaneous clearance of HCV in patients with chronic hepatitis C [27]. However, there have been very few reports of long-term follow-up of HCV antibody status after eradication of HCV by IFN therapy. Only Lefrere et al. [28, 29] reported the long-term changes of HCV antibody status of patients with chronic hepatitis C after eradication of HCV. They observed decreases in various HCV antibody titers in patients in whom HCV had been eradicated, including in 1 patient who experienced the eradication of HCV by IFN therapy [28, 29].

Patients with SVR are more frequently lost to follow-up after the end of IFN therapy than are other patients [12], because the eradication of HCV is often considered to be a complete cure of chronic hepatitis. Therefore, regular and long-term follow-up of patients with SVR is sometimes difficult, and that is why there are few studies of changes in laboratory data for patients with chronic hepatitis C after eradication of HCV.

In the present study, we prospectively observed HCV anti-

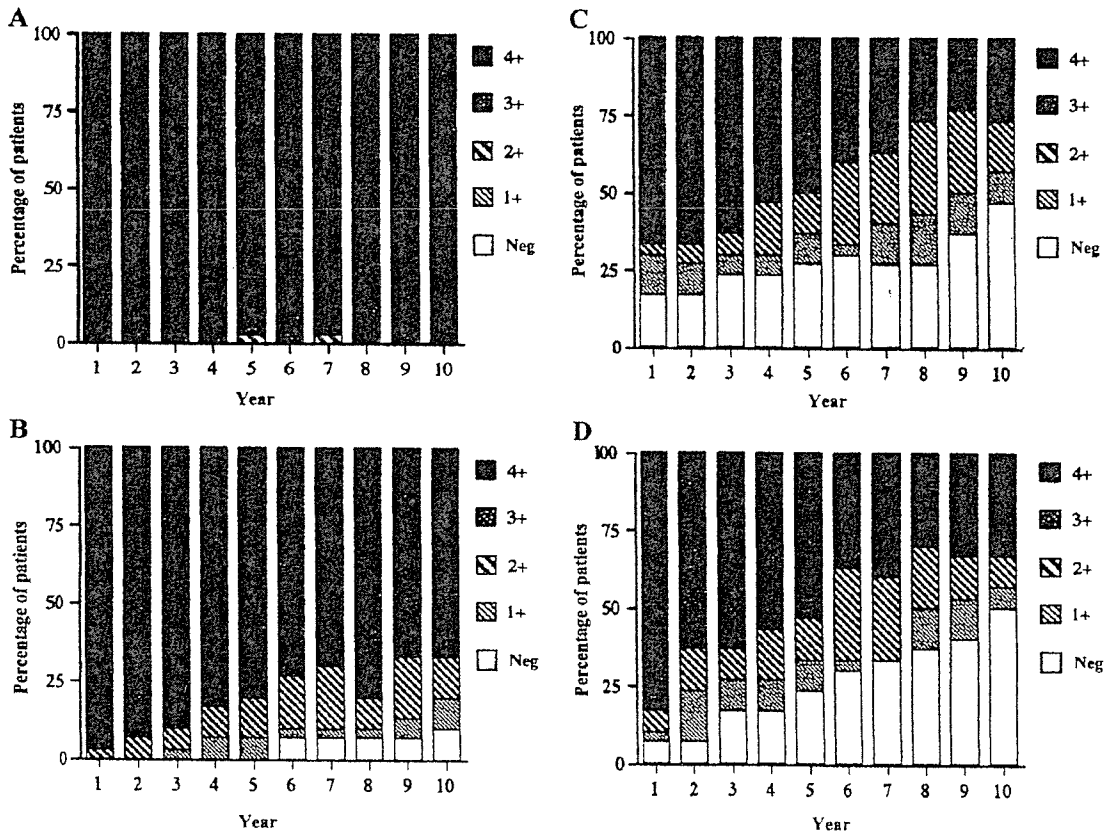

Figure 1. Annual changes in semiquantitative titer of antibody against hepatitis C virus (HCV) after eradication of HCV, as measured by third-generation recombinant immunoblot assay (Chiron RIBA HCV Test 3.0; Chiron). Antibody titers were graded as negative, 1+, 2+, 3+, and 4+, according to the manufacturer's instructions. *A*, Antibody against HCV core protein (c22p). *B*, Antibody against HCV NS3 protein (c33c). *C*, Antibody against HCV NS4 protein (c100p). *D*, Antibody against HCV NS5 protein (NS5). Neg, negative; Year, year after the end of IFN treatment.

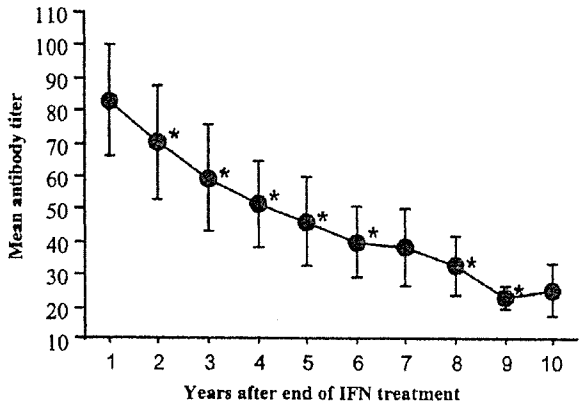

Figure 2. Annual changes in titer of antibody specific for hepatitis C virus (HCV) core protein (c22-3). *$P < .05$ by Student's t test in comparison with the previous year.

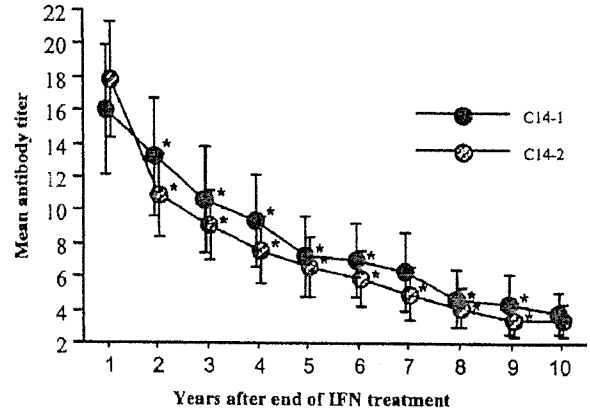

Figure 3. Annual changes in titers of genotype-specific antibodies against hepatitis C virus (HCV) NS4 protein (C14-1 and C14-2) after eradication of HCV. *$P < .05$ by Student's t test in comparison with the previous year.

body status for antigens specific to HCV core protein and to nonstructural proteins 3–5. On the basis of the results of RIBA, antibody against HCV core protein remained strongly positive (4+, according to semiquantitation) in most patients even 10 years after eradication of HCV, whereas antibodies against HCV nonstructural proteins (NS3, NS4, and NS5) weakened consecutively. Antibodies against NS4 and NS5 were absent in approximately one-half of patients 10 years after the eradication of HCV. The titer of HCV core antibody (c22-3), however, showed an annual decrease. Therefore, titers of HCV antibodies decrease regardless of their specific targets. In a more recent study, Wiegand et al. [27] reported the lack of a decrease in HCV antibody titer after eradication of HCV by IFN therapy in patients with chronic hepatitis C—in contrast to a marked decrease in patients with acute hepatitis C treated with IFN therapy—in a study involving patients who were observed for up to 80 weeks after completion of IFN therapy. In contrast, Lefrere et al. [28] observed a disappearance of HCV antibodies, except for antibody to HCV core protein. Although the antibody discussed in the study by Wiegand et al. [27] is different from that reported in ours, our study provided evidence of a decrease in HCV antibody following eradication of HCV even in patients with chronic HCV infection.

Pawlotsky et al. [30, 31] reported a difference in the reactivity of antibodies between HCV genotypes with use of a second-generation RIBA kit but not with a third-generation RIBA kit. In keeping with their findings, we observed no difference in reactivity between genotypes and no difference in the rate of decrease of antibody titers between patients with HCV genotypes 1 (1b) and 2 (2a or 2b) in an assessment with a third-generation RIBA kit. In addition, we found no patient pre-

Table 1. Changes in hepatitis C virus (HCV) genotype-specific antibody titers and determination of serotype for 2 patients, by year after IFN treatment.

Variable	Pretreatment	Year after IFN treatment									
		1	2	3	4	5	6	7	8	9	10
Patient 1											
C14-1 antibody titer	32.73	4.20	3.72	3.25	3.10	3.05	2.75	3.00	2.86	2.84	2.46
C14-2 antibody titer	21.02	20.70	14.71	12.37	9.62	7.88	8.20	6.38	5.69	4.43	2.71
Serological type[a]	1	2	2	2	2	2	2	2	2	1 and 2	1 and 2
Patient 2											
C14-1 antibody titer	1.89	1.40	1.16	1.29	1.19	1.38	1.55	1.65	1.57	1.30	1.10
C14-2 antibody titer	3.78	1.90	1.46	0.91	0.60	0.47	0.46	0.31	0.29	0.26	0.22
Serological type[a]	2	1 and 2	1 and 2	1	1	1	1	1	1	1	1

NOTE. Patient 1 was a 54-year-old man with HCV genotype 1b. Patient 2 was a 41-year-old man with HCV genotype 2a.

[a] Serological type 1 included samples with a C14-1/C14-2 antibody titer cut-off index (COI) ratio >2 or samples with that were positive for C14-1 antibody and negative for C14-2 antibody. Serological type 2 included samples with a C14-2/C14-1 antibody COI ratio >2 or samples that were negative for C14-1 antibody and positive for C14-2 antibody. The serological type was classified as 1 and 2 when the sample was positive for both C14-1 and C14-2 antibodies and the COI ratio of C14-1 antibody to C14-2 antibody was <2.

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-