TABLE 1. CORRELATION BETWEEN THE ACTIVITY OF HEPATITIS AND THE DEGREE OF FIBROSIS IN PATIENTS WITH CHRONIC HEPATITIS C

	Degree of fibrosis			
Activity of hepatitis	*F0*	*F1*	*F2*	*F3*
A0	30	12	0	0
A1	60	171	52	3
A2	2	42	80	41
A3	0	0	6	5

Note. $P < 0.0001$ for A0 or A1/A2 or A3 vs. F0 or F1/F2 or F3.

0.0058) and no difference between younger and older patients with A0/A1 (younger, 84.0 ± 63.4 IU/L, vs. older, 80.7 ± 66.8 IU/L; $P = 0.6492$) (Figure 4). Thus, the difference in serum ALT by sex was observed only when the activity of hepatitis was low, whereas the difference by age was observed only when the activity of hepatitis was high.

Multivariate Analysis for Influence of Age, Sex, and Degree of Liver Fibrosis on Serum ALT Levels. Multivariate analysis showed that all three factors (age, sex, and degree of liver fibrosis) independently affected serum ALT levels (>70 IU/L) (degree of liver fibrosis, $P = 0.0043$; age, $P = 0.0038$; sex, $P = 0.0184$), in addition to activity of hepatitis ($P = 0.0366$). When patients were stratified by activity of hepatitis, a high degree of liver fibrosis (F2 or F3) ($P = 0.0229$) and male sex ($P = 0.0153$) independently increased serum ALT levels in patients with A0 or A1. In patients with A2 or A3, age greater than 50 years independently increased serum ALT levels ($P = 0.0002$).

Influence of Age, Sex, and Degree of Liver Fibrosis in Patients with Persistently Normal ALT Levels or with Persistently Mild ALT Elevation. In considering patients in whom serum ALT levels were persistently less than 70 IU/L for more than 6 months before liver biopsy, which is less than twice the normal limit of ALT levels in our hospital, only 10.8% of patients with F0/F1 fibrosis had A2/A3 activity of hepatitis. In contrast, 67.1% of patients with F2/F3 fibrosis had A2/A3 activity of hepatitis ($P < 0.0001$; Table 2). There was no difference in the proportion of patients with moderate or severe activity of hepatitis (A2/A3) between men and women ($P = 0.9943$). However, 39.0% of patients >50 years old had A2/A3 activity of hepatitis, whereas only 11.8% of patients ≤50 years old had A2/A3 activity of hepatitis ($P < 0.0001$; Table 3). In addition, even in patients with persistently normal ALT levels (<35 IU/L), 6 of 13 patients (46.2%) with F2/F3 fibrosis had A2/A3 activity of hepatitis and 10 of 42 patients (23.8%) >50 years old had A2/A3 activity of hepatitis.

DISCUSSION

Initial evaluation of a patient with chronic HCV infection focuses on assessment of the activity and stage of liver disease, based on the histology of a liver biopsy specimen. Liver histology is frequently considered the gold standard for assessing the progression of chronic hepatitis in terms of the activity of hepatitis and fibrosis. However, it is usually difficult to monitor patients by serial histological evaluation because liver biopsy has potential complications.

Liver fibrosis is usually irreversible, except in patients in whom HCV has been eradicated by antiviral treatment, and it progresses slowly, over years. In contrast, the activity of hepatitis (i.e., liver inflammation) is reversible and is likely to fluctuate. Therefore, routine follow-up of patients with chronic hepatitis C focuses on the fluctuation in the activity of hepatitis, and patients are usually evaluated by laboratory tests.

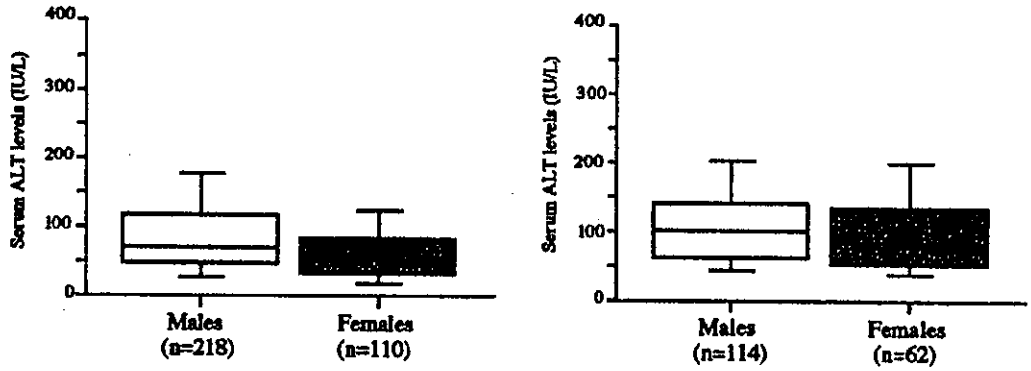

Fig 3. Serum alanine aminotransferase (ALT) levels per sex. Left: Comparison among patients with A0/A1 activity. Right: Comparison among patients with A2/A3 activity. Male patients had higher serum ALT levels than female patients among patients with A0/A1 activity (males, 90.9 ± 68.8 IU/L, vs. females, 66.0 ± 52.8 U/L; $P = 0.0010$).

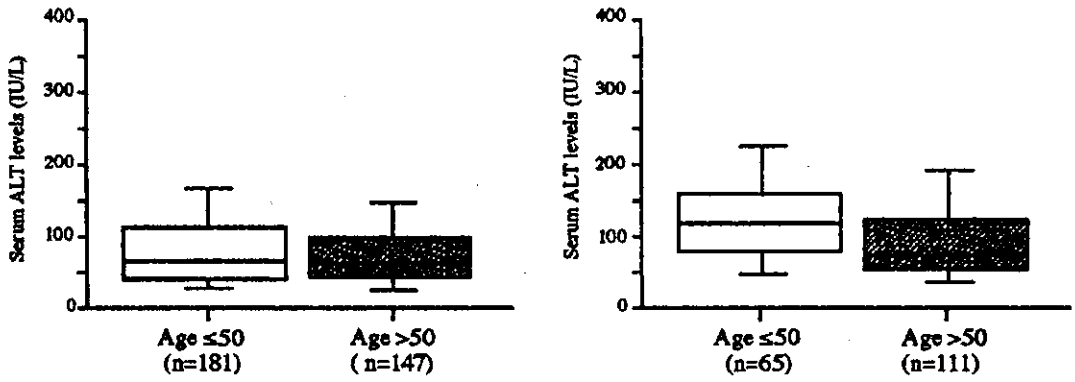

Fig 4. Serum alanine aminotransferase (ALT) levels per age. Left: Comparison among patients with A0/A1 activity. Right: Comparison among patients with A2/A3 activity. Younger patients (≤50 years old) had higher serum ALT levels than older patients (>50 year old) among patients with A2/A3 activity (younger, 128.6 ± 75.7 IU/L, vs. older, 98.2 ± 65.8 IU/L; $P = 0.0058$).

In the clinical setting, ALT is generally considered the most reliable indicator among laboratory measures of active liver injury. The highest concentration of ALT in the body is in the liver, and levels of this enzyme are therefore the most specific indicators of liver injury. ALT is reportedly released into the blood in increasing amounts when the liver cell membrane is damaged (1). An old study showed that elevations in serum aminotransferase levels are markers of liver cell injury (10), and another study showed a close correlation between elevated transaminases and histologic necroinflammation in biopsied specimens from patients with chronic liver disease (11, 12).

Most patients with low serum ALT levels show histologically low activity of hepatitis and low fibrosis (3, 13–16). Some previous studies, however, yielded a poor correlation between serum ALT levels and histological findings of liver-cell damage (13, 14, 17, 18). The volume of residual hepatocytes is reduced in patients with severe fibrosis because of the accumulation of fibrotic tissue caused by chronic liver disease; this reduction in the volume of intact hepatocytes may modify the correlation between liver inflammation and serum ALT levels. As a result, the elevation in serum ALT levels in association with the increase in the activity of hepatitis can be suppressed in patients with severe fibrosis. In addition, serum ALT levels reportedly vary according to sex and body mass index (BMI) in healthy individuals (19, 20).

In the present study, we evaluated the influence of age, sex, and degree of liver fibrosis on the elevation in serum ALT in patients with chronic hepatitis C. We found that patients with a higher degree of liver fibrosis had higher serum ALT levels, mainly because patients with severe fibrosis had a higher activity of hepatitis than that of patients with mild fibrosis. More importantly, however, we found that a large proportion of patients with severe fibrosis also had high activity of hepatitis (A2 or A3), even those with persistently normal ALT levels or those with persistently mild elevation in serum ALT (less than twice the normal value for more than 6 months). This should be taken into consideration when physicians evaluate the activity of hepatitis on the basis of serum ALT levels.

The increase in serum ALT levels in association with the activity of hepatitis seems to differ between the sexes. Two previous studies reported lower ALT levels in healthy females than in healthy males (19, 20). Thus, there is a trend toward lower ALT levels in females without liver

TABLE 2. DISTRIBUTION OF THE ACTIVITY OF HEPATITIS ACCORDING TO THE DEGREE OF FIBROSIS IN PATIENTS WITH SERUM ALT LEVELS ≤70 IU/L

	Degree of fibrosis	
Activity of hepatitis	*F0/F1*	*F2/F3*
A0/A1	157	23
A2/A3	19	47

Note. $P < 0.0001$.

TABLE 3. DISTRIBUTION OF THE ACTIVITY OF HEPATITIS ACCORDING TO AGE IN PATIENTS WITH SERUM ALT LEVELS ≤70 IU/L

	Age of patients	
Activity of hepatitis	*≤50 yr*	*>50 yr*
A0/A1	97	83
A2/A3	13	53

Note. $P < 0.0001$.

disease. This difference in serum ALT levels in healthy individuals seems to be maintained when the activity of hepatitis and fibrosis of the liver is mild. However, this difference disappears when the activity is severe.

Interestingly, in contrast, the age of patients influenced the increase in serum ALT levels when the activity of hepatitis became severe. This suggests a reduced elevation in ALT in response to liver injury in older patients. Liver morphology reportedly undergoes changes such as hepatocyte ploidy as a person ages (21), which may account for the difference in ALT elevation between younger and older patients observed in our study. In addition, 39.0% of older patients with a minor elevation in serum ALT (less than twice the normal value) had higher activity of hepatitis (A2 or A3). Thus, physicians should also consider patient age in the interpretation of serum ALT levels.

In the present study, we did not evaluate the influence of BMI because of a lack of data. BMI reportedly influences serum ALT levels in healthy individuals (19, 20). Therefore, the influence of BMI on serum ALT levels in patients with chronic hepatitis C will require further study.

In summary, age, sex, and degree of liver fibrosis independently influence serum ALT levels in patients with chronic hepatitis C. Physicians should consider these factors during routine follow-up of this population. Especially in older patients or patients with severe fibrosis, the activity of hepatitis can be severe even with mild ALT elevation. Serum ALT is still the most commonly used indicator of liver cell injury, and there is no current alternative method of monitoring fluctuation in liver inflammation that is more reliable than serum ALT. Therefore, further studies are needed to evaluate factors influencing the association between liver inflammation and serum ALT for a better understanding of the degree of liver injury.

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Journal of Medical Virology 73:195–199 (2004)

Prevalence and Clinical Implications of Occult Hepatitis B Viral Infection in Hemophilia Patients in Japan

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The prevalence and clinical implications of occult hepatitis B virus (HBV) infection were investigated in the Japanese patients with hemophilia in whom a high prevalence of infection with transfusion-transmissible viruses has been reported. HBV DNA was detected in the sera of 22 of 43 (51.2%) patients with hemophilia who were negative for HBV surface antigen (HBs), indicating that these patients had occult HBV infection. No factor, including age, type or severity of hemophilia, presence of HBs or HBV core (HBc) antibody, or coinfection with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) was associated with occult HBV infection, except for high anti-HBc titer and/or coinfection with HCV genotype 1 (1a or 1b). In general, occult HBV infection did not appear to have significant clinical implications. However, in patients in whom HBV was detected by PCR specific for the surface (S)-region, higher alanine aminotransferase levels were observed. The genotype of the occult HBV in the present study was exclusively the domestic type indigenous to Japan (genotype C), suggesting a different route of transmission for HBV in comparison to HCV and HIV in this population. ***J. Med. Virol. 73:195–199, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: hemophilia; hepatitis B virus; occult infection; genotype; route of transmission

INTRODUCTION

Patients with hemophilia are at high risk of infection with parenterally transmissible viruses due to the frequent use of blood products. High prevalence of infection with hepatitis C virus (HCV) [Makris et al., 1990; Troisi et al., 1993], human immunodeficiency virus (HIV) [Tsuchie et al., 1985; Kroner et al., 1994], and GB virus C (GBV-C) [Hanley et al., 1998; Toyoda et al., 1998] has been reported. A few studies have also been carried out on hepatitis B virus (HBV) infection in this population by serological evaluation [Kumar et al., 1993; Goedert et al., 2002].

Recently, several studies have reported occult HBV infection in subjects without HBV surface (HBs) antigen (HBsAg), and its clinical implications are suggested [Cacciola et al., 1999; Brechot et al., 2001; Torbenson and Thomas, 2002]. In addition to a history of frequent use of blood products, a large number of hemophilia patients are infected with HIV, which can result in dynamic changes in the immune status. Reactivation of HBV in association with changes in the immune status can occur and can cause liver damage, which sometimes results in liver failure [Xunrong et al., 2001]. Thus, hemophilia patients are a population in which the assessment of occult HBV infection is important, especially for those patients with HIV.

In the present study, we attempted to clarify the prevalence and clinical importance of occult HBV infection in the Japanese hemophilia patients without HBs antigen. As a result, occult HBV infection was found in around one-half of the patients.

PATIENTS AND METHODS

Patients

Among 44 patients with hemophilia who had been followed-up as outpatients at Nagoya University Hospital and who were admitted regularly to the hospital during 2002, 1 patient had HBs antigen and the other

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Accepted 18 February 2004
DOI 10.1002/jmv.20075
Published online in Wiley InterScience (www.interscience.wiley.com)

43 patients were negative for HBs antigen. These 43 patients were enrolled in this study. All were males with a mean age of 34.0 ± 12.1-years-old. Thirty-four patients had hemophilia A, and the remaining 9 had hemophilia B. Thirty-seven patients had severe, 4 patients had moderate, and 2 patients had mild hemophilia. All 24 patients with HIV infection were receiving HAART therapy at the time of sampling serum. Eight of 38 patients with HCV infection had a history of interferon therapy, but no patients were treated with interferon at the time serum was sampled.

Evaluation for Coinfection With HIV and HCV

HIV infection was confirmed by anti-HIV1 antibody detected by particle agglutination (SERODIA-HIV, Fuji Rebio, Tokyo, Japan). Serum HIV RNA concentration was measured by the Amplicor HIV Monitor test (Roche Diagnostics K.K., Tokyo, Japan). The presence of HCV was confirmed by both an HCV antibody assay (2nd generation, Dinabot; Tokyo, Japan), and detection of HCV RNA by nested reverse transcription-polymerase chain reaction (RT-PCR) [Okamoto et al., 1990]. HCV genotypes, according to Simmonds et al. [1994] classification, were determined by RT-PCR with genotype-specific primers [Okamoto et al., 1996]. Serum HCV RNA concentrations were measured by Amplicor Monitor assay (Roche Diagnostics K.K., Tokyo).

Serological Tests for HBV Infection and Detection of HBV DNA

HBV serum markers (HBs antigen, HBs antibody, and HBV core [HBc] antibody) were examined by means of commercial immunoenzyme assays (Abbott Laboratories, North Chicago, IL). For detection of HBV DNA, extracted DNA was amplified by nested touchdown PCR [Don et al., 1991] with three independent primer sets specific for HBV surface (s)–(sense: 5′-CTCTTGTCCTCCAATTTGTCCT-3′ and antisense: 5′-CAGCAAAGCCCAAAAGACCCAC-3′ for the first PCR, and sense: 5′-AGGTA-TGTTGCCCGTTTGTCCT-3′ and antisense: 5′-GGGTTTAAATGTATACCCA-3′ for the second PCR), core (c)–(sense: 5′-ACTGTTCAAGCCTCCAAGCT-3′ for the first and second PCR, antisense: 5′-GGAATACTAACATTGAGATTCCCGAG-3′ for the first PCR, and antisense: 5′-AGTGCGAATCCACACTC-3′ for the second PCR), and X-regions (sense: 5′-TGCCAAGTGTTTGCTGACGC-3′ for the first PCR, sense: 5′-CTGCCGATCCATACTGCGGAAC-3′ for the second PCR, and antisense: 5′-TTCCTGCAGTGGAGACCACCGTGAACG-3′ for the first and second PCR). HBV DNA was amplified from 100 ng of extracted DNA in a total volume of 50 μl, in the presence of 10 pmol of each primer, 125 μM dNTP, and 2.5 U Taq polymerase (Toyobo, Tokyo, Japan). PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). The PCR program consisted of 20 cycles of denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec with a 0.5°C decrease per one cycle (55.5°C at final cycle), and extension at 70°C for 3 min with an initial denaturation at 94°C for 1 min, and a subsequent 20 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 70°C for 3 min with a final extension at 70°C for 10 min. The same PCR program was used for both the first and second PCR amplifications. Amplified PCR products were analyzed by electrophoresis on 1.0% agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia, Buckinghamshire, UK). The amplified products were detected by hybridization with a specific probe based on the entire HBV sequences. This probe was generated with a DIG DNA Labeling and Detection kit (Roche Diagnostics, Mannheim, Germany). Results were considered to be valid only if identical results were obtained in at least two separate experiments.

The patients who were positive for both the S- and C-regions were considered to be positive for HBV. The patients who were positive for only one of the two regions examined were then referred to the result of PCR specific for the X-region, and HBV infection was confirmed according to this result.

Genotyping of HBV DNA

Genotyping of occult HBV was performed in seven patients in whom HBV DNA was detected by PCR specific for the S-region. For genotyping of HBV DNA, the PCR product from amplification of the S-region was sequenced directly, and phylogenetic analysis was performed with the neighbor-jointing and bootstrap methods.

The entire protocol was approved by the hospital ethics committee and carried out in compliance with the Helsinki Declaration.

RESULTS

Serological Prevalence of Hepatitis B Viral Markers

Among the 43 patients with hemophilia but without HBs anitgen, HBs antibody was detected in 27 patients (62.8%), and HBc antibody was detected in 37 patients (86.0%). There was no correlation between the type and/or severity of hemophilia and serological prevalence of HBV. There was no association between coinfections with HIV and/or HCV and HBV serological status.

Detection of HBV DNA in Serum

HBV DNA was detected in 22 of the 43 hemophilia patients (51.2%). HBV was detected by PCR specific for the S-region in 7 patients and by PCR specific for the C-region in 19 patients. In 4 patients HBV DNA was detected by both methods. In the 18 patients in whom HBV DNA was detected by only one of the PCR methods, additional PCR targeting the X-region was performed and all patients were positive for this region.

We compared the background, serological markers for HBV, and coinfection status between HBV DNA-positive and -negative patients (Table I). There were no significant differences in the background of patients, including age, and type or severity of hemophilia. Both

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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Journal of Medical Virology 74:54–61 (2004)

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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Accepted 20 April 2004
DOI 10.1002/jmv.20145
Published online in Wiley InterScience (www.interscience.wiley.com)

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

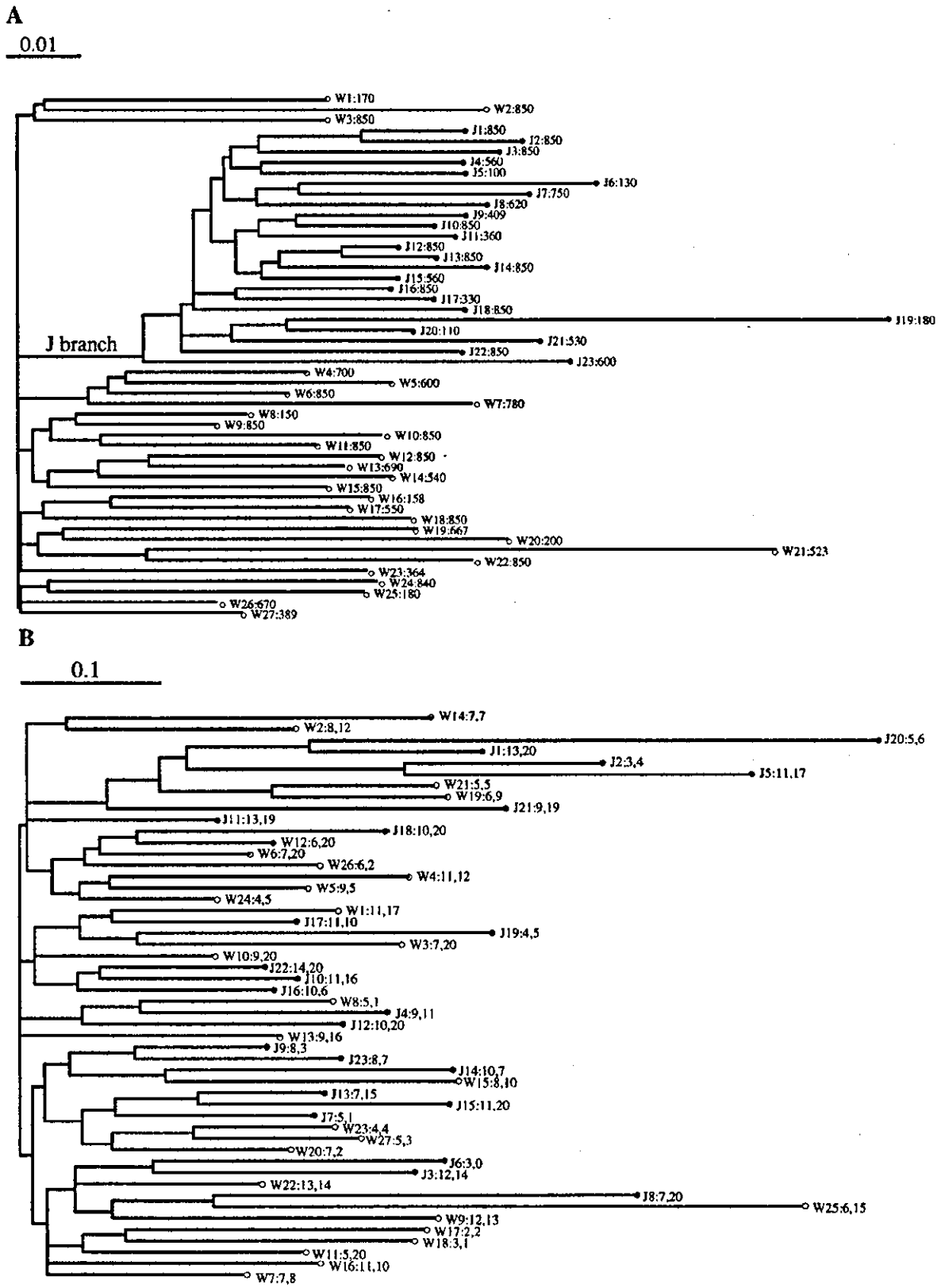
A
0.01
W1:170
W2:850
W3:850
J1:850
J2:850
J3:850
J4:560
J5:100
J6:130
J7:750
J8:620
J9:409
J10:850
J11:360
J12:850
J13:850
J14:850
J15:560
J16:850
J17:330
J18:850
J19:180
J20:110
J21:530
J22:850
J23:600
J branch
W4:700
W5:600
W6:850
W7:780
W8:150
W9:850
W10:850
W11:850
W12:850
W13:690
W14:540
W15:850
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.

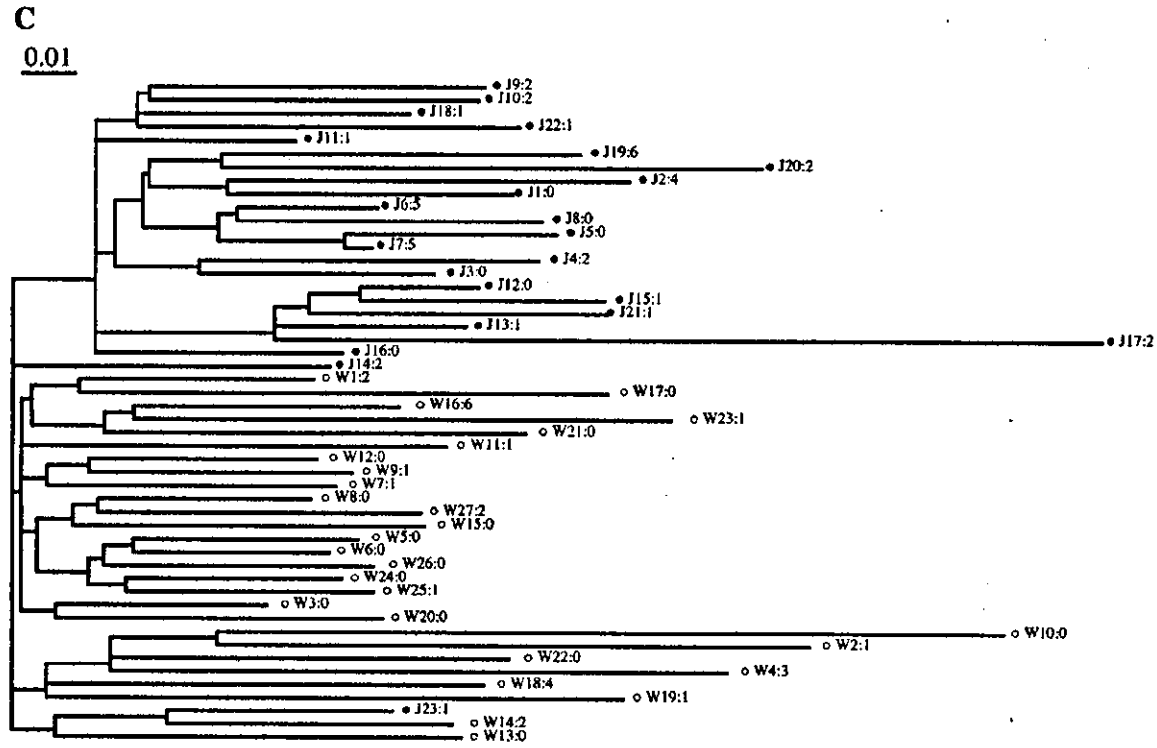

Fig. 1. A: Phylogenetic analysis and subtype determination. Classification of J and W types was performed as described in "Materials and Methods." Fifty chronic hepatitis patients with HCV genotype 1b were classified into 23 J type patients (closed circles; J1–J23) and 27 W type patients (open circles; 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*
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.
*$P < 0.05$.

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.
$^aP < 0.05$.
$^bP < 0.01$.

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

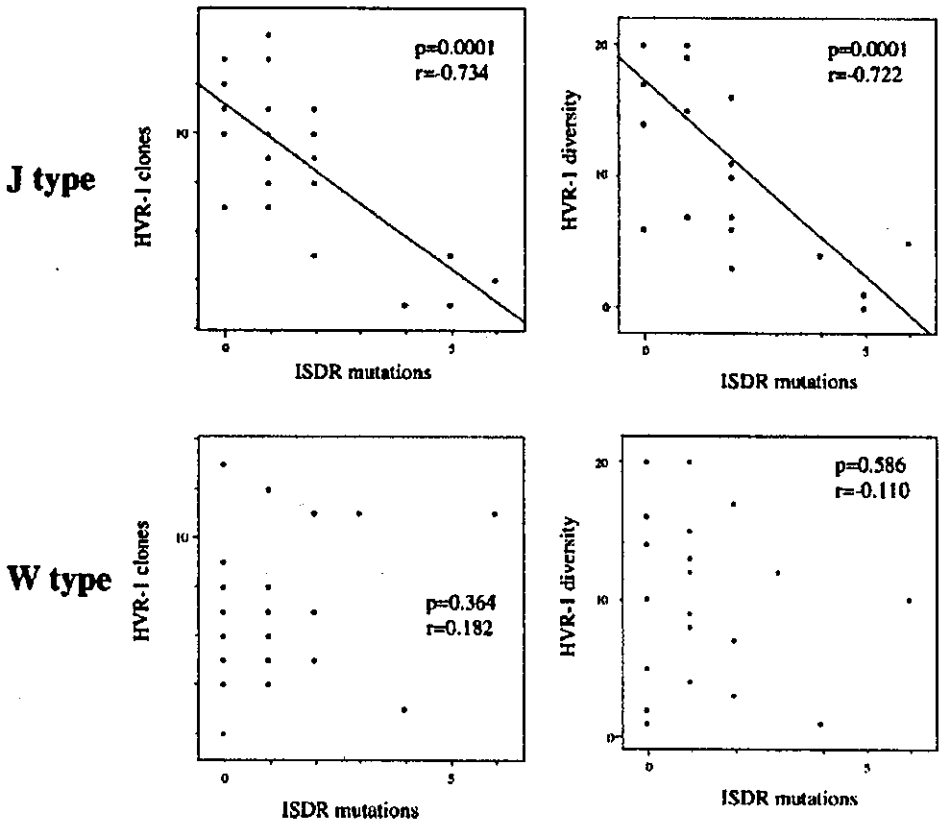

Fig. 2. Correlation between HVR-1 complexity and ISDR mutations. Scattergrams of HVR-1 clone number (left panels, vertical axes) and diversity (right panels, vertical axes), and ISDR mutation number (horizontal axes) for J type (upper panels) and W type (lower panels) HCV 1b are shown. Correlation values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

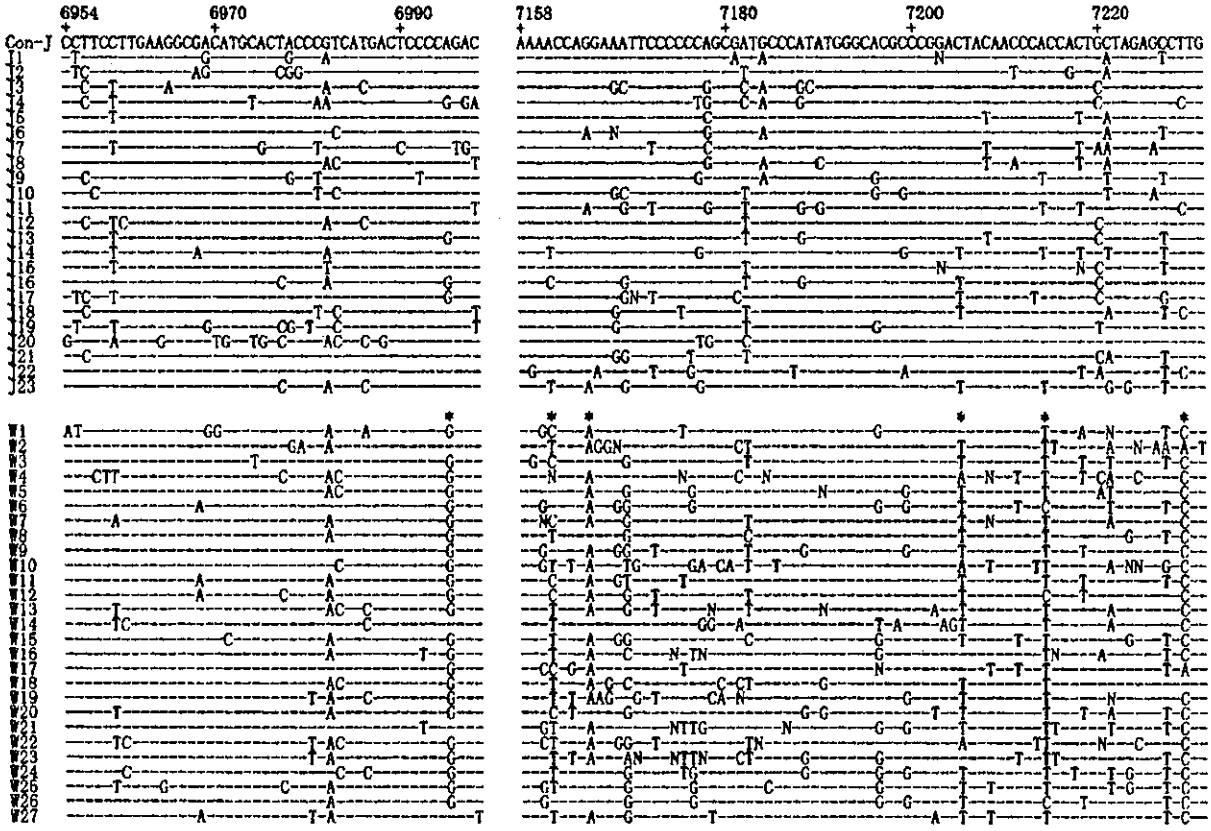

Fig. 3. Nucleotide sequence alignments of the NS5A region. The relatively conserved regions are removed. The top sequence, Con-J, is a consensus sequence deduced from J type patients J1–J23. Dashes indicate nt residues identical to those of the Con-J sequence. The six stars (*) indicate the nucleotide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

ACKNOWLEDGMENTS

We thank Ms. Matsumi Hata for her technical assistance with sequencing.

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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.

Received 11 October 2004; accepted 17 November 2004; electronically published 18 February 2005.

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Clinical Infectious Diseases 2005; 40:e49–54

1058-4838/2005/4006-00E1$15.00

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-

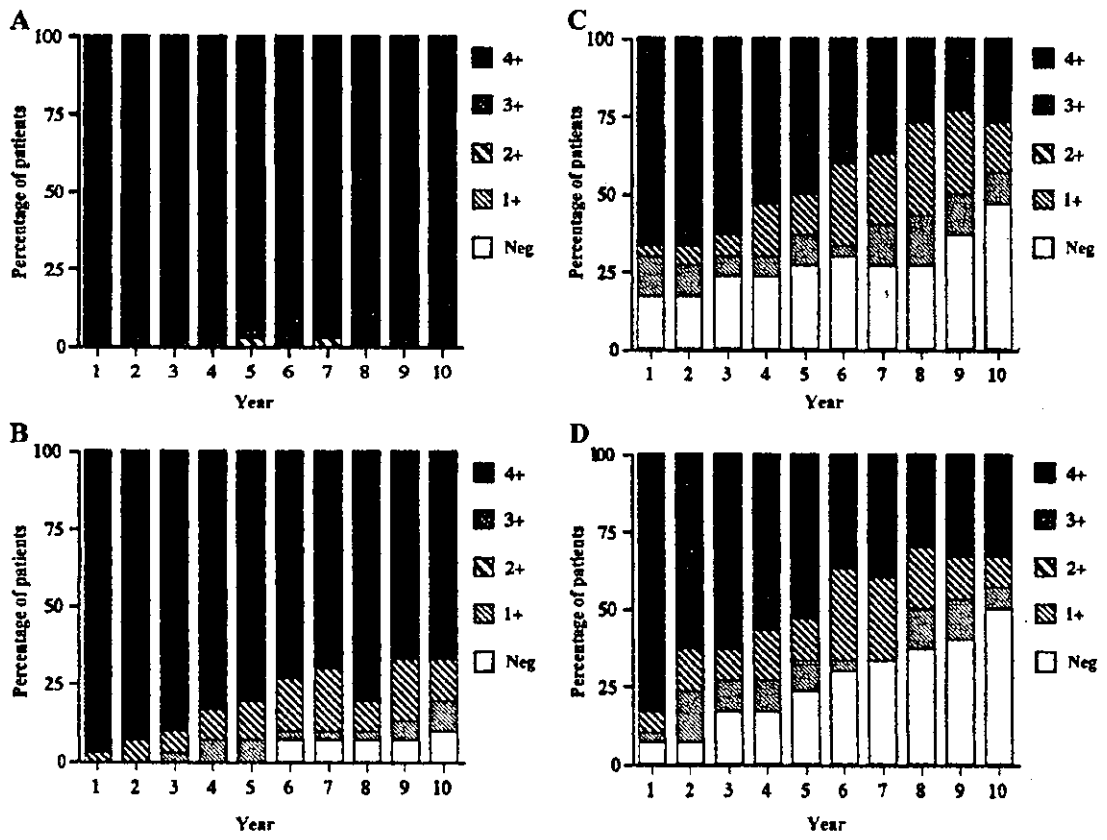

Figure 1. Annual changes in semiquantitative titer of antibody against hepatitis C virus (HCV) after eradication of HCV, as measured by third-generation recombinant immunoblot assay (Chiron RIBA HCV Test 3.0; Chiron). Antibody titers were graded as negative, 1+, 2+, 3+, and 4+, according to the manufacturer's 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.

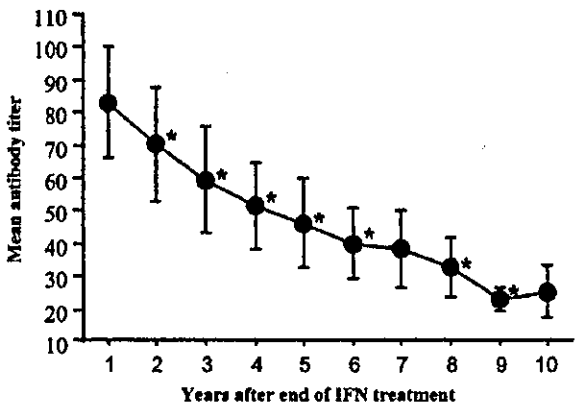

Figure 2. Annual changes in titer of antibody specific for hepatitis C virus (HCV) core protein (c22-3). *$P < .05$ by Student's t test in comparison with the previous year.

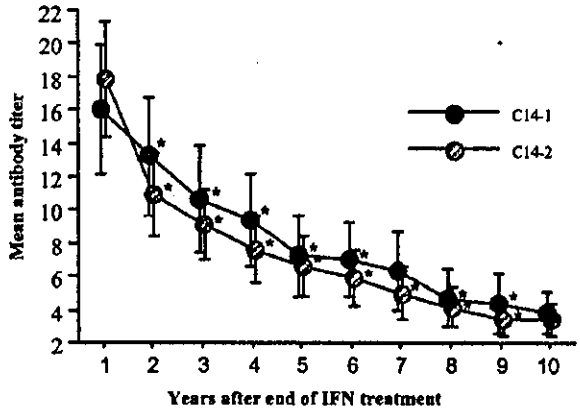

Figure 3. Annual changes in titers of genotype-specific antibodies against hepatitis C virus (HCV) NS4 protein (C14-1 and C14-2) after eradication of HCV. *$P < .05$ by Student's t test in comparison with the previous year.

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.