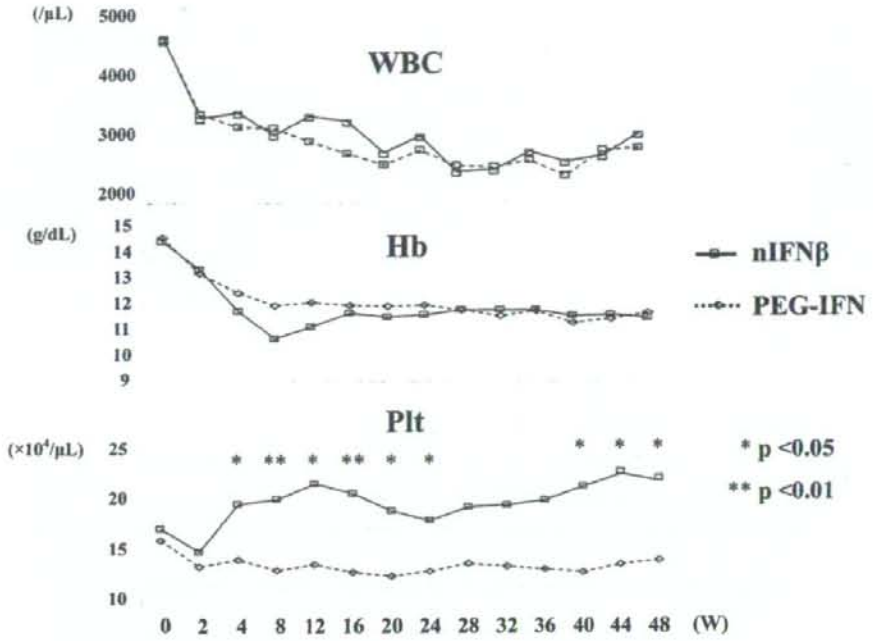

Figure 1. **Mean leukocyte count, hemoglobin concentration and platelet count during the 48 weeks of treatment of patients with chronic HCV infection, genotype 1b and high viral load. Solid lines: nIFNβ plus RBV group (group A), broken lines: PEG-IFN plus RBV group (group B). P values by Mann-Whitney U test.**

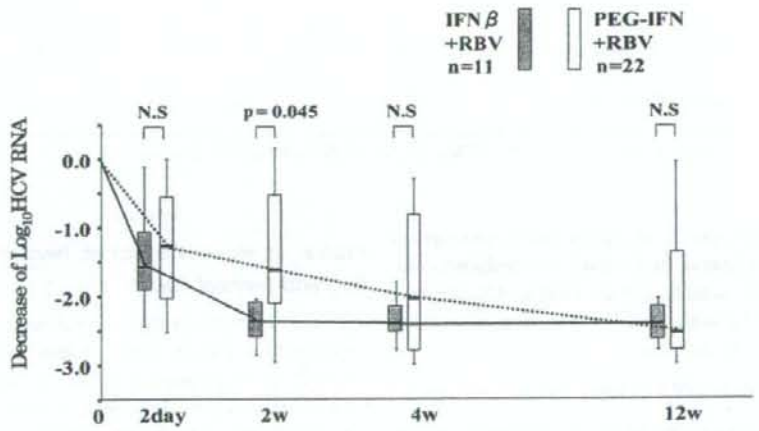

Figure 2. **Log changes in viral load from baseline during the initial 12 weeks of treatment in patients with chronic HCV infection, genotype 1b and high viral load. Gray-boxes and solid lines: nIFNβ plus RBV group (group A), light gray filled boxes and broken lines: PEG-IFN plus RBV group (group B). Bars within the boxes indicate the median value of log changes in viral load. The boxes denote the 25 to 75 percentiles, the lower and upper bars the 10 and 90 percentiles, respectively.**

Early viral kinetics

Figure 2 shows the early viral kinetics in groups A and B. Viral load was assessed at day 2, week 2, week 4 and week 12. At week 2, the median decrease in log viral load in group A was more than in group B (median, -2.34 logs vs. -1.80 logs, respectively; p = 0.045). However, the median decrease in log viral load was not significant between groups

A and B at other points.

Discussion

The frequent occurrence of psychiatric illness as a side effect of IFNα is well known; the reported incidences of IFNα- and IFNβ-induced depression range from 22 to 35% (1-3) and 10 to 21% (20-23), respectively. In general, however, IFNβ is a safe drug, but there is little or no information on its effects on patients with chronic HCV infection and depression or those with IFNα-induced depression.

In the present study, among 8 patients who developed mental disorders, only one patient stopped the therapy due to exacerbation of depression. One patient received antidepressant medication, 2 patients received anti-anxiety drugs at the start of the treatment. Moreover, during the therapy, 1 patient received anti-anxiety drugs and another one received an anti-depressant. The remaining 3 patients did not need any medications. Recently, Schaefer et al (24) reported that among 22 psychiatric patients treated with PEG-IFN plus RBV, 3 (13.6%) required antidepressants at the start of the treatment, 15 (68.2%) received antidepressants during anti-HCV therapy, and 1 (4.5%) discontinued PEG-IFN plus RBV treatment due to psychiatric disorders. The results of the above study and those of the present study indicate that the dropout rate due to psychiatric illness of patients treated with IFNβ is similar to that in patients on PEG-IFN. However, in our study, weaker anti-anxiety drugs were used during IFNβ therapy compared with the above report, suggesting that IFNβ-induced mental disorders are milder than those induced by PEG-IFN. However, the sample size of the present study is too small to make a firm conclusion and further studies are needed to specifically compare these two agents.

In the present study, the platelet count during administration of IFNβ plus RBV increased above baseline after week 4. A previous study reported that platelet count did not increase above the baseline during administration of nIFNβ monotherapy (21). In our study, at week 4, 64% (7/11) of patients were switched to 3 times a week administration. The increased platelet count after week 4 might be due to this switching and thus related to the study protocol. Interestingly, another previous study reported that platelet count increased after the time of daily administration of IFNα, but not above the baseline value (25). Such an increase of the platelet count above baseline after week 4 may be specific to only nIFNβ plus RBV combination therapy. Although the exact mechanism is not clear at this stage, nIFNβ plus RBV therapy may be useful for patients with thrombocytopenia who are not suitable for PEG-IFN plus RBV therapy. Further studies are required to clarify the mechanism of increased platelet count during nIFNβ plus RBV therapy.

The reported SVR rate of patients with genotype 1b and high viral load treated with nIFNβ monotherapy ranges from 0 to 11% (11, 12). Recent trials of rhIFNβ-1a plus RBV reported improved SVR rate. For example, the SVR reported by Chan et al (14) was 32.7% (18/55) in their patients with genotype 1b and high viral load treated with 44 μg rhIFNβ-1a (equivalent to 12 MU) 3 times weekly plus RBV for 24 weeks. Furthermore, the SVR reported by Pellicano et al (13) was 12.1% (4/33) in their patients with genotype 1 (viral titer was not mentioned) treated with 22 μg rhIFNβ-1a daily plus RBV for 24 weeks. In the present study, the SVR rate in our patients with genotype 1b and high viral load treated with 6 MU nIFNβ plus RBV for 48 weeks was 27% (3/11). This result ranked well with the above previous reports. We performed case-control study in patients treated with PEG-IFN plus RBV and the SVR rate was 41% (9/22). Although the SVR rate of the case group was lower than that of the control group, the difference was not statistically significant, suggesting that nIFNβ plus RBV combination therapy for 48 weeks is also efficacious.

The viral load of the case group decreased rapidly at week 2; the log drop was greater than that of the control group. However, after week 2, there were no differences in the rate of drop of viral load between the two groups at weeks 4 and 12. In the case group, nIFNβ was administrated daily for 2 weeks in 6 patients, for 4 weeks in 1 patient and for 8 weeks in 4 patients. These viral kinetics suggest that daily administration of nIFNβ plus RBV is more effective against HCV than PEG-IFN plus RBV, and that three times a week nIFNβ administration regimen might be less effective than PEG-IFN plus RBV. The fact that none of the patients dropped out in our study even during the 8-week daily administration of nIFNβ, suggests good tolerability of the combination therapy. Prolongation of daily administration might improve treatment outcome of nIFNβ plus RBV. However, our results, based on a small sample size, need to be confirmed in another large-scale study, including determination of the most appropriate duration of daily administration.

In conclusion, nIFNβ plus RBV therapy carries sufficient tolerability and efficacy in patients with mental disorders. nIFNβ plus RBV could be considered an efficacious and safe second-line therapy for subpopulations of patients who are not eligible for PEG-IFN plus RBV. Further studies in a larger group of will be necessary.

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Correlation Between Serum Hepatitis B Virus Core-Related Antigen and Intrahepatic Covalently Closed Circular DNA in Chronic Hepatitis B Patients

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Nucleos(t)ide analogues are utilized for the treatment of chronic HBV infection, and HBe seroconversion and HBV DNA levels are commonly used as markers of viral status and as primary treatment endpoints. Recently, a new assay was prepared for the detection of serum HBV core-related antigen (HBcrAg), consisting of HBcAg, HBeAg, and p22cr, which is a precore protein from amino acid −28 to at least amino acid 150, by coding the precore/core region. In this study, we examined the correlation between serum HBcrAg concentration and viral status by the analysis of serum HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic covalently closed circular DNA (cccDNA) in 57 chronic hepatitis B patients. Intrahepatic cccDNA was detected in all 57 patients, 42 patients were HBcrAg-positive, and serum HBcrAg concentration level was closely correlated with cccDNA. Additionally, positive HBcrAg concentration level results were observed in 6 out of 13 HBsAg seroclearance patients and 20 out of 31 HBV DNA-negative patients. Moreover, the correlation between HBcrAg and cccDNA in these 31 HBV DNA-negative patients was statistically significant ($r = 0.482$, $P = 0.006$). These data suggest that serum HBcrAg concentration is well correlated with intrahepatic cccDNA level, and that the measurement of serum HBcrAg may be clinically useful for monitoring intrahepatic HBV viral status, especially in patients under treatment with nucleos(t)ide analogues. ***J. Med. Virol. 81:27–33, 2009.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: HBV DNA; HBcrAg; cccDNA; HBsAg; lamivudine; entecavir

INTRODUCTION

Hepatitis B virus (HBV) is an important causative agent for liver disease such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Recently, several nucleos(t)ide analogues such as lamivudine [Dienstag et al., 1995], adefovir dipivoxil [Chin et al., 2001], and entecavir [Colonno et al., 2001] have been found to consistently produce rapid and dramatic decreases in viremia [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. For the serological monitoring of chronic hepatitis patients under treatment with nucleos(t)ide analogues, improvement of alanine transaminase level, seroconversion from HBe antigen (HBeAg)-positive to anti-HBe antibody (HBeAb)-positive, and peripheral HBV DNA concentration are used as markers in chronic active hepatitis, and both HBeAg seroconversion and HBV DNA levels below the detection limit and/or of 10^5 copies/ml are commonly used as primary treatment endpoints [Lok et al., 2004]. In addition, HBV surface antigen (HBsAg) seroclearance has been linked to a good prognosis, including improvement of liver histopathology and liver function, and prolongation of survival [Arase et al., 2006], although spontaneous HBsAg seroclearance and/or remission occurred in only a small proportion of patients during the natural history of chronic HBV infections.

However, a major problem with long-term lamivudine treatment is the potential development of drug-resistance, mainly caused by the mutation of the YMDD motif of reverse transcriptase [Chayama et al., 1998]. We previously reported the efficacy of lamivudine therapy and factors associated with the emergence of resistance in chronic HBV infection in Japan [Suzuki et al., 2003].

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The measurement of the predictive serum markers of residual intrahepatic HBV DNA and/or covalently closed circular DNA (cccDNA), which is intrahepatic HBV replicated intermediate, is more important than the measurement of peripheral HBV DNA for monitoring the viral status of hepatitis patients [Sung et al., 2005]. Additionally, the amount of cccDNA in serum is reported to be higher in patients who develop YMDD mutants than in patients who do not [Yuen et al., 2005]. Recently, it was established that HBV RNA is detectable in serum and the elevation of HBV RNA is a predictor of early occurrence of viral mutation during lamivudine therapy [Rokuhara et al., 2006; Hatakeyama et al., 2007]. However, these HBV DNA, HBV RNA, or cccDNA detection assay methods remain complicated and difficult to perform. Therefore, simple methods of viral status evaluation are required for routine assays rather than for nucleic acid assays.

Recently, a new assay was performed for the detection of hepatitis B core-related antigen (HBcrAg) consisting of HBV core antigen (HBcAg), HBeAg, and 22 kDa precore protein (p22cr) coded with precore/core gene [Kimura et al., 2002, 2005]. p22cr is a precore protein from amino acid −28 to at least amino acid 150, containing an uncleaved signal sequence and lacking the C-terminal arginine-rich domain. p22cr is found in empty and HBV DNA negative virus particles; the production of empty particles is not dependent on the formation of HBV DNA [Kimura et al., 2005]. Several reports indicate that the concentration of serum HBcrAg is closely correlated with peripheral HBV DNA in untreated patients [Rokuhara et al., 2003; Tanaka et al., 2006]. Additionally, HBcrAg is considered as a prospective marker of the appearance of drug-resistant HBV mutants and of the identification of patients with low risk of HBV reactivation after discontinuation of lamivudine administration, while peripheral HBV DNA does not qualify as a prospective marker in these patients [Rokuhara et al., 2005; Shinkai et al., 2006; Tanaka et al., 2006; Matsumoto et al., 2007]. The relationship between HBcrAg and intrahepatic cccDNA levels has not yet been clarified.

In this study, we examined the correlation between HBcrAg and viral status by the analysis of HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic cccDNA in patients with chronic hepatitis B.

MATERIALS AND METHODS

Patients and Samples

Serum samples and biopsy specimens were obtained from 57 chronic hepatitis B patients at Toranomon Hospital under informed consent. The median age of patients was 49 (range, 25–71 years). Out of 57 patients, 28 underwent nucleos(t)ide analogue administration (17 patients of lamivudine, 7 patients of both lamivudine and adefovir dipivoxil, 4 patients of entecavir), and 13 were HBsAg-negative/HBs-seroclearance patients with more than 12 months of being HBsAg-positive before HBs-seroclearance.

Routine Laboratory Tests

HBsAg, HBeAg, and HBeAb were routinely measured by the commercially available Chemiluminescent Enzyme Immunoassay (CLEIA) (Lumipulse System, Fujirebio, Inc., Tokyo, Japan).

HBcrAg Test

Serum HBcrAg was measured by CLEIA HBcrAg assay kit (Fujirebio, Inc.) with a fully automated analyzer system (Lumipulse System, Fujirebio, Inc.). Briefly, 150 μl of serum was incubated with 150 μl of pretreatment solution containing 15% sodium dodecyl sulfate at 60°C for 30 min. After heat treatment, 120 μl of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61, and HB114) against denatured HBcAg, HBeAg, and p22cr. After 10 min incubation at 37°C and washing, further incubation was done for 10 min at 37°C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg, and p22cr. After washing, 200 μl of substrate solution [AMPPD: 3-(2′-spiroadamantan)-4-methoxy-4- (3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA) was added to the test cartridge which was then incubated for 5 min at 37°C. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg (amino acids: −10 to 183 of precore/core gene product). HBcrAg concentration was expressed as units/ml (U/ml), which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, HBcrAg value was expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analysis, HBcrAg-negative cases were calculated as 3.0 log U/ml.

HBV DNA Assay

HBV DNA in serum was measured by polymerase chain reaction (PCR) assay kit (Amplicor HBV monitor test, Roche Molecular Systems, Inc., Branchburg, NJ). Values under or over the detection range were calculated as 2.6 log copies/ml or as 7.6 log copies/ml, respectively.

Measurement of cccDNA

Liver biopsy specimens were taken and stored at −80°C before DNA extraction. HBV DNA was extracted using QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on absorbance at 260 nm. For this study, two oligonucleotide primers, cccF2 (5′-cgtctgtgccttctcatctga-3′, nucleotides 1,424–1,444), cccR4 (5′-gcacagcttggaggcttgaa-3′, nucleotides 1,755–1,737), and a probe cccP2 (5′-VIC-accaatttatgcctacag-MGB-3′, nucleotides 1,672–1,655), were designed using Primer Express™ software (Applied Biosystems, Foster City, CA) to flank the direct

repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4 oligonucleotide primers spanning the direct repeat region of the HBV genome allows the PCR of native viral DNA in the Dane particle to block the amplification of products, because the partially double-stranded HBV DNA is disrupted in the direct repeat region [Mason et al., 1998]. Twenty-five microliters of the extracted DNA (0.5 μg) were detected with the sequence detector system (ABI 7900HT, Applied Biosystems) in 50 μl of a PCR mixture containing TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nmol of each primer, and 250 nmol of the probe. After initial activation of uracil-*N*-glycosylase at 50°C for 2 min, AmpliTaq Gold was activated at 95°C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 90 sec per cycle (SRL, Inc., Tokyo, Japan).

Statistical Analysis

The statistical analysis of the correlation data between serum HBcrAg, HBsAg, HBV DNA, and/or cccDNA was performed by SPSS software (version 14.0J, SPSS Japan Inc., Tokyo, Japan), and the statistical significance between the two sides was taken as *P*-value lower than 0.05.

RESULTS

Serological and Genetic Assay Results

We classified the 57 patients according to assay results of HBsAg, HBeAg, serum HBV DNA, intrahepatic HBV cccDNA, and serum HBcrAg. Positive results were observed with all 57 patients in the cccDNA assay, in 44 patients with the HBsAg test, in 16 patients with the HBeAg test, in 26 patients with the HBV DNA assay, and in 42 patients with the HBcrAg assay (Table I). Among the 13 patients with negative results with HBsAg, HBeAg, and HBV DNA but positive results with cccDNA assay, six patients showed HBcrAg-positive results, although the serum HBcrAg concentration value was low (mean value ± standard deviation: 3.23 ± 0.27 log U/ml) in comparison to that of the group with positive results with HBsAg, HBeAg, and HBV DNA (6.91 ± 1.06 log U/ml) tests. Among the 28 patients with HBsAg-positive but HBeAg-negative results, 20 patients were HBcrAg-positive and 10 out of these 20 patients showed negative HBV DNA assay results.

Next, assay results were analyzed according to presence/absence of nucleos(t)ide analogue treatment, HBsAg-positive/negative results, and HBeAg-positive/negative results by the combination with treated/untreated subgroups (Table II). When patients were classified into two groups, namely 28 patients treated with nucleos(t)ide analogues and 29 untreated patients, no difference was observed in average mean value of HBcrAg, HBV cccDNA, and HBV DNA (data not shown). However, since the 13 HBsAg-negative patients were clinically stable, we further analyzed the 44 HBsAg-positive patients by grouping them according to pres-

TABLE I. Summary of HBcrAg Concentration in Positive and/or Negative Patients by HBsAg, HBeAg, HBV DNA, and HBcrAg Assay

All cases		Classified by assay results with:							
		HBsAg		HBeAg		HBV DNA		HBcrAg	
Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)
All (N = 57)	4.61 (1.64)	Positive (N = 44)	5.05 (1.62)	Positive (N = 16)	6.53 (1.14)	Positive (N = 12)	6.91 (1.06)	Positive (N = 12)	6.91 (1.06)
								Negative (N = 0)	
						Negative (N = 4)	5.40 (0.38)	Positive (N = 4)	5.40 (0.38)
								Negative (N = 0)	
				Negative (N = 28)	4.20 (1.18)	Positive (N = 14)	4.29 (1.20)	Positive (N = 10)	4.81 (1.02)
								Negative (N = 4)	<3.00
						Negative (N = 14)	4.11 (1.20)	Positive (N = 10)	4.55 (1.14)
								Negative (N = 4)	<3.00
		Negative (N = 13)	3.11 (0.21)	Positive (N = 0)		Positive Negative (N = 0)		Positive Negative (N = 0)	
				Negative (N = 13)	3.11 (0.21)	Positive (N = 0)		Positive Negative (N = 0)	
						Negative (N = 13)	3.11 (0.21)	Positive (N = 6)	3.23 (0.27)
								Negative (N = 7)	<3.00

Mean value: log U/ml ± Standard deviation.

TABLE II. Classification of HBcrAg, cccDNA and HBV DNA Assay Results According to Presence/Absence of Treatment, HBeAg Test and HBsAg Test

Item	Category	N	HBcrAg (log U/ml) Mean (SD)	HBcrAg (log U/ml) P value	cccDNA (log copy/mg) Mean (SD)	cccDNA (log copy/mg) P value	HBV DNA (log copy/ml) Mean (SD)	HBV DNA (log copy/ml) P value
Unclassified	All	57	4.61 (1.64)		4.25 (0.91)		3.67 (1.59)	
Treatment[a]	With	26	4.77 (1.49)	NS[c]	4.41 (0.68)	NS[c]	3.20 (1.08)	<0.001
	Without	18	5.45 (1.75)		4.54 (1.09)		5.13 (1.78)	
HBsAg	Positive							
	Total	44	5.05 (1.62)		4.46 (0.87)		3.99 (1.69)	
	Negative			<0.001		0.001		<0.001
	Total	13	3.11 (0.21)		3.52 (0.68)		2.60 (0.00)	
HBeAg[b]	Positive							
	Total	16	6.53 (1.14)		4.88 (1.06)		5.17 (1.92)	
	Negative			0.001		0.015		0.002
	Total	28	4.20 (1.18)		4.23 (0.64)		3.32 (1.09)	
	Positive							
	Treated	8	6.21 (1.09)	NS[c]	4.76 (0.95)	NS[c]	3.83 (1.64)	0.005
	Untreated	8	6.85 (1.18)		5.00 (1.21)		6.51 (1.02)	
	Negative							
	Treated	18	4.13 (1.16)	NS[c]	4.25 (0.49)	NS[c]	2.93 (0.57)	0.016
	Untreated	10	4.33 (1.26)		4.18 (0.88)		4.02 (1.45)	

[a]In the with/without treatment group, 44 HBsAg-positive patients were analyzed.
[b]HBsAg-positive 44 patients were further separated into HBeAg-positive and HBeAg–negative groups. In addition, these groups were further separated into treated and untreated groups; the mean value of each assay was calculated and a statistical analysis was done.
[c]NS: statistically not significant.

ence/absence of treatment of nucleos(t)ide analogues (Table II). HBcrAg concentration was 4.77 ± 1.49 log U/ml in 26 treated patients and 5.45 ± 1.75 log U/ml in 18 untreated patients (not statistically significant). Similar results in cccDNA levels were observed in both treated and untreated groups (4.41 ± 0.68 log copy/μg and 4.54 ± 1.09 log copy/μg, not statistically significant). In contrast, lower HBV DNA was observed in the treated group (3.20 ± 1.08 log copy/ml) as compared with the untreated group (5.13 ± 1.78 log copy/ml, $P < 0.001$).

Statistically significant results were observed under grouping according to HBsAg assay results; namely, HBcrAg was 5.05 ± 1.62 log U/ml in the HBsAg-positive group and 3.11 ± 0.21 log U/ml in the HBsAg-negative group ($P < 0.001$), HBV cccDNA was 4.46 ± 0.87 log copies/μg in the HBsAg-positive group and 3.52 ± 0.68 log copies/μg in the HBsAg-negative group ($P < 0.001$), HBV DNA was 3.99 ± 1.69 log copies/ml in the HBsAg-positive group and <2.60 log copies/ml in the HBsAg-negative group ($P < 0.001$).

Similar results were observed under grouping according to HBeAg results. In this analysis, HBsAg-negative patients were omitted because HBeAg-negative patients included both HBsAg-positive/negative patients, whereas all HBeAg-positive patients were HBsAg-positive. The mean values of HBcrAg, HBV cccDNA, and HBV DNA in the HBeAg-positive group were higher than those of the HBeAg-negative group; namely, HBcrAg concentration was 6.53 ± 1.14 log U/ml in the HBeAg-positive group and 4.20 ± 1.18 log U/ml in the HBeAg-negative group ($P < 0.001$), cccDNA was 4.88 ± 1.06 log copy/μg in the HBeAg-positive group and 4.23 ± 0.64 log copies/μg in the HBeAg-negative group ($P = 0.015$), HBV DNA was 5.17 ± 1.92 log copies/ml in the HBeAg-positive group and 3.32 ± 1.09 log copies/ml in the HBeAg-negative group ($P = 0.002$). When the HBeAg-positive group was further separated into treated and untreated groups, these three markers in eight treated patients were lower than in eight untreated patients; namely, HBcrAg (6.21 ± 1.09 log U/ml vs. 6.85 ± 1.18 log U/ml), cccDNA (4.76 ± 0.95 log copy/μg vs. 5.00 ± 1.21 log copy/μg), and HBV DNA (3.83 ± 1.64 log copy/ml vs. 6.51 ± 1.02 log copy/ml), although HBcrAg and cccDNA values between the treated and untreated groups were not statistically significant. In the HBeAg-negative group, statistically significant ($P = 0.016$) lower values of HBV DNA but not of HBcrAg and cccDNA were observed in 18 treated patients by comparison with 10 untreated patients. The HBV DNA level of 8 out of 10 untreated patients was less than 5 log copies/ml. These patients underwent a liver biopsy for progress follow-up. Therefore, there was a relatively small difference in HBV DNA level between treated and untreated patients.

Correlation Between HBcrAg, HBV DNA and/or cccDNA

The correlation between HBcrAg, HBV DNA, and/or cccDNA in all 57 patients was summarized in Figure 1. A statistically significant positive correlation was observed in all analyses, namely HBcrAg versus HBV cccDNA (Fig. 1a, $r = 0.692$, $P < 0.001$), HBcrAg versus HBV DNA (Fig. 1b, $r = 0.713$, $P < 0.001$), and HBV cccDNA versus HBV DNA (Fig. 1c, $r = 0.637$, $P < 0.001$).

Next, HBcrAg concentration in 31 HBV DNA-negative patients was measured; 20 patients showed levels greater than 3.0 log U/ml. A statistically significant correlation between HBcrAg and cccDNA in these 31 patients was observed (Fig. 2, $r = 0.482$, $P = 0.006$),

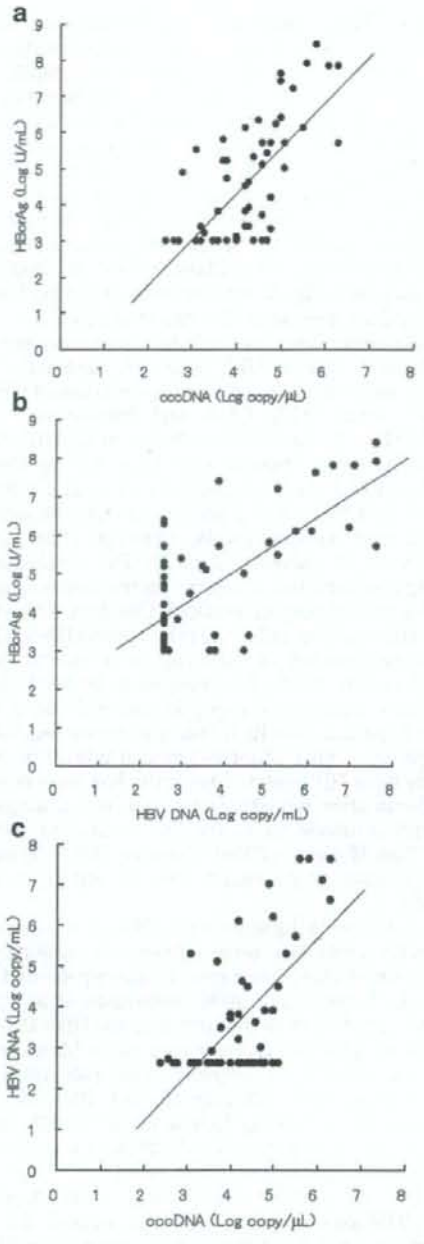

Fig. 1. Correlation between serum HBcrAg and intrahepatic HBV cccDNA in 57 patients with chronic hepatitis B (**a**: $y = 1.25 \times -0.69$, $r = 0.692$, $P < 0.001$), HBcrAg and serum HBV DNA (**b**: $y = 0.74 \times +1.91$, $r = 0.713$, $P < 0.001$), and serum HBV DNA and intrahepatic cccDNA (**c**: $y = 1.11 \times -1.05$, $r = 0.637$, $P < 0.001$). Straight lines indicate the correlation between each other.

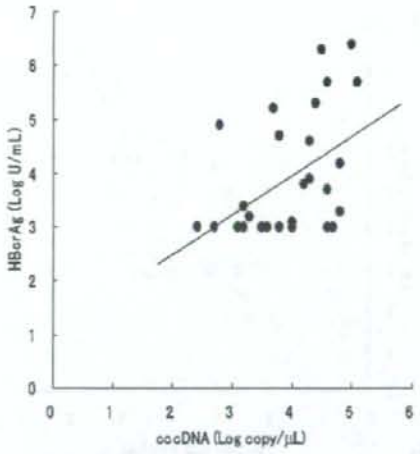

Fig. 2. Correlation between HBcrAg and cccDNA in 31 HBV DNA negative patients ($y = 0.73 \times +1.00$, $r = 0.482$, $P = 0.006$).

suggesting that HBcrAg measurement may be a useful marker in HBV DNA-negative patients as a substitute for cccDNA assay.

When 44 HBsAg positive patients were grouped according to whether they were HBeAg-positive or HBeAg-negative, HBcrAg concentration was correlated with cccDNA in both 16 HBeAg-positive patients (Fig. 3a, $r = 0.687$, $P = 0.003$) and 28 HBeAg-negative patients (Fig. 3a, $r = 0.542$, $P = 0.003$), and with HBV DNA in the HBeAg-positive (Fig. 3b, $r = 0.681$, $P = 0.004$) but not in the HBeAg-negative group (Fig. 3b, $r = 0.311$, $P = 0.107$). A positive correlation between HBV DNA and cccDNA was also observed in both the HBeAg-positive group (Fig. 3c, $r = 0.588$, $P = 0.017$) and the HBeAg-negative group (Fig. 3c, $r = 0.442$, $P = 0.018$).

DISCUSSION

Nucleos(t)ide analogues have a suppressive effect on the transcription of pregenomic RNA, and the administration of these agents can induce a rapid and dramatic decrease in peripheral HBV DNA, seroclearance of HBeAg, and remission of chronic hepatitis B [Dienstag et al., 1995, 1999; Lai et al., 1998]. However, these nucleos(t)ide analogues are unable to induce an adequate and complete elimination of HBV. Therefore, the measurement of intrahepatic HBV DNA and/or HBV cccDNA is important for monitoring the viral status of hepatitis patients [Sung et al., 2005], although these assays involve the physical stress of needle biopsy.

Several reports indicate that the level of HBcrAg, which is a complex of HBeAg, HBcAg, and p22cr coding precore/core gene [Kimura et al., 2002, 2005], reflects the natural course of viral loads in patients under treatment with nucleos(t)ide analogues, and that the reduction rate of HBcrAg is slower than that of serum

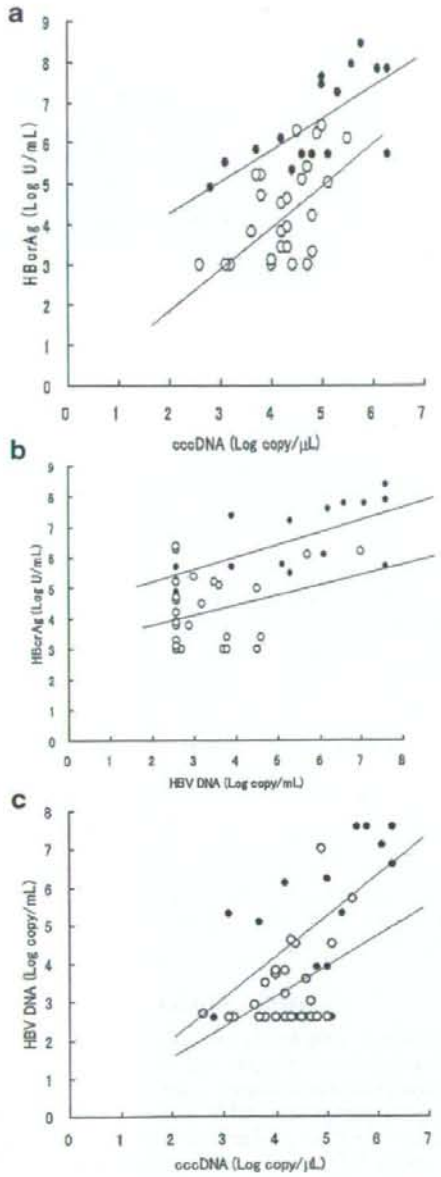

Fig. 3. Correlation between HBcrAg, cccDNA and HBV DNA in 44 HBsAg-positive with 16 HBeAg-positive and 28 HBeAg-negative patients. **a**: Correlation between HBcrAg and cccDNA (HBeAg-positive cases as closed circle; $y = 0.74 \times +2.91$, $r = 0.687$, $P = 0.003$, HBeAg-negative cases as open circle; $y = 1.00 \times -0.02$, $r = 0.542$, $P = 0.003$). **b**: Correlation between HBcrAg and serum HBV DNA (HBeAg-positive patients as closed circle; $y = 0.41 \times +4.43$, $r = 0.681$, $P = 0.004$, HBeAg-negative patients as open circle; $y = 0.34 \times +3.09$, $r = 0.311$, $P = 0.107$). **c**: Correlation between serum HBV DNA and intrahepatic cccDNA (HBeAg-positive cases as closed circle; $y = 1.07 \times -0.03$, $r = 0.588$, $P = 0.017$, HBeAg-negative patients as open circle; $y = 0.76 \times +0.12$, $r = 0.442$, $P = 0.018$).

HBV DNA [Rokuhara et al., 2003; Tanaka et al., 2006]. Similar results were observed in patients infected with genotypes B and C of HBV [Rokuhara et al., 2005]. This phenomenon may be explained by the fact that the production of HBcrAg depends on the transcription of mRNA from cccDNA, and that cccDNA still remains in high levels after treatment with these nucleos(t)ide analogues. Therefore, several reports suggest that HBcrAg may be a predicting marker for relapse after cessation of lamivudine therapy in chronic HBV infection [Shinkai et al., 2006; Matsumoto et al., 2007] and that it may also help identify patients who are at low risk of lamivudine resistance [Tanaka et al., 2006].

In this study, we analyzed the correlation between HBcrAg and several HBV markers, especially HBV cccDNA. Results indicated a good correlation of HBcrAg against serum HBV DNA and intrahepatic HBV cccDNA (Fig. 1). In addition, 20 out of 31 HBV DNA-negative patients showed more than 3.0 log U/ml in HBcrAg. All of these 20 patients were also cccDNA-positive, and there was a positive correlation between HBcrAg and cccDNA levels, although HBcrAg was negative in 11 patients (Fig. 2). The production of HBcrAg is considered to depend on the transcription of mRNA from intrahepatic cccDNA. Our data showed that serum HBcrAg may reflect intrahepatic cccDNA. Therefore, measurement of HBcrAg as a substitute for cccDNA may be useful for monitoring chronic hepatitis B patients. Recently, the acquisition of de novo HBV-related hepatitis after liver transplantation has become an important cause of morbidity and mortality. Moreover, de novo HBV-related hepatitis has been reported in patients after hematopoietic stem cell transplantation and cytotoxic chemotherapy treatment [Dhedin et al., 1998; Hui et al., 2006]. Therefore, HBcrAg may be a useful marker of occult HBV infection in these patients.

Several reports indicate that HBsAg seroclearance confers favorable long-term outcomes in patients without hepatocellular carcinoma or decompensated liver cirrhosis [Arase et al., 2006; Kobayashi et al., 2006]. However, studies show that intrahepatic HBV DNA still remains in HBsAg seroclearance cases [Arase et al., 2006], and that 10–20% of patients have 50–100 copies/ml of serum HBV DNA for 5 and 10 years after seroclearance of HBsAg [Arase et al., 2007]. In this study, 6 out of 13 patients with HBsAg seroclearance showed HBcrAg-positive results (3.23 ± 0.27 log U/ml), and all 13 patients remained cccDNA-positive (3.52 ± 0.68 log copy/μg). These data suggest that HBV remains present for a prolonged period after HBsAg seroclearance, further studies are thus necessary to clarify the mechanism of HBcrAg production and/or the regulation of mRNA in chronic hepatitis with HBsAg seroclearance.

Meanwhile, positive correlations between HBV DNA and HBcrAg were not observed in the HBsAg-positive and HBeAg-negative group (Fig. 3b), although HBcrAg concentration was correlated with cccDNA in HBeAg-negative patients (Fig. 3a). This finding shows that

measurement of HBcrAg as a substitute for cccDNA may be useful for monitoring patients in HBsAg-positive and HBeAg-negative groups.

In conclusion, serum HBcrAg concentration appears to be well correlated with intrahepatic cccDNA level, and the measurement of serum HBcrAg as substitute for cccDNA and/or serum HBV DNA may be clinically useful for the monitoring of intrahepatic HBV viral status.

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Change of Hepatitis B Virus Genotypes in Acute and Chronic Infections in Japan

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During 35 years from 1971 to 2005, 153 patients with acute and 4,277 with chronic HBV infection visited the Toranomon Hospital in Tokyo, Japan. They were grouped into seven 5-year periods, and HBV genotypes/subgenotypes were determined. Patients with acute HBV infection were younger ($P = 0.046$), predominantly male ($P = 0.004$), possessed higher alanine aminotransferase levels ($P < 0.001$), positive more frequently for HBeAg ($P < 0.001$), and had lower HBV DNA loads ($P = 0.014$) than those with chronic infection. Sexual transmission was more frequent in patients with acute than chronic HBV infection (67% vs. 3%, $P < 0.001$). The number of patients with acute infection increased throughout 1971–2005. Patients with chronic infection increased since 1971, peaked in 1986–1990 and then decreased. The number of patients increased since 1990–2000 again, however, reflecting recent boost of acute HBV infection. The distribution of HBV genotypes was considerably different between patients with acute and chronic infections (A, B, and C: 28.6%, 10.3%, and 59.5% vs. 3.0%, 12.3%, and 84.5%, respectively, $P < 0.001$). Since 1991, genotype A foreign to Japan started to increase sharply in patients with acute infection, and gradually in those with chronic infection. There was a trend for the foreign subgenotype B2/Ba to increase recently ($P < 0.05$). Despite immunoprophylaxis of high-risk babies born to carrier mothers with hepatitis B e antigen, implemented nationally since 1986, acute and chronic infections with HBV have been increasing in Japan. Based on genotypes/subgenotypes changing with time, the resurgence of hepatitis B could be attributed to infections, with foreign HBV genotypes/subgenotypes, spreading swiftly by sexual contact. ***J. Med. Virol. 80: 1880–1884, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: acute hepatitis; chronic hepatitis; genotypes; hepatitis B virus: subgenotypes

INTRODUCTION

Worldwide, an estimated 350 million people are infected persistently with hepatitis B virus (HBV), and approximately a third develop serious liver disease such as decompensated cirrhosis and hepatocellular carcinoma (HCC) during the lifetime [Lee, 1997]. Universal vaccination of newborns has been implemented successfully in Taiwan [Ni et al., 2001], the United States [MMWR, 2002] and elsewhere. Catch-up vaccination is extended to children and adults for preventing HBV infection further.

Japan is unique in that, since 1986, passive and active immunopropylaxis has been performed annually on some 4,000 babies born to mothers who are infected with HBV and have hepatitis B e antigen (HBeAg) in the serum [Koyama et al., 2003; Noto et al., 2003]. This policy is based on a high risk of such babies to develop persistent HBV infection, in contrast to a low risk of babies born to carrier mothers with antibody to HBeAg (anti-HBe) [Okada et al., 1976]. As a result, the prevalence of HBV infection has been decreasing in Japan during past decades, which is reflected in the age-specific frequency of hepatitis B surface antigen (HBsAg) among first-time blood donors. The prevalence of HBsAg is low in blood donors born after 1981 at 0.23%, in remarkable contrast to 1.5% in those born between 1941 and 1950 [Tanaka et al., 2004].

The high incidence of HBV infection in men aged 20 years or older has been noted in the United States since 1999 [MMWR, 2004], forecasting continued new

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infections in the next two decades. There is a possibility that Japan, as well as any country in the world, will suffer from resurgent HBV infection that might be inapparent in the general population. During 35 years from 1971 to 2005, a city hospital in the Metropolitan Tokyo was visited by 4,430 patients infected with HBV. Patients with acute and chronic infections increased since 1996, thereby indicating that HBV infection has not been controlled efficiently in Japan.

MATERIALS AND METHODS

Patients

During 35 years from 1971 through 2005, 4,430 patients with HBV infection visited the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo, including 153 with acute and 4,277 with chronic HBV infection. Genotypes were A in 158 (3.6%) patients, B in 521 (11.8%), C in 3,564 (80.5%), D in 7 (0.2%), F in 3 (0.06%), H in 2 (0.04%) and not typeable in the remaining 175 (3.9%) patients. The median age of the patients was 37 years (range: 0.1–83) at the presentation, and included 3,210 (72.1%) men. Acute infection was diagnosed by high-titered antibody to hepatitis B core antigen of the IgM class and/or the development of HBsAg in previously seronegative individuals. Chronic hepatitis was diagnosed by liver biopsy carried out by laparoscopy and/or ultrasonic images, and liver cirrhosis by liver biopsy and/or ultrasonographic images plus laparoscopic findings. The number of patients with acute and chronic hepatitis B changed through 35 years, and the genotypes/subgenotypes were surveyed for predicting future trends of HBV infection in Japan. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the Ethics Committee of the institution. Every patient gave an informed consent for this study.

Markers of HBV Infection

HBsAg and the corresponding antibody (anti-HBs) were determined by hemagglutination (MyCell, Institute of Immunology Co., Ltd., Tokyo, Japan), and HBeAg by enzyme-linked immunosorbent assay (F-HBe, Sysmex, Kobe, Japan). HBV DNA was determined by the polymerase chain reaction (PCR) followed by hybridization (Amplicor HBV Monitor, Roche Molecular Systems, Inc., Branchburg, NJ) and the results were expressed in log copies/ml over a range from 2.6 to 7.6. HBV genotypes (A–H) were determined by enzyme-linked immunosorbent assay (HBV GENOTYPE EIA, Institute of Immunology) [Usuda et al., 1999, 2000] and PCR-Invader assay with genotype-specific probes [Tadokoro et al., 2006]. Subgenotypes of A, B and C were determined by sequence analysis, restriction fragment length polymorphism [Sugauchi et al., 2004a, 2004b; Tanaka et al., 2005] and PCR-Invader assay [Tadokoro et al., 2006].

Statistical Analysis

Frequencies were compared between groups by the Chi-squared test and Fisher's exact test, and medians by the Mann–Whitney's *U*-test. Analysis of data was conducted with the computer program SPSS ver.11.0 (SPSS Inc., Chicago, IL). The trend of subgenotypes B1/Bj and B2/Ba was analyzed by the Cochran-Armitage trend test with SAS version 9.1.3 software (SAS Institute, Inc., Cary, NC). A P value less than 0.05 was considered significant.

RESULTS

Patients With HBV Infection During 35 Years (1971–2005)

During 35 years from 1971 through 2005, the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo was visited by 4,430 patients infected with HBV, including 153 with acute and 4,277 with chronic infection. Table I compares the demographic, clinical and virological characteristics between the patients with acute and chronic HBV infection at the baseline. Patients with acute HBV infection were younger ($P = 0.046$), predominantly male ($P = 0.004$), had higher alanine aminotransferase levels ($P < 0.001$),

TABLE I. Baseline Characteristics of Patients Infected With HBV Who Visited Toranomon Hospital During 35 Years (1971–2005)

Features[a]	Acute infection (n = 153)	Chronic infection (n = 4,277)	Differences (P value)
Age in years	34 (19–69)	38 (0.1–83)	0.046
≤39	99 (65%)	2,358 (55%)	
40–59	49 (32%)	1,642 (38%)	
≥60	5 (3%)	277 (7%)	
Men	125 (82%)	3,067 (72%)	0.004
ALT (IU/L)	1,460 (19–6,876)	58 (12–3,520)	<0.001
Sexual transmission	102 (67%)	129 (3%)	<0.001
Liver disease			
Symptom-free	0	1,035 (24%)	
Chronic hepatitis	0	2,617 (61%)	
Cirrhosis	0	405 (10%)	
Hepatocellular carcinoma	0	220 (5%)	
HBeAg	100 (65%)	2,131 (50%)	<0.001
HBV DNA (log copies/ml)	5.9 (<2.6 to >7.6)	6.4 (<2.6 to >7.6)	0.014

[a]Data are expressed in number of patients with percentage in parentheses or the median value with a range in parentheses.

were positive more frequently for HBeAg ($P < 0.001$), and had lower HBV DNA loads ($P = 0.014$) than those with chronic infection. Sexual transmission was more frequent in patients with acute than chronic HBV infection (67% vs. 3%, $P < 0.001$).

The number of new patients presenting with acute and chronic HBV infections during a 5-year period was compared during 1971 through 2005 (Fig. 1). In the initial four 5-year periods (1971–1990), both patients with acute and chronic HBV infections increased linearly. In the fifth 5-year period (1991–1995), however, patients with acute or chronic HBV infection decreased to less than those in the previous 5-year period (1986–1990). In the next 5-year period (1996–2000), nevertheless, patients with acute HBV infection began to increase while a decrease in chronic HBV infection was observed. In the seventh 5-year period (2001–2005), patients with acute HBV infection kept increasing. In addition, there was a small but appreciable increase of patients with chronic HBV infection in comparison with the previous 5-year period (1996–2000). Taken altogether, acute HBV infection resurged since 1991 accompanied by an increase in chronic HBV infection since 2001.

HBV Genotypes in Patients Infected With HBV

HBV was typeable in 126 of the 153 (82.4%) patients with acute and 4,121 of the 4,277 (96.4%) with chronic HBV infection (Table II). Genotype A, foreign to Japan, was more frequent in acute than chronic HBV infection (28.6% vs. 3.0%, $P < 0.001$). There were no differences in the distribution of endemic genotypes B and C; combined, they accounted for 69.8% and 96.8%, respectively, in patients with acute and chronic HBV infections. Foreign genotypes other than A (D–H) were detected in 2 (1.6%) and 10 (0.24%) patients with acute and chronic HBV infections, respectively. One each genotype D and H were found in patients with acute HBV infection; and 6 with genotype D, 3 genotype F and 1 genotype H in those chronic infection. Among patients with chronic HBV infection, genotype B was more frequent (566/3,481 [16.3%] vs. 28/508 [5.5%], $P < 0.001$), while genotype C was less common (2,915/3,481 [83.7%] vs. 480/508 [94.5%], $P < 0.001$), in those with chronic hepatitis than cirrhosis and/or HCC.

TABLE II. Distribution of Genotypes in Patients With Acute and Chronic HBV Infections

Genotypes[a]	Acute (n = 126)	Chronic (n = 4,129)	Differences (*P* value)
A	36 (28.6%)	122 (3.0%)	<0.001
B	13 (10.3%)	508 (12.3%)	NS
C	75 (59.5%)	3,489 (84.5%)	NS
D	1 (0.8%)	6 (0.1%)	NS
E	0	0	NS
F	0	1 (0.02%)	NS
G	0	0	NS
H	1 (0.8%)	3 (0.07)	NS

[a]Data are expressed in number of patients with percentage in parentheses.

Subgenotypes of HBV

Subgenotypes of A, B, and C were determined in patients with HBV infection. Of the 158 patients infected with genotype A, 15 (9.5%) were classified into subgenotype A1/Aa and 121 (76.6%) into A2/Ae; the remaining 22 (13.9%) were not typeable. Likewise, of the 521 patients with genotype B, 388 (74.5%) were infected with the domestic subgenotype B1/Bj and 102 (19.6%) with foreign subgenotype B2/Ba; subgenotypes in the remaining 31 (6%) patients could not be determined. Figure 2 compares the proportion of these subgenotypes among the seven 5-year periods. By the trend analysis, subgenotype B2/Ba was increasing recently ($P < 0.05$). Subgenotypes of C were domestic C2/Cs in all the 1,610 HBV isolates tested. The foreign subgenotype C1/Ce was not detected in any patient infected with HBV genotype C.

Change in the Distribution of Genotypes in Patients Infected With HBV

Figure 3 illustrates distributions of genotypes A–C in patients with acute and chronic HBV infection during

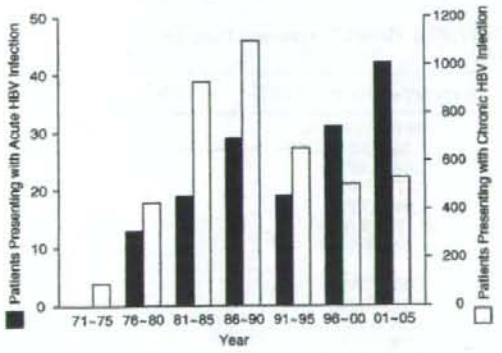

Fig. 1. Patients with acute and chronic HBV Infection who visited Toranomon Hospital during 35 years from 1971 to 2005. Numbers are indicated in different scales for patients with acute and chronic HBV infections for seven 5-year periods.

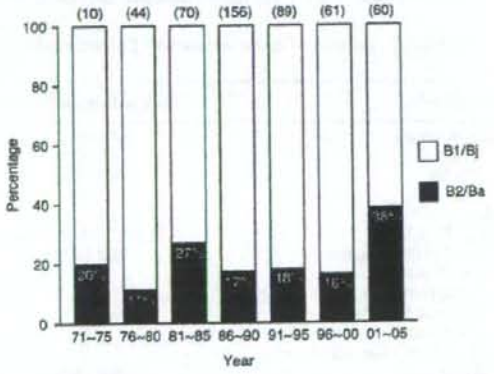

Fig. 2. Distribution of subgenotypes of genotype B shifting during 1971 through 2005. The number of patients is shown in parentheses for each seven 5-year period.

seven 5-year periods. The genotype distribution in patients with acute HBV infection changed through seven 5-year periods. The proportion of genotype A started to increase sharply in the fifth 5-year period (1996–1995) and accounted for 43% and 40%, respectively, in the sixth (1996–2000) and seventh (2001–2005) 5-year periods. The proportion of genotype A during the fifth and seventh 5-year periods (1991–2005) was significantly higher than that during the second through fourth 5-year periods (1976–1990) (39% [33/84] vs. 8% [3/40], $P < 0.001$). Before 1995, genotype A was detected in men <35 years but not found in those >36 years (7/21 [33%] vs. 0/25 [0%]). However, genotype A became comparably frequent since 1996 (15/29 [52%] vs. 14/32 [44%]).

By remarkable contrast, the distribution of genotypes in patients with chronic HBV infection remained fairly constant, although the proportion of genotypes A kept increasing constantly. Thus the proportion of genotype A during the fifth through seventh 5-year periods (1991–2005) was greater than that during the first to fourth 5-year periods (1971–1990) (4.3% [70/1,638] vs. 2.4% [51/2,128], $P < 0.001$).

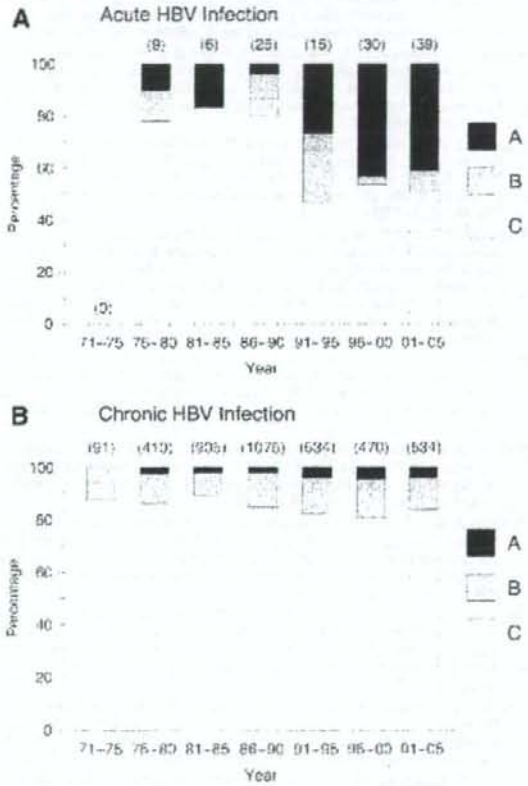

Fig. 3. Time-dependent distribution of HBV genotypes in patients with acute and chronic HBV infections during 1971 through 2005. Distribution of genotypes A–C in patients with acute HBV infection (A) and those with chronic HBV infection (B) are shown for seven 5-year periods. The number of patients is shown in parentheses for each seven 5-year period.

DISCUSSION

The Department of Hepatology at the Toranomon Hospital was visited by 153 with acute and 4,277 patients with chronic HBV infection during 35 years from 1971 through 2005. Patients with acute HBV infection were younger, more commonly male and had been infected by sexual contact more frequently than those with chronic infection. Patients were grouped by the year when they visited the department, and they were compared among seven 5-year periods spanning 1971–2005, for the purpose of estimating time-dependent trends of acute and chronic HBV infections in Japan.

Remarkably, patients presenting with acute HBV infection increased during the past 35 years (Fig. 1). Patients with chronic HBV infection peaked in 1986–1990 and then decreased until 1996–2000. They did not decrease further, but instead, increased slightly in the 21st century. Such a recent increase in chronic HBV infection would reflect resurgence of acute infection, which is supported by the analysis of genotypes.

The distribution of HBV genotypes was much different between patients with acute and chronic infections. Of note, infection with genotype A was much more frequent in acute than chronic infection (28.6% vs. 3%, $P < 0.001$). HBV genotypes have distinct geographic distribution [Miyakawa and Mizokami, 2003; Fung and Lok, 2004; Norder et al., 2004]. The Japanese have been infected with genotypes B and C since the prehistoric era [Yamashita et al., 1975], and foreign genotypes represented by A (both subgenotypes A1/Aa and A2/Ae) were introduced by travelers and immigrants after the end of World War II. Since 1991, foreign genotypes have been increasing in acute HBV infection in Japan [Sugauchi et al., 2006]. As for chronic HBV infection, genotype C was more prevalent in patients with cirrhosis and/or HCC than in those with chronic hepatitis (480/508 [94.5%], vs. 2,915/3,481 [83.7%], $P < 0.001$), standing in corroboration with previous studies [Kao et al., 2000; Orito et al., 2001].

There was a dramatic change in the distribution of HBV genotypes in patients with acute HBV infection during the past 35 years. This change is attributed to ever increasing infection with genotype A in them. It accounted for only 8.1% before 1990, in marked contrast to 39.3% after 1991 ($P < 0.001$). The recent resurgence of acute infection in Japan could be due to increase in the transmission with HBV of foreign genotypes. The gradual increase of genotype A, in patients with chronic HBV infection since 2001, would be accounted for by an increase of acute infection with this genotype in Japan. In support of this view, infection with genotype A tends to persist, infection even in adulthood, and becomes chronic in 10% of infected adults [Suzuki et al., 2005; Kobayashi et al., 2006]. In an outbreak transmitted by a surgeon, 5 of the 16 (31%) patients infected with genotype A became HBV carriers [Harpaz et al., 1996].

There are two types of risk for exposure to HBV. One is avoidable and mediated by promiscuous sexual contacts and the use of illicit intravenous drugs. The other is not preventable and can involve citizens without high-risk behaviors. For instance, HBV can be transmitted from patient to patient in dental care [Redd et al., 2007]. HBV can spread from carrier surgeons who are negative for serum HBeAg [Perry et al., 2006]. In 2002, the largest outbreak of HBV involving 38 patients occurred in a physician's office in New York City by multidose vials contaminated with HBV [Samandari et al., 2005]. There is a pressing need to investigate and determine the risk of HBV transmission in the health care setting [Allos and Schaffner, 2007]. Fortunately, risks of HBV infection can be avoided by vaccination. Mass vaccination of newborns and catch-up vaccination, such as those conducted in the United States [MMWR, 2002], Taiwan [Ni et al., 2001] and elsewhere, would need to be considered in Japan. The ultimate national protection would be universal vaccination of all age groups.

In conclusion, acute HBV infection is increasing in Japan in spite of immunoprophylaxis of high-risk babies implemented nationally since 1986. Based on genotypes/subgenotypes changing with time, the increase may be attributed to infections with HBV of foreign genotypes/subgenotypes predominantly by sexual contact. Since HBV genotype A, with a high propensity to persist, prevailed in acute infection, chronic infection would increase in the foreseeable future. Effective measures have to be taken for preventing HBV transmission among young men at high risk in Japan.

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ORIGINAL ARTICLE

Poor Response to Pegylated Interferon and Ribavirin in Older Women Infected with Hepatitis C Virus of Genotype 1b in High Viral Loads

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Abstract *Background* Response to treatment in patients with chronic hepatitis C, with reference to age and gender, has not been examined fully. *Aim* The influence of gender and age on treatment with pegylated interferon (PEG-IFN) and ribavirin was evaluated in a retrospective study. *Methods* PEG-IFN and ribavirin were given for 48 weeks to 179 men and 121 women infected with hepatitis C virus (HCV) of genotype 1b in high viral loads (>100 kIU/ml). *Results* Sustained virological response at 24 weeks after treatment was poorer in women than men who were aged ≥50 years (22% vs 53%, $P < 0.001$). Among the patients aged ≥50 years who had received ≥80% of the doses of PEG-IFN, ribavirin, or both, women responded less often than men (26% vs 64%, $P < 0.001$; 33% vs 61%, $P = 0.022$; and 32% vs 63%, $P = 0.016$; respectively). In multivariate analysis, male gender, retention of indocyanine green, ribavirin dose and compliance with therapy increased sustained virological response. *Conclusions* Response to combined PEG-IFN and ribavirin is poorer in female than male patients with hepatitis C who are aged ≥50 years, irrespective of compliance with treatment. Low estrogen levels in older women could be responsible for their impaired response to PEG-IFN and ribavirin.

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Keywords Aging · Women · Chronic hepatitis C · Genotypes · Interferon · Ribavirin

Introduction

There are an estimated 170 million people worldwide that are chronically infected with hepatitis C virus (HCV) [1]. HCV can persist in 70–80% of individuals who have been exposed to it, and it can induce chronic liver disease, through cirrhosis to hepatocellular carcinoma (HCC) in approximately 30% of them until 30–40 years after they were infected [2–4]. A number of viral and host factors influence the velocity of fibrosis progression in chronic hepatitis C. Thus, stage and grade of hepatitis are more severe in patients who are infected with HCV genotype 1 in high viral loads [5–7]. Male gender, age and intake of alcohol accelerate fibrosis, as well [8–10].

Interferon (IFN) combined with ribavirin has been the most effective and favored treatment of chronic hepatitis C to date. The combined treatment with the standard IFN can terminate HCV-1 infection with high viral loads in approximately 20% [11], and that with pegylated IFN (PEG-IFN) in >40% [12]. Owing to hemolytic side effects, however, women are less tolerant to ribavirin [13]. Although the response to combined treatment has been shown to be better in women than in men in previous studies, there remains a possibility that it could be influenced by age. Hence, there is a need for the comparison of the response between men and women in different age groups.

Virological response to PEG-IFN and ribavirin at the end of a 48-week treatment (ETR), as well as sustained virological response (SVR) 24 weeks after the completion of therapy, was compared between 179 men and 121

women who were infected with HCV-1b in high viral loads. In patients aged ≥50 years, both ETR and SVR were poorer in women than in men, irrespective of the total dose of IFN, ribavirin or both.

Methods

Study Population

From December 2001 to February 2006, 490 consecutive patients with chronic hepatitis C received combination therapy with PEG-IFN and ribavirin at the Department of Hepatology in the Toranomon Hospital in Metropolitan Tokyo. The following inclusion criteria were met by 300 (61%) patients: they were (1) positive test results for antibodies to HCV (anti-HCV) and for HCV RNA genotype 1b by qualitative methods, and not co-infected with HCV of other genotypes; (2) negative test results for hepatitis B surface antigen or antibodies to human immunodeficiency virus type-1 (HIV-1); (3) confirmed findings of high HCV RNA levels ≥100 kIU/ml, which is the Japanese definition of high viral loads [14, 15], within the past 2 months; (4) no cirrhosis diagnosed by laparoscopy and ultrasonography, and with platelet counts $>80 \times 10^3/mm^3$; (5) body weight ≥40 kg and not pregnant or lactating; (6) total alcohol intake <500 kg in the past; (7) no HCC, hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic hepatitis or autoimmune hepatitis; (8) no treatment with antivirals or immunosuppressants during the previous 3 months; and (9) with the wish to comply with the treatment protocol for 48 weeks. None of them received growth factors before or during the study period.

The 300 patients, comprising 179 men and 121 women, received PEG-IFN and ribavirin for 48 weeks and were followed for at least 24 weeks after completion of this combination therapy. Informed consent was obtained from each patient, and the study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Serum Markers of HCV Infection

Anti-HCV was determined by third-generation enzyme-linked immunosorbent assay (ELISA) by commercial kits (Ortho HCV Ab ELISA Test 3; Chiron Cooperation, Emeryville, CA, USA). HCV RNA was determined quantitatively by polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor ver. 2.0, Roche Diagnostics, Tokyo, Japan) in serum diluted tenfold at the baseline, as well as at least monthly during and after treatment; it has a dynamic range between 5 kIU/ml and 5,000 kIU/ml. Sera negative for HCV RNA (<5 kIU/ml) by quantitative assay were tested by qualitative PCR (Amplicor, Roche Molecular Systems, Inc., Branchburg, NJ, USA) with a detection limit at 100 copies/ml.

Combined PEG-IFN and Ribavirin Therapy

Patients underwent subcutaneous administration of PEG-IFN-α2b (PEG-Intron, Schering-Plough Corp, Kenilworth, NJ, USA), weekly, at a median dose of 1.4 μg/kg (range 0.8–1.9 μg/kg), together with ribavirin orally, at a median daily dose of 11 mg/kg (range 3.7–14.2 mg/kg) for 48 weeks. The dose of ribavirin was adjusted by body weight: 600 mg for patients weighing ≤60 kg; 800 mg for those between >60 kg and <80 kg; and 1,000 mg for those ≥80 kg. It was tapered in the 99 (33%) patients in whom hemoglobin levels decreased below 10 g/dl during the combination therapy.

Statistical Analysis

Variables were compared between groups by the chi-square test, Fisher's exact probability test and the Mann–Whitney U test. Differences in the loss of HCV RNA from the serum between groups was evaluated with the Kaplan–Meier life table with use of the log rank test. The influence of various factors on the response to PEG-IFN/ribavirin was evaluated by logistic regression in univariate and multivariate analyses. Analysis of all data was performed with the computer program SPSS software (SPSS Inc., Chicago, IL, USA), and a P value less than 0.05 was considered significant.

Results

Baseline Characteristics of Male and Female Patients Infected with HCV-1b in High Loads

PEG-IFN and ribavirin were given for 48 weeks to 179 men and 121 women who had been infected with HCV-1b in high viral loads (>100 kIU/ml). Table 1 compares baseline characteristics between them. Women were older, had lower hemoglobin values and platelet counts, and lower levels of albumin, gamma-glutamyl-transpeptidase (γ-GTP) and ferritin, than men. The stage of fibrosis was lower in women than in men, although their alanine aminotransferase (ALT) levels were comparable. Three months before the start of combination therapy, IFN had been given to 75 (42%) of the 179 male patients, comparably frequently to 40 of the 121 (33%) female patients. Age distribution for men and women is shown in Fig. 1. The proportion of patients ≥60 years was higher in women than in men (39% vs 19%, $P < 0.001$).

Table 1 Baseline characteristics of 300 patients with chronic hepatitis with high-titers of HCV-1b RNA who had received PEG-IFN and ribavirin for 48 weeks and were followed for 48 weeks or longer

Characteristic[a]	Men ($n = 179$)	Women ($n = 121$)	Differences P
Age (years)	50 (19–66)	57 (30–69)	<0.001
Previous IFN treatment	75 (42%)	40 (33%)	0.146
Hemoglobin (g/dl)	15.2 (11.5–17.8)	13.5 (11.2–15.1)	<0.001
Platelets ($\times 10^3/mm^3$)	176 (88–366)	165 (91–331)	0.025
Albumin (g/dl)	3.9 (3.2–4.6)	3.8 (3.0–4.6)	0.004
ALT (IU/l)	77 (23–504)	68 (19–391)	0.078
γ-GTP (IU/l)	78 (14–409)	37 (11–171)	0.011
LDL (mg/dl)	98 (50–176)	99 (57–168)	0.920
Ferritin (mg/l)	186 (<10–1,327)	95 (<10–4 42)	<0.001
ICG_{15} (%)	14 (4–41)	13 (2–31)	0.969
Stage (F0-1/F2-3)	80/66 (50 unknown)	42/55 (57 unknown)	0.050

ALT alanine aminotransferase, *γ-GTP* gamma-glutamyl transpeptidase, *LDL* low density lipoprotein, ICG_{15}, retention of indocyanine green at 15 min

[a] The means (ranges) are given

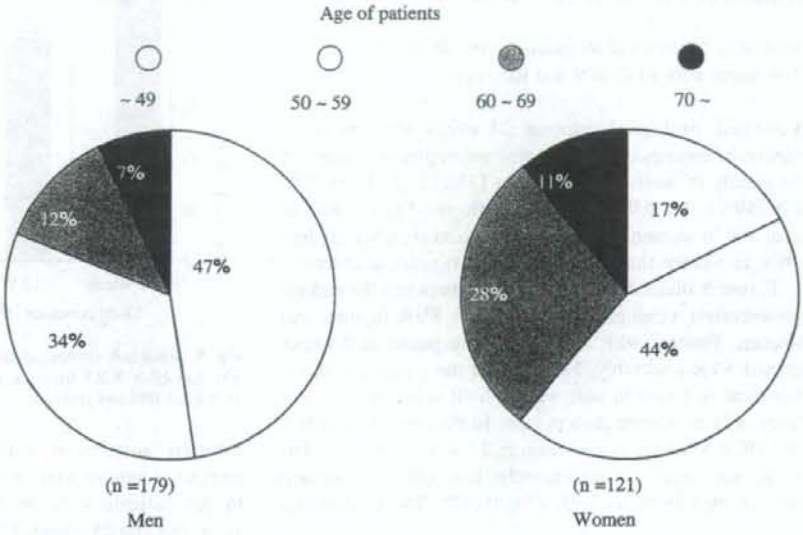

Fig. 1 Distribution of ages in the male and female patients with chronic hepatitis C who were infected with HCV-1b in high loads

Virological Response During the 48-Week Treatment with PEG-IFN and Ribavirin

On-treatment response to the combined treatment is compared between men and women in Fig. 2. Through 48 weeks on treatment, women gained a virological response less frequently than did men. ETR was achieved by 58% of women as against 83% of men ($P < 0.001$). Restricted to the patients who gained ETR, women lost HCV RNA from serum later than men did [median (range) 24.1 (2.0–36.4) vs 13.0 (2.0–48.0) weeks, $P < 0.001$]. Figure 3 depicts the on-treatment virological response in patients <50 years and those ≥50 years separately. The virological response was no different between men and women <50 years. However, it was poorer in women than

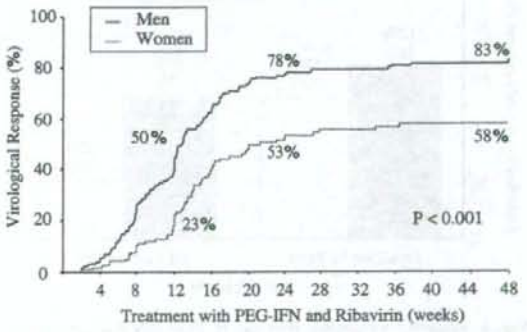

Fig. 2 On-treatment virological responses to PEG-IFN and ribavirin in male and female patients infected with HCV-1b in high viral loads

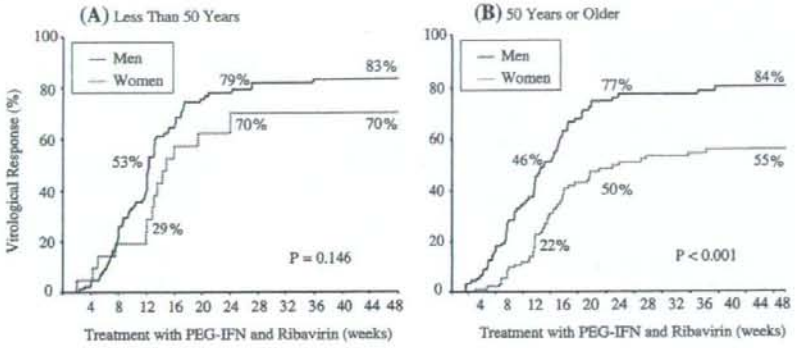

Fig. 3 On-treatment virological responses to combined IFN and ribavirin in male and female patients infected with HCV-1b in high viral loads who were less than 50 years (**a**) or 50 years or older (**b**)

in men ≥50 years. Differences between men and women in total (Fig. 2), therefore, were attributed to a poorer response of women ≥50 years to the combined treatment.

Sustained Virological Response to the 48-Week Treatment with PEG-IFN and Ribavirin

Sustained virological response 24 weeks after the completion of combined treatment was accomplished much less frequently in women than in men [33/121 (27%) vs 105/179 (59%), $P < 0.001$]. SVR was influenced by age both in men and in women (Fig. 4). It was found significantly less often in women than men who were 50 years or older.

Figure 5 illustrates the relationship between the earliest on-treatment virological response and SVR in men and women. Patients with a virological response at 4 weeks gained SVR invariably. However, in the patients with virological response in later weeks, SVR was achieved less frequently in women than in men. In the patients who had lost HCV RNA from the serum at 12 weeks, in particular, SVR was achieved significantly less often in women than in men (63% vs 88%, $P = 0.012$). The relationship

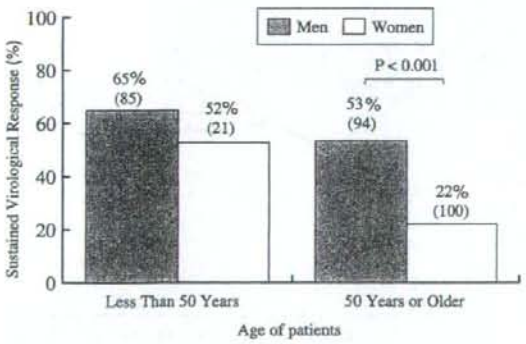

Fig. 4 Sustained virological response to PEG-IFN and ribavirin in male and female patients stratified by age. The number of patients is indicated in parentheses in each column

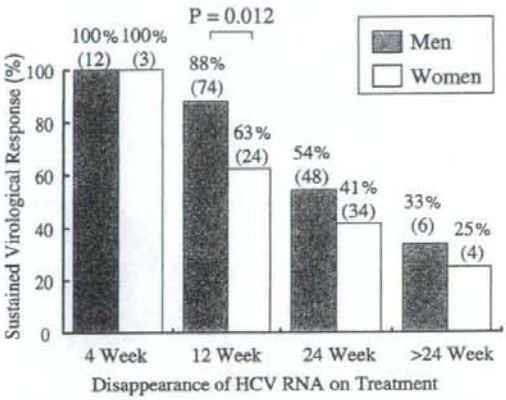

Fig. 5 Sustained virological response in male and female patients who lost HCV RNA from the serum at various weeks on treatment with PEG-IFN and ribavirin

between on-treatment virological response and SVR was compared among women in different age groups (Fig. 6). In the patients with the earliest virological response at 12 weeks and 24 weeks, SVR was achieved less frequently in women aged ≥50 years than in those <50 years, but the difference fell short of being significant due to the small numbers of patients in the comparison.

SVR and Compliance with PEG-IFN Therapy, Ribavirin Therapy, or Both

Table 2 compares compliance with the combined treatment between men and women. Either or both of PEG-IFN and ribavirin were tolerated to a lesser extent by women than by men. Thus, doses ≥80% were reached less frequently in women than in men for PEG-IFN or ribavirin, or both. The initial dose of ribavirin was no different between men and women.

SVR was achieved less frequently in women than in men who had received ≥80% of the dose of PEG-IFN

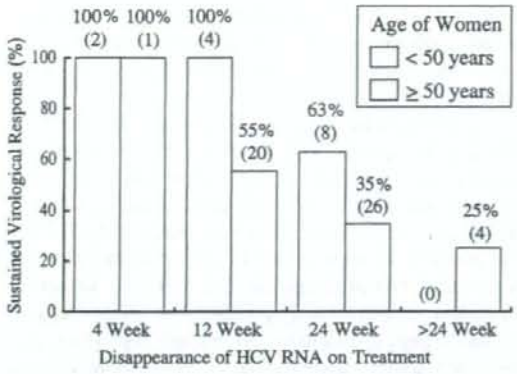

Fig. 6 Sustained virological response to PEG-IFN and ribavirin in female patients stratified by age who lost HCV RNA from serum at various weeks on treatment

Table 2 PEG-IFN and ribavirin received by patients with chronic hepatitis with high-titers of HCV-1b RNA

PEG-IFN and ribavirin	Men ($n = 179$)	Women ($n = 121$)	Differences P
Initial ribavirin dose (mg/kg body weight)	11.1 (5.0–14.1)	11.2 (3.7–14.3)	0.735
Total dose			
PEG-IFN ≥ 80%	139 (78%)	94 (53%)	<0.001
Ribavirin ≥ 80%	117 (65%)	42 (35%)	<0.001
Both ≥ 80%	110 (61%)	32 (27%)	<0.001
Withdrawn	28 (16%)	28 (23%)	0.131

[20/64 (31%) vs 92/138 (67%), $P < 0.001$], ribavirin [19/42 (45%) vs 79/120 (66%), $P = 0.027$] or both [13/32 (41%) vs 72/109 (66%), $P = 0.013$]. Again, differences were observed only in patients ≥50 years (Fig. 7). In the patients <50 years, in contrast, the rate of SVR was no different between women and men who had received ≥80% of the dose of PEG-IFN, ribavirin, or both.

The influence of age was compared between male and female patients in different age groups. SVR was achieved significantly more frequently in the men aged ≥60 years [88/145 (61%) vs 17/74 (37%), $P = 0.001$] and <60 years [17/34 (50%) vs 6/48 (13%), $P < 0.001$]. Likewise, SVR was more common in male than female patients aged 50–59 years [33/60 (55%) vs 16/53 (30%), $P = 0.013$].

Multivariate Analysis for Factors Accelerating the Response to PEG-IFN and Ribavirin Therapy

In univariate analysis, age, gender, hemoglobin, albumin, ICG_{15}, ribavirin dose and compliance with PEG-IFN therapy, ribavirin therapy, or both, influenced SVR. In multivariate analysis, only male gender, ICG_{15}, ribavirin dose and compliance with PEG-IFN, as well as both PEG-IFN and ribavirin, accelerated the chance of SVR (Table 3).

Discussion

In a retrospective study, response to PEG-IFN and ribavirin for 48 weeks was compared between 179 men and 121 women with chronic hepatitis C who had been infected with HCV-1b in high viral loads by the Japanese definition (>100 kIU/ml) [14, 15]. Loss of HCV RNA from serum occurred less often in women than in men throughout the 48 weeks of treatment. Both ETR (55% vs 83%, $P < 0.001$) and SVR (27% vs 59%, $P < 0.001$) were achieved significantly less frequently in women than in men. The observed low response to PEG-IFN and ribavirin stands at odds with the better response to antiviral treatments and slow progression of fibrosis in women than in men [9, 16, 17]. There are, however, viral and host factors other than gender that can influence the course of chronic hepatitis C and, by inference, the response to antiviral treatments.

Viral factors such as HCV genotypes and infection load affect the course of chronic hepatitis C. Thus, hepatitis is

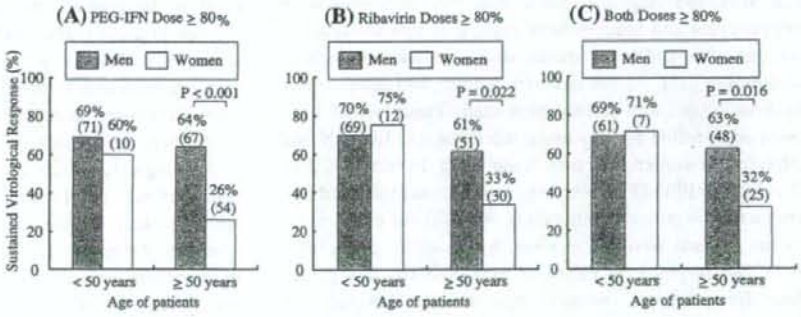

Fig. 7 Sustained virological response to PEG-IFN and ribavirin in patients who had received 80% or more of the dose of IFN (**a**), ribavirin (**b**) or both of them (**c**). Results are shown for men and women in two age groups