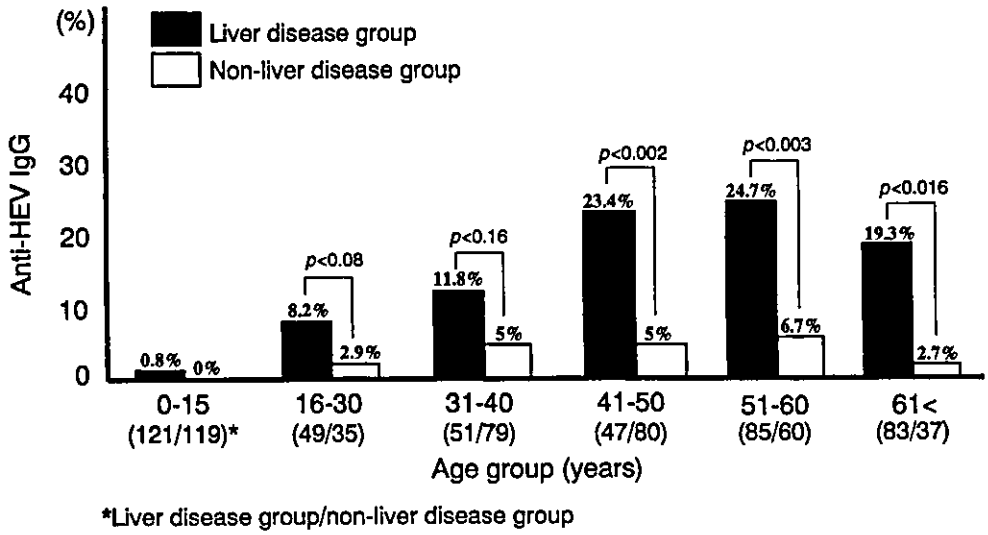

Fig. 1. Age-specific prevalence of IgG class antibody to HEV among residents living in Tokyo. *, Number of individuals in liver disease group/non-liver disease group. Number of cases shown in this figure is not all cases tested due to an indistinct age in some cases.

difference of anti-HEV IgG prevalence between healthy populations and liver disease patients were statistically significant ($P < 0.01$). Among liver disease patients with non-B, non-C hepatitis, anti-HEV IgG was detected in 17.6% (35/199); 29.4% (5/17) in fulminant hepatitis, 17% (15/88) in acute hepatitis and 16% (15/94) in chronic hepatitis, respectively. Furthermore, anti-HEV IgG was detected in 23.6% (21/89) of hepatitis B virus (HBV)-infected and 7.9% (12/152) of hepatitis C virus (HCV)-infected patients, respectively. The prevalence of antibody in HBV-infected patients was significantly higher than that of HCV-infected patients ($P < 0.001$) and healthy individuals ($P < 0.001$). In addition, anti-HEV IgG was present in 30% (18/60) of hemodialysis patients and 9.2% (8/87) of hospital workers and these results were significantly higher than in the healthy population ($P < 0.01$). The infection rate of HEV increased with age in the liver disease patient group, but not in non-liver disease group: the infection rate in the liver disease group was 0.8% in the age group of less than 15 years, 8.2% in the 16–30 year group, 11.8% in the 31–40 year group, 23.4% in the 41–50 year group, 24.7% in the 51–60 year group and 19.3% in the age group of over 61 years (Fig. 1). The rate of HEV infection was significantly more prevalent in males than in females ($P < 0.001$).

3.2. Anti-HEV IgM prevalence

Anti-HEV IgM was detected only in one infant with acute hepatic failure of unknown etiology and gastrointestinal dysfunction. This case was also positive for IgG class of anti-HEV (OD492 = 0.261 for IgM and 0.904 for IgG, respectively). We obtained the serum sample after onset of acute hepatitis in this case. None of the individuals with HEV antibody had a recent history of visiting countries known to be endemic for hepatitis E.

3.3. HEV RNA detection by PCR

We tested HEV RNA by nested RT-PCR in serum samples from 106 patients, including one infant who was positive for anti-HEV IgM, 17 cases of non-B, non-C fulminant hepatitis and 88 cases of non-B, non-C acute hepatitis. However, none of serum samples tested was positive for HEV RNA in this study.

4. Discussion

HEV is the leading cause of water-borne epidemics of hepatitis in many developing countries in Asia, South America and Africa [1–3]. Recently, it has become clear that sporadic hepatitis E occurs in persons in industrialized countries and who have no evidence of exposure to HEV strains from countries where the infection is endemic [5–9]. It is said that Japan is not an endemic area of HEV infection, because acute hepatitis patients infected with HEV are rare, and most patients with acute hepatitis E have recently traveled to countries where HEV is highly endemic. However, the actual conditions of HEV epidemiology in Japan have not yet been adequately clarified. Recently, Li et al. [10,11] developed a new method for anti-HEV assay by an ELISA that is reliably sensitive and specific enough to use for a seroepidemiological study of HEV infection. In their preliminary study, the prevalence of the anti-HEV IgG in Japan was varied from 1.9 to 14%, depending on the geographical areas [11]. Using the same method, Tanaka et al. [5] also reported that the

prevalence of anti-HEV IgG in Nagano Prefecture, Japan was around 4.6–6.7%. Our results showed that prevalence of anti-HEV IgG was 3% in healthy individuals residing in Tokyo; however, it was significantly higher in patients with liver diseases. Surprisingly, 29.4% of non-B, non-C fulminant hepatitis patients were positive for anti-HEV IgG. Very recently, Suzuki et al. [12] reported that three of 18 fulminant hepatitis patients in Japan were positive for anti-HEV IgM by ELISA. However, we were not able to catch both of IgM class of antibody and HEV RNA in our fulminant hepatitis cases although sera obtained after onset. We need further investigation using serum samples obtained at the different time point to clarify the relation of both. Interestingly, the prevalence of antibody in HBV-infected patients was significantly higher than in HCV-infected patients. These were unexpected results, because we had not expected any difference in the detection rate of anti-HEV among the individuals of different categories. The reason is that HEV is mainly transmitted feco-orally and causes only acute resolving hepatitis. To address these questions, it is necessary to examine the efficiency of HEV exposure including past infection in chronic hepatitis patients with or without HBV/HCV infection.

The infection rate of HEV increased with age, but in the liver disease patient group only. Furthermore, it is noteworthy that a high prevalence of anti-HEV IgG was found among hemodialysis patients (30%) suggesting blood-borne transmission, and in hospital workers (9.2%) suggesting nosocomial infection, respectively. On the other hand, the IgM class of anti-HEV was detected only in one case of infant tested in this study. The specificity of anti-HEV IgM was confirmed by an absorption test (data not shown). This infant was diagnosed as acute hepatic failure of unknown etiology with gastrointestinal dysfunction. This indicates that HEV infection should be considered in such cases.

All individuals with anti-HEV antibody in the present study had no recent history of visiting countries where HEV is endemic. This indicates that they had contracted the HEV infection in Japan. However, the infection route of these patients remains unknown. Several recent studies have suggested the existence of indigenous HEV strains in Japan as several HEV strains were recovered from Japanese patients with acute hepatic failure of unknown etiology, but who had not traveled abroad [6–9]. The route of HEV infection in those patients is still unknown. It has been reported that swine [13,14] and rodents [15–17] may be reservoirs of HEV, but the exact role of animals in the transmission of HEV to humans remains unclear. Very recently, we found the evidence for widespread infection of HEV among wild rats and Japanese monkeys living in Japan [18,19]. It is said until recently that Japan is a non-endemic country of hepatitis E, however, our study presented here indicated that HEV are prevailing unexpectedly in Japan. The role of transmission of HEV to humans from these animals should be considered to solve these important problems.

In conclusion, our results suggest that HEV is circulating in Japan, even if Japan is not endemic for HEV. Clarification of the infection route and establishment of prevention measures are needed in Japan.

Acknowledgements

We thank Tetsutaro Sata at the National Institute of Infectious Diseases, for his continuous encouragement during this study, and Shin-ichi Aotsuka at the Research Institute of International Medical Center of Japan, for his kindly collaboration. This study was supported in part by grants-in-aid for Science Research of the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan and the International Medical Cooperation Research Grant in Japan.

References

[1] Bradley DW. Hepatitis E: epidemiology, aetiology, and molecular biology. Rev Med Virol 1992;2:19–28.
[2] Krawczynski K. Hepatitis E. Hepatology 1993;17:932–41.
[3] Worm HC, van der Poel WHM, Brandstatter G. Hepatitis E: an overview. Microb Infect 2002;4:657–66.
[4] Mast EE, Kuramoto IK, Favorov MO, et al. Prevalence of and risk factors for antibody to hepatitis E virus seroreactivity among blood donors in Northern California. J Infect Dis 1997;176:34–40.
[5] Tanaka E, Takeda N, Li TC, et al. Seroepidemiological study of hepatitis E virus infection in Japan using a newly developed antibody assy. J Gastroenterol 2001;36:317–21.
[6] Takahashi K, Iwata K, Watanabe N, et al. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 2001;287:9–12.
[7] Takahashi M, Nishizawa T, Yoshikawa A, et al. Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. J Gen Virol 2002;83:1931–40.
[8] Takahashi K, Kang JH, Ohnishi S, et al. Genetic heterogeneity of hepatitis E virus recovered from Japanese patients with acute sporadic hepatitis. J Infect Dis 2002;185:1342–5.
[9] Mizuo H, Suzuki K, Takikawa Y, Sugai Y, et al. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J Clin Microbiol 2002;40:3209–18.
[10] Li TC, Yamakawa Y, Suzuki K, et al. Expressin and self-assembly of empty virus-like particles of hepatitis E virus. J Virol 1997;71:7207–13.
[11] Li TC, Zhang J, Shinzawa H, et al. Empty virus-like particle-based enzyme-linked immunosorbent assay for antibodies to hepatitis E virus. J Med Virol 2000;62:327–33.
[12] Suzuki K, Aikawa T, Okamoto H. Fulminant hepatitis E in Japan. New Engl J Med 2002;347:1456.
[13] Meng XJ, Purcell RH, Halbur PG, et al. A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci USA 1997;94:9860–5.
[14] Okamoto H, Takahashi M, Nishizawa T, Fukai K, Muramatsu U, Yoshikawa A. Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. Biochem Biophys Res Commun 2001;289:929–36.
[15] Kabrane-Lazizi Y, Fine JB, Elm J, et al. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. Am J Trop Med Hyg 1999;61:331–5.

[16] Favorov MO, Kosoy MY, Tsarev SA, Childs JE, Margolis HS. Prevalence of antibody to hepatitis E virus among rodents in the United States. J Infect Dis 2000;181:449–55.
[17] Arankalle VA, Joshi MV, Kulkarni AM, et al. Prevalence of anti-hepatitis E virus antibodies in different Indian animal species. J Viral Hepatitis 2001;8:223–7.
[18] Hirano M, Ding X, Li TC, et al. Evidence for widespread infection of hepatitis E virus among wild rats in Japan, Hepatol. Res., in press.
[19] Hirano M, Ding X, Tran T-TH, et al. Prevalence of antibody against hepatitis E virus in various species of non-human primates-evidence of widespread infection in Japanese monkeys (*Macaca fuscata*). Jpn J Infect Dis 2003;56:8–11.

Digestive Diseases and Sciences, Vol. 47, No. 10 (October 2002), pp. 2163–2169 (© 2002)

Lower Th-1/Th-2 Ratio Before Interferon Therapy May Favor Long-Term Virological Responses in Patients with Chronic Hepatitis C

NAOHIKO MASAKI, MD, SUGANO FUKUSHIMA, MD, and SHIGEKI HAYASHI, MD

It is still controversial whether alterations in helper-T cell subpopulations contribute to the pathogenesis and clinical characteristics of chronic hepatitis C. The aim of this study was to clarify this issue, particularly in relation to interferon therapy. Thirty-one patients with histologically proven chronic hepatitis C were treated by conventional interferon (IFN) monotherapy for 6 months, and virological responses were evaluated by polymerase chain reaction 6 months later. Helper-T cell subpopulations, Th-1 and Th-2, were determined in peripheral blood by intracellular cytokine assay using flow cytometry. In chronic hepatitis C, the percentage of Th-1 and Th-2 subpopulations in peripheral blood were significantly increased, by 1.4-fold as compared with normal controls. Serum levels of ALT were inversely proportional to the percentage of Th-1 subpopulations, while directly proportional to that of Th-2 subpopulations. In nonresponders ($N = 16$) to interferon therapy, the percentage of Th-1 subpopulations and Th-1/Th-2 ratio were significantly higher than those in complete responders ($N = 15$). By multivariate logistic regression analysis, HCV genotype non-1b, HCV viral load less than 500 kilocopies/ml, and the lower Th-1/Th-2 ratio could independently merit favorable long-term virological responses. Helper-T cell subpopulations, Th-1 and Th-2, seem to contribute to progression of chronic hepatitis C in a reciprocal fashion. The imbalance between the two subpopulations may determine the final outcome of interferon therapy as one of the host factors.

KEY WORDS: hepatitis C virus; helper-T cell subpopulations; Th-1/Th-2 imbalance; interferon.

Interferon (IFN) therapy has been introduced worldwide in the treatment of chronic hepatitis C (CH-C) for almost a decade. However, a complete response rate by standard regimen has still remained as low as 30% on average. In Japan, HCV genotype 1b is the most common (70%) among patients with CH-C, and it is well known that a complete response rate to IFN monotherapy with this genotype remains less than 20%, the lowest value compared with other genotypes. In addition, it should be emphasized that the standard regimen of IFN therapy for 6 months in Japan costs at least US$ 20–50 thousands per patient. Considering cost and benefit of this therapy, it would be practically acceptable to select proper candidates. Under these circumstances, many clinical studies have been conducted to elucidate the determinants of the final outcome of this therapy, including host factors as well as those of the hepatitis C virus (HCV) itself. As for viral factors, both genotype and viral

Manuscript received August 16, 2001; accepted January 13, 2002.
From the Division of Gastroenterology, International Medical Center of Japan, Tokyo, Japan.
Address for reprint requests: Dr. Naohiko Masaki, Division of Gastroenterology, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan.

load have been established as important parameters influencing biochemical and virological responses (1–3). In addition, the case of genotype 1b, the number of mutations in the NS5A region of HCV genomes has been reported to correlate with the therapeutic responses (4–6). As for host factors, it has been reported that patient age (7) and the extent of chronic liver disease evaluated by histological fibrosis scores (8–11) are independently significant.

Recently, the imbalance among helper-T cell subpopulations, Th-1 (producing IFN- γ and IL-2) and Th-2 (producing IL-4 and IL-10), has been proposed to play a pivotal role in the development of chronic viral infections (12) and autoimmune diseases (13–15), chiefly based on studies using experimental animal models. With respect to CH-C, however, it is still controversial which helper-T cell subpopulation, Th-1 or Th-2, may contribute to its pathogenesis. Napoli et al (16) showed that IFN- γ and IL-2 mRNA expression in liver tissues from CH-C were significantly up-regulated, and correlated with histological fibrosis and portal tract inflammation. On the other hand, the intrahepatic expression of IL-10 mRNA was decreased. They concluded that the progressive liver injury in CH-C is associated with the up-regulation of intrahepatic Th-1-like cytokines and the down-regulation of IL-10, a Th-2-like cytokine. In contrast, Cacciarelli et al (17) demonstrated that serum levels of Th-2-cytokines were significantly increased in patients with CH-C and that those were decreased by interferon therapy in parallel with a decrease in HCV RNA load. Similarly, Tsai et al (18) proposed that activation of Th-2 responses, evaluated by the hypersecretion of IL-4 and IL-10 from HCV antigen-stimulated $CD4^+$ T cells, may play a role in the development of HCV chronicity. Such conflicting data may arise from the situations in which different methodologies are applied to evaluate cytokine levels alone. Recently, identification of helper-T cell subpopulations, Th-1 or Th-2, at the single cell level has become practical with development of intracellular cytokine assay using flow cytometry (19). These circumstances prompted us to conduct a prospective human study in IFN-treated patients with CH-C, in particular, from the viewpoint of Th-1/Th-2 imbalance and long-term virological responses.

MATERIALS AND METHODS

Thirty-one patients with CH-C (*M/F* = 19/12; 49.0 ± 13.0 years old) were consecutively enrolled and treated with IFN for 6 months according to standard regimen (6–10 MU IFN-α or 6 MU IFN- β, every day for the initial 2 weeks, then 3 times a week for the rest of 22 weeks). Th-1 (ratio of IFN- γ^+ cells to $CD4^+$ T cells) and Th-2 (ratio of IL-4^+ cells to $CD4^+$ T cells) were determined before and at 4 weeks after the start of the therapy, as reported elsewhere (19) and detailed below. As a control, blood samples were collected from 35 healthy volunteers with normal liver function (*M/F* = 17/18; 34.0 ± 10.0 years old). The outpatient doctors in charge of each patient were not informed of these data until the date of final analysis. Patients were retrospectively divided into 2 groups according to the absence (complete responders: CR) or presence (nonresponders: NR) of HCV RNA at 6 months after the end of IFN therapy.

Antibodies and Reagents. Fluorescein isothiocyanate (FITC) -conjugated monoclonal antibody (MAb) against human IFN-γ (mouse IgG_{2b}) and a phycoerythrin (PE) -conjugated MAb against human IL-4 (mouse IgG_1) were purchased from Becton Dickinson (San Jose, California, USA). A *(R)*-phycoerythrin covalently linked to cyanin 5.1 (PC5) -conjugated MAb against CD4 (mouse IgG_1) was generated at Beckman Coulter. For the negative controls, FITC- or PE-conjugated MAbs (mouse IgG_{2b} and IgG_1; Becton Dickinson and Company, Franklin Lakes, New Jersey, USA) against keyhole limpet hemocyanin (KLH), an antigen not expressed on human cells, were used. Phorbol 12-myristate 13-acetate (PMA), ionomycin, and brefeldin A were obtained from Sigma Chemical Co (St. Louis, Missouri, USA).

Cell Culture and Staining. Peripheral whole blood, diluted with 2× RPMI-1640 with 2 mM L-glutamine (Wako Junyaku Co., Ltd., Osaka, Japan), was stimulated with 25 ng/ml PMA and 1 μg/ml ionomycin for 4 h in the presence of 10 μg/ml brefeldin A at 37°C in 7% CO_2. After stimulation, 20 μl of PC5-conjugated anti-CD4 MAb was added to 500 μl of diluted blood in the dark at room temperature for 15 min, then 4 ml of FACS Lysing Solution (Becton Dickinson and Company) were added for 5 min to lyse the erythrocytes. Cells were centrifuged at 500 *g* for 5 min, and the supernatant was removed, One and a half ml of FACS Permeabilizing Solution (1.5 ml; Becton Dickinson and Company) was added for 10 min at room temperature to make cells permeable. Cells were washed with 3 ml of phosphate-buffered saline (Wako Junyaku Co., Ltd.) and centrifuged at 500 *g* for 5 min. For the immunostaining of intracellular cytokines, the supernatant was removed and 20 μl of FITC-conjugated MAb against human IFN- γ and PE-conjugated MAb against human IL-4 were added for 30 min in the dark at room temperature. For the negative controls, cells were stained with FITC- or PE-conjugated anti-KLH MAb. Finally, cells were resuspended and fixed in 500 μl of 1% paraformaldehyde (Wako Junyaku Co., Ltd.).

Flow Cytometry. A FACScan flow cytometer (Becton Dickinson and Company) equipped with a 15 mW argon ion laser and filter settings for FITC (530 nm), PE (585 nm) and PC5 emitting in deep red (670 nm) were used. Cells (50,000–100,000) were acquired in the list mode and analyzed with CELLQuest software (Becton Dickinson and Company). Analysis gates were set for lymphocytes according to forward and side-scatter properties. Results are expressed as the percentage of each cytokine-producing cells in the $CD4^+$ cell population.

TABLE 1. DEMOGRAPHIC FEATURES AND LABORATORY DATA OF COMPLETE RESPONDERS AND NONRESPONDERS TO INTERFERON THERAPY

Parameters	*Complete responders (N = 15)*	*Nonresponders (N = 16)*	*Significance**
Age	49.8 ± 12.9	48.3 ± 13.5	NSa
Gender (M/F)	10/5	9/7	NSb
WBC (/μl)	5293 ± 1855	5471 ± 1208	NSa
PLT (× 10^4/μl)	16.8 ± 5.7	16.2 ± 4.6	NSa
ICG_{R15} (%)	10.3 ± 4.4	15.2 ± 10.1	NSa
Alb (g/dl)	4.3 ± 0.4	4.2 ± 0.4	NSa
ALT (units/liter)	183 ± 146	87 ± 67	$P = 0.019$a
Grading score (A1/A2/A3)	4/10/1	6/8/2	NSc
Staging score (F0/F1/F2/F3/F4)	0/11/4/0/0	1/8/4/2/1	NSc
HCV viral load (kilocopies/ml)	195 ± 217	831 ± 853	$P < 0.001$a
HCV genotype (1b/2a/2b)	6/7/2	12/1/1	$P = 0.033$c
IFN type (α/ β/ $\alpha + \beta$)	11/3/1	15/0/1	NSc
IFN total dose (MU)	667 ± 300	783 ± 304	NSa

*a, Mann-Whitney *U*-test; b, Fisher's exact test; c, Pearson's χ^2 test.

Statistical Analysis. Differences between the two groups were tested using Mann-Whitney *U* test, Fisher's exact test, or Pearson's χ^2 test. Correlation of serum ALT levels with Th-1 or Th-2 subpopulations before IFN therapy was assessed by Pearson's correlation coefficient. To find out the variables to affect the final outcomes, logistic regression analysis was performed using age, gender, peripheral white blood cell counts (WBC), platelet counts (PLT), serum albumin (Alb), ALT, ICG_{R15} (indocyanin green clearance test), histological scores (grading, staging), HCV RNA genotype, serum viral load (Amplicor HCV monitor), total dose of IFN, Th-1, Th-2, and Th-1/Th-2 ratio.

RESULTS

Comparisons Between Complete Responders and Nonresponders to IFN Therapy. As shown in Table 1, the patients were finally divided into 15 complete responders (CR group) and 16 nonresponders (NR group) at 6 months after the end of IFN therapy. Serum ALT levels were significantly higher in the CR group, almost twice as much as those in the NR group. Pretreatment viral load was significantly higher in the NR group, and genotype 1b was more frequent in the NR group. Other parameters including the type and total dose of used IFN were not different between the two groups.

Th-1 and Th-2 Subpopulations in CH-C. As shown in the upper panel of Figure 1, the percentage of Th-1 subpopulations was significantly increased in CH-C, almost by 1.4-fold as compared with that in normal control (23.2 ± 7.2 vs 17.0 ± 5.8, $P < 0.001$). Similarly, the percentage of Th-2 subpopulations was significantly increased in CH-C as compared with that in normal control (the middle panel: 3.4 ± 1.7 vs 2.4 ± 1.0, $P < 0.05$). Consequently, Th-1/Th-2 ratio before IFN therapy in CH-C was similar to that in normal control (the lower panel). The percentage of Th-1 and Th-2 subpopulations and Th-1/Th-2 ratio were unchanged at 4 weeks after the start of IFN therapy in both CR and NR (data not shown). Serum ALT levels were marginally in inverse proportion to the percentage of Th-1 subpopulations before IFN therapy (Figure 2, the upper panel; $r = -0.351$, $P = 0.053$), while they are directly proportional to that of Th-2 subpopulations (Figure 2, middle panel; $r = 0.384$, $P = 0.033$). Thereby, the Th-1/Th-2 ratio before IFN therapy was inversely correlated with serum ALT levels (the lower panel; $r = -0.444$, $P = 0.012$).

In order to investigate whether the final outcome of IFN therapy may be affected by pretreatment levels of helper-T cell subpopulations, differences in Th-1, Th-2, and the Th-1/Th-2 ratio between CR and NR were retrospectively analyzed. As shown in the upper panel of Figure 3, the percentage of Th-1 subpopulations in NR (26.6 ± 6.7) was significantly higher than that in CR (19.5 ± 5.8; $P < 0.01$) and in normal control (17.0 ± 5.8; $P < 0.001$). In contrast, the percentage of Th-2 subpopulations in NR (2.8 ± 1.1) was not different from that in CR (3.9 ± 2.1) and in normal control (2.4 ± 1.0). However, the percentage of Th-2 subpopulations in CR was significantly higher than that in normal control ($P < 0.05$; Figure 3, middle panel). Thus, as shown in the lower panel of Figure 3 the Th-1/Th-2 ratio in NR (10.1 ± 2.8) was significantly higher than that in CR (6.5 ± 3.5; $P < 0.01$) and in normal control (8.1 ± 3.4; $P < 0.05$). The Th-1/Th-2 ratio in CR was not different from that in normal controls. Such differences between CR and NR were also confirmed for Th-1 subpopulations and the Th-1/Th-2 ratio at 4 weeks after the start of IFN therapy, although it was less significant than those at

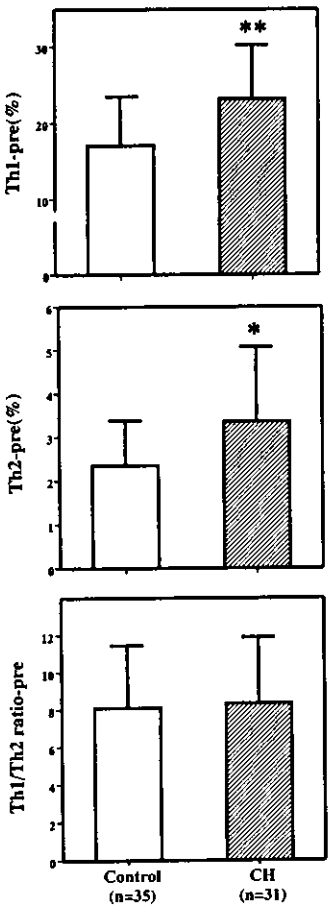

Fig 1. Th-1 and Th-2 subpopulations in chronic hepatitis C. Th-1 subpopulations (upper panel), Th-2 subpopulations (middle panel) and Th-1/Th-2 ratio (lower panel) at pretreatment in patients with chronic hepatitis C (CH, $N = 31$) were compared with those in normal controls ($N = 35$). Data are shown as the mean (column) and standard deviation (bar) in each group. Both Th-1 and Th-2 subpopulations in CH were significantly higher than those in normal controls (**$P < 0.001$ and *$P < 0.05$, respectively, by Mann-Whitney U test).

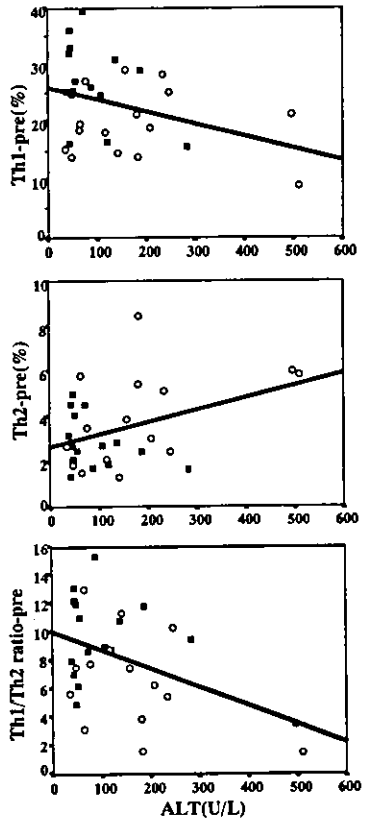

Fig 2. Correlation of helper-T cell subpopulations with serum ALT levels. Serum ALT levels were in marginally inverse proportion to the percentage of Th-1 subpopulations before IFN therapy (upper panel; $r = -0.351$, $P = 0.053$), but were directly proportional to that of Th-2 subpopulations (middle panel; $r = 0.384$, $P = 0.033$). Hence, the Th-1/Th-2 ratio was inversely proportional to serum ALT levels (the lower panel; $r = -0.444$, $P = 0.012$). Open circle (○) and closed square (■) denote virological complete responders and nonresponders to interferon therapy, respectively.

pretreatment. At 4 weeks, the percentage of Th-1 subpopulations in NR (26.2 ± 7.7) was marginally higher than that in CR (20.3 ± 7.2; $P = 0.072$). Furthermore, the Th-1/Th-2 ratio in NR (8.3 ± 1.7) was significantly higher than that in CR (6.2 ± 3.9; $P = 0.022$).

Factors Affecting Final Outcome of IFN Therapy. To find out the variables contributing to the long-term virological responses to IFN therapy, logistic regression analysis was performed. The cutoff for HCV viral load was set at its mean serum levels before IFN therapy (500 kilocopies/ml). HCV genotypes were categorized into 1b and 2a + 2b, because of the small number in the latter two genotypes. By univariate analysis, eight variables (ALT, HCV genotype, HCV viral load, Th-1-pre, Th-2-pre, Th-1/Th-2 ratio-pre, Th-1-4wk, and Th-1/Th-2 ratio-4wk) achieved P values less than 0.1 (Table 2). These eight variables were subjected to multivariate analysis with forward stepwise selection, and three variables—HCV genotype, HCV viral load, and pretreatment Th-1/Th-2 ratio—were found to be independent predictors for final outcome of IFN therapy. As compared with genotypes 2a and 2b, genotype 1b does not merit the long-term virological responses (odds ratio = 42.769, $P = 0.041$). In addition, more than 500 kilocopies/ml of HCV viral load (odds ratio = 44.723, $P = 0.036$) and the higher Th-1/Th-2 ratio before IFN therapy (odds ratio for 1.0 increment = 2.292, $P = 0.035$) are also unfavorable predictors (Table 3).

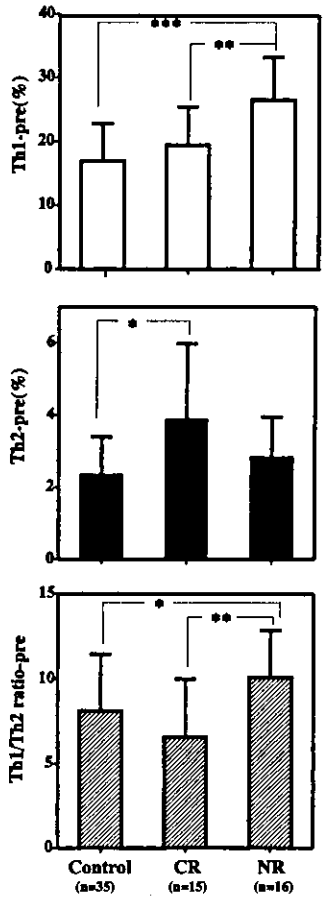

Fig 3. Comparisons of helper-T cell subpopulations among normal control, virological complete responders (CR), and nonresponders (NR) to interferon therapy. As shown in the upper panel, the percentage of peripheral Th-1 subpopulations in NR was significantly higher than that in CR and in normal controls. The percentage of Th-2 subpopulations in CR was significantly higher than that in normal controls (middle panel). Hence, as shown in the lower panel, the Th-1/Th-2 ratio in NR was significantly higher than that in CR and in normal controls. Data are shown as the mean (column) and standard deviation (bar) in each group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, by Mann-Whitney U test).

DISCUSSIONS

The present study is the first report to document that both helper-T cell subpopulations, Th-1 and Th-2, are significantly increased in peripheral blood of patients with CH-C, as compared with normal controls. It should be emphasized that our determinations of helper-T cell subpopulations depend on the ability of T lymphocytes to produce intracellular cytokines in response to PMA and ionomycin. Hence, individual helper-T cells can be classified at the single cell level, according to their potentiality to produce one of the two kind of cytokines, IFN- γ (Th-1 cytokine) or IL-4 (Th-2 cytokine). As shown in Figure 2, serum ALT levels were correlated with the percentage of Th-1 and Th-2 subpopulations in a reciprocal fashion. This might suggest that Th-1 and Th-2 subpopulations play different roles in progression of CH-C. Th-1/Th-2 cytokines in CH-C have been evaluated by various parameters such as serum protein levels, intrahepatic mRNA expressions or *in vitro* cytokine responses to recombinant HCV antigens. First, Cacciarelli et al (17) showed that serum levels of Th-1 cytokines (IFN- γ, IL-2) and Th-2 cytokines (IL-4, IL-10) were elevated and that, in particular, the extent of the increase in the latter cytokines was more prominent. Reiser et al (20) also showed that serum levels of Th-2 cytokines (IL-4, IL-10) were increased as a systemic response, not correlating with numbers of the locally infiltrating Th-2 subpopulations in the liver. Secondly, Napoli et al (16) reported that intrahepatic mRNA expressions of IFN- γ and IL-2 were up-regulated and correlated with histological fibrosis and portal tract inflammation, while those of IL-10 were down-regulated. Finally, Tsai et al (18) and Eckels et al (21) demonstrated that *in vitro* cytokine responses to recombinant HCV antigens were confined to IL-4 and IL-10, and proposed that such Th-2 predominance might be conducive to viral persistence. Taken together, the imbalance among helper-T cell subpopulations in CH-C could be schematically hypothesized, as detailed in Figure 4. Briefly, in HCV chronically infected liver, cytokine profiles are shifted to Th-1 predominance. In contrast, in peripheral blood, they are shifted to Th-2 predominance. Since it is well documented that Th-1 and Th-2 are mutually inhibitory (22), such a reciprocal status of Th-1 and Th-2 cytokines in each compartment would be possible. Furthermore, if such a mirror image of cytokine

TABLE 2. VARIABLES CONTRIBUTING TO LONG-TERM VIROLOGICAL RESPONSES TO INTERFERON THERAPY (UNIVARIATE LOGISTIC REGRESSION ANALYSIS)

Variable	B	*Exp(β)*	P
ALT	−0.010	0.990	0.054
HCV genotype			
1b	*		
2a + 2b	−1.872	0.154	0.024
HCV viral load (kilocopies/ml)			
≤ 500	*		
> 500	1.872	6.500	0.040
Th-1-pre	0.180	1.198	0.013
Th-2-pre	−0.417	0.659	0.097
Th-1/Th-2-pre	0.368	1.445	0.012
Th-1-4wk	0.106	1.112	0.057
Th-1/Th-2-4wk	0.251	1.285	0.088

*Reference group.

TABLE 3. VARIABLES CONTRIBUTING TO LONG-TERM VIROLOGICAL RESPONSES TO INTERFERON THERAPY (MULTIVARIATE LOGISTIC REGRESSION ANALYSIS)

Variable	B	*Wald* χ^2	P *value*	*Exp* (β)	*95% CI*
HCV genotype					
1b	3.756	4.190	0.041	42.769	1.173–1559.122
2a + 2b	*				
HCV viral load (kilocopies/ml)					
≤ 500	*				
> 500	3.800	4.407	0.036	44.723	1.287–1554.180
Th-1/Th-2-pre	0.829	4.450	0.035	2.292	1.061–4.954

*Reference group.

profiles between the two compartments exists, our findings that serum ALT levels were inversely correlated with peripheral Th-1/Th-2 ratio as well as the percentage of Th-1 subpopulations might be consistent with Th-1 predominance in the liver with the more active inflammation.

It should be emphasized that the final outcome of IFN therapy could be affected by host helper-T cell imbalance at pretreatment. As shown in Figure 3, the Th-1/Th-2 ratio as well as the percentage of Th-1 subpopulations were significantly lower in CR than those in NR. By multivariate logistic regression analysis, besides HCV genotype and viral load as viral factors, only Th-1/Th-2 ratio at pretreatment was selected as a host factor to determine long-term virological responses. Our findings that the lower Th-1/Th-2 ratio in peripheral blood merits the more favorable responses to IFN therapy would suggest that relative activation of humoral immunity over cellular immunity (Th-2 predominance in peripheral blood) could be a prerequisite to achieve complete eradication of HCV by IFN therapy. Again, considering the probable mirror image between peripheral blood and the liver, this finding could indicate that more activated cell-mediated immunity (Th-1 predominance) in the liver is beneficial to HCV clearance. This hypothesis would be supported by our findings that serum ALT levels in CR were significantly higher than those in NR (Table 1), since it has been well documented that hepatocytotoxicity in CH-C is causally related to enhanced immune recognition of viral antigens through activation of $CD8^+$ T cells and NK cells (cytotoxic T lymphocytes in the liver) by Th-1 cytokine (23). At present, however, its implication has not been clarified, since IFN administration did not affect the percentage of peripheral helper-T cell subpopulations at all in this study, at least at 4 weeks after the start of IFN therapy (data not shown). Similar observation was recently reported by Saito et al (24), that the pretreatment $CD11^+$, $CD8^-$ cell population was significantly lower in responders than in nonresponders and was selected as an independent factor, besides pretreatment HCV viral load, to affect final response to IFN therapy. However, in their studies, the biological role of this cell population could not be defined. In addition, Yee et al recently reported that polymorphisms of the IL-10 promoter region could be closely associated with virological responses to a combination of interferon and ribavirin (25), whereas they did not provide any data about serum levels of IL-10 at pretreatment. Steady-state expressions of Th-1 and Th-2 cytokines at pretreatment and their changes by antiviral therapy should be quantitatively determined in the liver as well as in peripheral blood by further investigations.

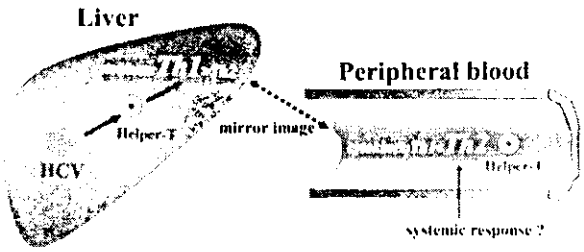

Fig 4. Proposed schema for Th-1/Th-2 cytokine imbalance in chronic hepatitis C. As reviewed in the Discussion, previous studies from other laboratories strongly suggest that, in HCV chronically infected liver, cytokine profiles are shifted to Th-1 predominance. On the other hand, they are shifted to Th-2 predominance in peripheral blood. There seems to be a mirror image between the two compartments in patients with chronic hepatitis C.

In conclusion, pretreatment determinations of helper-T cell subpopulations may aid in predicting the final outcome of IFN therapy and could be one of the useful tools to select proper candidates for IFN therapy.

REFERENCES

1. Martinot-Peignoux M, Marcellin P, Pouteau M, Castelnau C, Boyer N, Poliquin M, Degott C, Descombes I, Le Breton V, Milotova V, Benhamou JP, Erlinger S: Pretreatment serum

hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. Hepatology 22:1050–1056, 1995
2. Martinot-Peignoux M, Boyer N, Pouteau M, Castelnau C, Giuily N, Duchatelle V, Auperin A, Degott C, Benhamou JP, Erlinger S, Marcellin P: Predictors of sustained response to alpha interferon therapy in chronic hepatitis C. J Hepatol 29:214–223, 1998
3. Knolle PA, Kremp S, Hohler T, Krummenauer F, Schirmacher P, Gerken G: Viral and host factors in the prediction of response to interferon-alpha therapy in chronic hepatitis C after long-term follow-up. J Viral Hepatitis 5:399–406, 1998
4. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C: Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b: sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest 96:224–230, 1995
5. Enomoto N, Sakuma J, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C: Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81, 1996
6. Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Kumada H: Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. Hepatology 25:745–749, 1997
7. Vandelli C, Renzo F, Braun HB, Tisminetzky S, Albrecht M, De Palma M, Ranzi A, Di Marco G, Stroffolini T, Baralle F, Ventura E, Michel G: Prediction of successful outcome in a randomised controlled trial of the long-term efficacy of interferon alpha treatment for chronic hepatitis C. J Med Virol 58:26–34, 1999
8. Tsubota A, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Kumada H: Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. Hepatology 19:1088–1094, 1994
9. Yoshioka K, Higashi Y, Yamada M, Aiyama T, Takayanagi M, Tanaka K, Okumura A, Iwata K, Kakumu S: Predictive factors in the response to interferon therapy in chronic hepatitis C. Liver 15:57–62, 1995
10. Shindo M, Arai K, Okuno T. The clinical value of grading and staging scores for predicting a long-term response and evaluating the efficacy of interferon therapy in chronic hepatitis C. J Hepatol 26:492–497, 1997
11. Kim SR, Hayashi Y, Yoon S, Taniguchi M, Yang MK, Kim KI, Kim MM, Saeki K, Nukata I, Imoto S: Prediction of efficacy of interferon treatment of chronic hepatitis C by multivariate analysis and a new classification. Pathol International 48:215–220, 1998
12. Gazzinelli RT, Makino M, Chattopadhyay SK, Snapper CM, Sher A, Hugin AW, Morse HC III: $CD4^+$ subset regulation in viral infection: preferential activation of Th2 cells during progression of retrovirus-induced immunodeficiency in mice. J Immunol 148:182–188, 1992
13. Healey D, Ozegbe P, Arden S, Chandler P, Hutton J, Cooke A: *In vivo* activity and *in vitro* specificity of $CD4^+$ Th1 and Th2 cells derived from the spleens of diabetic NOD mice. J Clin Invest 95:2979–2985, 1995
14. Peng SL, Moslehi J, Craft J: Roles of interferon-γ and interleukin-4 in murine lupus. J Clin Invest 99:1936–1946, 1997
15. Segal R, Bermas BL, Dayan M, Kalush F, Shearer GM, Mozes E: Kinetics of cytokine production in experimental systemic lupus erythematosus: involvement of T helper cell 1/T helper cell 2-type cytokines in disease. J Immunol 158:3009–3016, 1997
16. Napoli J, Bishop GA, McGuinness PH, Painter DM, McCaughan GW: Progressive liver injury in chronic hepatitis C infection correlates with increased intrahepatic expression of Th1-associated cytokines. Hepatology 24:759–765, 1996
17. Cacciarelli TV, Martinez OM, Gish RG, Villanueva JC, Krams SM: Immunoregulatory cytokines in chronic hepatitis C virus infection: pre- and posttreatment with interferon alfa. Hepatology 24:6–9, 1996
18. Tsai S-L, Liaw Y-F, Chen M-H, Huang C-Y, Kuo GC: Detection of type2-like T-helper cells in hepatitis C virus infection: implications for hepatitis C virus chronicity. Hepatology 25:449–458, 1997
19. Kawakami Y, Nabeshima S, Furusyo N, Sawayama Y, Hayashi J, Kashiwagi S: Increased frequency of interferon-γ-producing peripheral blood $CD4^+$ T cells in chronic hepatitis C virus infection. Am J Gastroenterol 95:227–232, 2000
20. Reiser M, Marousis CG, Nelson DR, Lauer G, Gonzalez-Peralta RP, Davis GL, Lau JYN: Serum interleukin 4 and interleukin 10 levels in patients with chronic hepatitis C virus infection. J Hepatol 26:471–478, 1997
21. Eckels DD, Tabatabail N, Bian T-H, Wang H, Muheisen SS, Rice CM, Yoshizawa K, Gill J: *In vitro* human Th-cell responses to a recombinant hepatitis C virus antigen: failure in IL-2 production despite proliferation. Hum Immunol 60:187–199, 1999
22. Abbas AK, Murphy KM, Sher A: Functional diversity of helper T lymphocytes. Nature 383:787–793, 1996
23. Rehermann B, Chisari FV: Cell mediated immune response to the hepatitis C virus. Curr Topics Microbiol Immunol 242:299–325, 2000
24. Saito H, Ebinuma H, Satoh I, Miyaguchi S, Tada S, Iwabuchi N, Kumagai N, Tsuchimoto K, Morizane T, Ishii H: Immunological and virological predictors of outcome during interferon-α therapy of chronic hepatitis C. J Viral Hepatitis 7:64–74, 2000
25. Yee LJ, Tang J, Gibson AW, Kimberly R, van Leeuwen DJ, Kaslow RA: Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. Hepatology 33:708–712, 2001

Long-Term Histologic and Virologic Outcomes of Acute Self-Limited Hepatitis B

Nobukazu Yuki,[1] Takayuki Nagaoka,[2] Masatoshi Yamashiro,[2] Kiyoshi Mochizuki,[3] Akira Kaneko,[1] Keiji Yamamoto,[1] Masao Omura,[2] Kazumasa Hikiji,[2] and Michio Kato[1]

The long-term impact of acute self-limited hepatitis B on the liver is unknown. Fourteen patients were recalled at a median of 4.2 years (range, 1.8-9.5 years) after the onset of acute hepatitis B. All showed clinical and serologic recovery with circulating hepatitis B surface antigen (HBsAg) clearance. Antibody to HBsAg (anti-HBs) had developed in 12 patients. Nine underwent liver biopsies at a median of 7.2 years, and histologic findings were evaluated using Ishak scores. Serum samples and frozen liver tissue were subjected to real-time detection polymerase chain reaction (PCR) to quantify the surface and X regions of the hepatitis B virus (HBV) genome and qualitative PCR to detect the covalently closed circular (ccc) HBV DNA replicative intermediate. Three patients had low levels of circulating HBV DNA up to 8.9 years after the onset, whereas both HBV DNA surface and X regions were found in the liver of all 9 patients examined, including 7 negative for serum HBV DNA. Liver viral loads assessed by the 2 regions showed a significant correlation ($r = 0.946$; $P = .008$), and all patients tested positive for ccc HBV DNA. Liver fibrosis and mild inflammation persisted in 8 patients. The fibrosis stage had relation to peak serum HBV DNA in the acute phase ($P = .046$) but not to liver viral loads in the late convalescent phase. In conclusion, occult HBV infection persists in the liver and is accompanied by abnormal liver histology for a decade after complete clinical recovery from acute self-limited hepatitis B. (HEPATOLOGY 2003;37:1172-1179.)

The clearance of circulating hepatitis B surface antigen (HBsAg) and appearance of antibody to HBsAg (anti-HBs) with normalization of liver function have been generally accepted as evidence of clinical and serologic recovery from acute hepatitis B. However, in chronic HBsAg carriers, there is growing evidence that hepatitis B virus (HBV) DNA sequences persist in the liver for years after seroclearance of HBsAg and seroconversion to anti-HBs.[1-3] Although the clinical and pathologic implications of occult HBV infection in the liver are unknown, viral eradication is unlikely to be achieved once chronic HBV infection has been established. The cytotoxic T-lymphocyte (CTL) response is weak or undetectable in chronic HBV infection. In contrast, a vigorous, polyclonal, and HBV-specific CTL response against multiple HBV epitopes is readily detectable during acute self-limited HBV infection.[4-7] HBV-specific CTLs further persist in the blood for several decades after recovery from acute hepatitis B.[7,8] In the face of an enhanced immune response leading to disease resolution, the virologic outcomes of acute self-limited hepatitis B may differ from those of HBsAg seroclearance and anti-HBs seroconversion in the course of chronic HBV infection.

At present, the long-term histologic and virologic impact of acute self-limited hepatitis B on the liver is unexplored. Studies using polymerase chain reaction (PCR) to detect HBV DNA sequences have shown that low levels of circulating HBV DNA can persist after clinical and serologic recovery from acute hepatitis B but tend to disappear after long-term follow-up.[9,10] Peripheral blood mononuclear cells are known as the site of persistent HBV infection long after recovery from acute hepatitis B[11-13] and may contribute to continuous priming of the HBV-specific CTL response. However, direct evidence is very limited for the possibility of HBV infection persisting in the liver in the late convalescent phase and exerting viru-

Abbreviations: HBsAg, hepatitis B surface antigen; anti-HBs, antibody to hepatitis B surface antigen; HBV, hepatitis B virus; CTL, cytotoxic T lymphocyte; PCR, polymerase chain reaction; anti-HBc, antibody to hepatitis B core antigen; ccc, covalently closed circular; WHV, woodchuck hepatitis virus.

From the [1]Department of Gastroenterology, Osaka National Hospital, Osaka, Japan; [2]SRL Inc., Hino, Japan; and [3]Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Japan.

Received April 18, 2002; accepted January 30, 2003.

Address reprint requests to: Nobukazu Yuki, M.D., Department of Gastroenterology, Osaka National Hospital, Hoenzaka 2-1-14, Chuo-ku, Osaka 540-0006, Japan. E-mail: yuki@onh.go.jp; fax: (81) 06-6943-6467.

0270-9139/03/3705-0027$30.00/0

doi:10.1053/jhep.2003.50171

lence. To address these issues, liver histologic and virologic outcomes were investigated in patients with a remote history of acute self-limited HBV infection.

Patients and Methods

Patients. Between December 1990 and August 1998, 19 patients with acute hepatitis B were admitted to our institution. A diagnosis of acute hepatitis B was based on elevated serum alanine aminotransferase activity, detection of HBsAg and immunoglobulin M antibody to hepatitis B core antigen (anti-HBc) in the serum, and the recent onset of jaundice and other typical symptoms. Coinfection with hepatitis A, C, D, or E; Epstein-Barr virus; or cytomegalovirus was ruled out because of the negative results of serologic tests. Confounding etiology of liver disease, human immunodeficiency virus infection, and other immunodeficient diseases were not found in any patient. In 2000, this complete series of patients with acute hepatitis B was contacted for follow-up. Fourteen patients (74%) revisited our institution and were reevaluated for clinical, serologic, histologic, and virologic recovery from the disease. The group comprised 11 men and 3 women ranging in age from 26 to 65 years (median, 43 years). All patients were negative for hepatitis C virus antibody by an enzyme-linked immunosorbent assay (Ortho Diagnostic Systems Co., Ltd., Tokyo, Japan) and serum hepatitis C virus RNA by PCR.[14] They had no history of administration of hepatotoxic drugs and showed no evidence of autoimmune liver disease. Their daily alcohol intake was reevaluated based on a detailed questionnaire and interview. Of the 14 patients, 5 were nondrinkers. Four patients did not have a habit of daily drinking, and their alcohol intake was less than 5 g/d. Alcohol consumption exceeded 5 g/d in the remaining 5 patients, but the levels were relatively low (10-15 g/d in 4 patients and 40-45 g/d in 1 patient). After a median of 4.2 years (range, 1.8-9.5 years) from the onset of the disease, the liver function of the 14 patients was reevaluated using blood chemistry and ultrasonography. Serum samples were obtained from all patients and subjected to serologic and virologic tests for HBV. Ultrasound-guided liver biopsies were performed on 9 patients at 1.8 to 9.5 years (median, 7.2 years) after resolution, and paired serum and liver samples were obtained. The study was approved by the local research ethics committee in accordance with the 1975 Declaration of Helsinki, and all patients provided written informed consent.

Serologic and Virologic Assays. Serum samples were tested for HBsAg, anti-HBs, hepatitis B e antigen, and antibody to hepatitis B e antigen with enzyme immunoassays (Abbott Laboratories, North Chicago, IL). Anti-HBc and immunoglobulin M class anti-HBc were measured with radioimmunoassays (Abbott Laboratories). Serum HBV DNA was quantitatively detected by real-time detection PCR based on TaqMan chemistry as previously reported.[15] Serial serum samples collected at 1- to 4-week intervals in the acute phase of infection and the late convalescent sera had been stored at −80°C without thawing and were subjected to HBV DNA PCR. In brief, total DNA was extracted from 100 μL of serum. Purified DNA was resuspended in 20 μL of distilled water, and a 10-μL aliquot of DNA solution (50-μL serum equivalent) was used for real-time detection PCR. Amplification was performed using primers corresponding to conserved sequences of the surface and X regions. A portion of the HBV surface region was amplified using set 2 primers: antisense primer HBSF2 (5′CTTCATCCTGCTGCTATGCCT3′, nucleotide positions 406-426) and sense primer HBSR2 (5′AAAGCCCAGGATGATGGGAT3′, nucleotide positions 608-627). Another portion of the HBV X region was further amplified using set 3 primers: antisense primer HBXF1 (5′ACGTCCTTTGTTTACGTCCCGT3′, nucleotide positions 1414-1435) and sense primer HBXR1 (5′CCCAACTCCTCCCAGTCCTTAA3′, nucleotide positions 1723-1744). Using serum samples obtained at the onset of the disease, HBV genotypes were further determined by restriction fragment length polymorphism analysis of the surface gene region as described previously.[16]

Percutaneous needle liver biopsies were performed using 14-gauge Tru-Cut needles (Hakko Medical Co., Ltd., Nagano, Japan), and biopsy specimens sufficient for histologic and virologic evaluation were obtained. Liver specimens for PCR testing were immediately frozen and then stored at −80°C until PCR testing. For the detection of HBV DNA, total DNA was extracted from liver tissue using a commercially available kit (SMI test EX R and D; Sumitomo Metal Industries, Tokyo, Japan). Purified total hepatic DNA was resuspended in 500 μL of distilled water. A 25-μL aliquot of DNA solution was subjected to quantitative real-time detection PCR using set 2 and set 3 primers, and the relative amounts of hepatic HBV DNA loads were obtained. In preliminary experiments, the efficacy of real-time detection PCR with 2 sets of primers (sets 2 and 3) was evaluated by quantitatively measuring sequential levels of synthetic standard HBV DNA. The detection limit of this system was as few as 10 DNA copies/reaction for each set of primers, and a linear standard curve was obtained between 10^1 and 10^8 DNA copies/reaction.[15] Without gel electrophoresis, the specificity of the assay was confirmed via hybridization with a target-specific fluorogenic probe. To further confirm the sizes of the amplified products, the assay was repeated

Table 1. Long-Term Virologic and Histologic Outcomes of Acute Self-Limited Hepatitis B

Patient No.	Sex	Age (y)	Peak ALT (ULN)	Peak Bilirubin (ULN)	Peak HBV DNA		HBV Genotype	Follow-up (y)	Alcohol Intake (g/d)	HBsAg /Anti-HBs	Serum HBV DNA		Liver HBV DNA			Histologic Outcome	
					S	X					S	X	S	X	cccDNA	Activity Grade	Fibrosis
1	M	53	23.0	26.4	8.0×10^3	4.7×10^3	C	9.5	<5	−/+	<200	<200	4.5×10^3	2.3×10^3	+	0.0.0.1	1
2	M	65	129.3	10.2	8.9×10^7	2.5×10^7	A	8.9	40-45	−/+	<200	<200	1.4×10^4	2.6×10^3	+	0.0.0.0	3
3	M	47	78.7	6.8	1.2×10^8	3.3×10^7	C	8.9	10-15	−/+	1.3×10^3	<200	1.6×10^4	3.3×10^3	+	0.0.0.1	3
4	F	43	27.5	1.0	5.3×10^7	1.6×10^7	C	7.8	0	−/+	2.4×10^4	7.7×10^3	4.4×10^4	1.4×10^4	+	0.0.0.1	3
5	M	60	37.1	2.2	3.0×10^8	1.1×10^8	A	7.3	10-15	−/+	<200	<200	NA	NA	NA	NA	NA
6	M	34	70.0	12.2	NA	NA	NA	7.2	<5	−/+	<200	<200	3.0×10^3	4.6×10^2	+	0.0.0.0	0
7	M	42	46.1	6.6	1.1×10^5	4.2×10^4	C	4.6	0	−/−	7.7×10^2	<200	NA	NA	NA	NA	NA
8	M	26	65.2	13.7	5.0×10^5	8.2×10^4	C	3.7	<5	−/+	<200	<200	NA	NA	NA	NA	NA
9	M	64	63.5	35.1	1.5×10^5	2.2×10^4	C	3.6	0	−/+	<200	<200	5.7×10^3	1.4×10^3	+	0.0.1.1	1
10	F	27	114.5	6.0	5.0×10^6	7.8×10^5	C	3.5	0	−/+	<200	<200	NA	NA	NA	NA	NA
11	M	29	48.3	18.6	2.0×10^6	2.1×10^5	C	2.9	10-15	−/+	<200	<200	1.6×10^3	1.9×10^2	+	0.0.0.1	3
12	M	31	88.5	9.3	3.6×10^7	8.0×10^6	C	2.6	<5	−/−	<200	<200	8.6×10^3	1.4×10^3	+	0.0.0.1	3
13	F	33	65.4	4.9	5.0×10^4	1.8×10^4	C	1.9	0	−/+	<200	<200	NA	NA	NA	NA	NA
14	M	51	80.7	12.2	1.4×10^5	3.6×10^5	C	1.8	10-15	−/+	<200	<200	5.1×10^4	1.8×10^4	+	0.0.0.1	3

NOTE. HBV DNA was detected by real-time detection PCR with 2 sets of primers derived from the surface (S) and X regions, and results are expressed in genome copies per milliliter serum or milligram liver. Ishak score is given to liver histology in the following order: piecemeal necrosis, 0-4; confluent necrosis, 0-6; focal necrosis, 0-4; portal inflammation, 0-4; and fibrosis, 0-6.

Abbreviations: ALT, alanine aminotransferase; ULN, upper limit of normal; NA, not available.

without the fluorogenic probe and the PCR products were run on 3% agarose gels with ethidium bromide and visualized under UV light.

Using PCR primers flanking the direct repeat region, the covalently closed circular (ccc) HBV DNA replicative intermediate in the liver was further detected by PCR. The methodology used was similar to that reported previously except that the unconserved 3′ nucleotide was deleted from the DRR3 primer.[17] In brief, the PCR was performed on a 5-μL aliquot of hepatic DNA solution using primers DRF1 (5′GTCTGTGCCTTCTCATCTGC3′, nucleotide positions 1553-1572) and modified DRR3 (5′AGTATGGTGAGGTGAGCAATG3′, nucleotide positions 2040-2060) for 30 cycles at 94°C for 1 minute, 53°C for 1.5 minutes, and 72°C for 3 minutes. Seminested PCR was then performed using primers DRF1 and DRR2 (5′ACAAGAGATGATTAGGCAGAGG3′, nucleotide positions 1830-1851) for 30 cycles at 94°C for 1 minute, 49°C for 1.5 minutes, and 72°C for 3 minutes. The products were analyzed on 3% agarose gels. In preliminary experiments, PCR products were observed for the liver from HBsAg-positive control patients but not from individuals without HBV infection. The incomplete Dane particle HBV DNA in sera was not amplified with the primers used, showing specificity for double-stranded DNA in the direct repeat region. To rule out the effect of DNA repair activity in liver nuclei on the specificity, DNA extraction was performed on normal liver tissue from 2 HBV-uninfected individuals in the presence of 10^2 to 10^7 copies of the partially double-stranded virion HBV DNA and purified DNA was subjected to the PCR procedure. All samples remained negative, thus confirming the specificity. Direct sequencing of the PCR amplicons was also performed bidirectionally by the dideoxy method with the DNA sequencing kit/ABI Prism Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Tokyo, Japan), and specific amplification was confirmed. The threshold levels of hepatic HBV DNA, which give positive results for ccc HBV DNA, were further determined based on serial 2-fold dilution of hepatic DNA solution containing ccc HBV DNA.

The real-time detection PCR method used is a simple and specific assay. The chance of contamination is reduced via monitoring and calculation of fluorescent signals in a single sample tube with a closed optical cap. To further avoid contamination in all PCR assays, the contamination avoidance measures of Kwok and Higuchi[18] were strictly applied throughout, and positive and negative controls were used.

Histologic Evaluation. Liver biopsy specimens for histologic evaluation were fixed in formalin and embedded in paraffin for routine staining with hematoxylin-eosin. All specimens were examined by the same experienced pathologist, who was unaware of the biochemical, serologic, and virologic data. Biopsy specimens were semiquantitatively evaluated by the modified histologic activity index described by Ishak et al.[19]

Statistical Analysis. Statistical analysis for group comparisons was performed using the Wilcoxon nonparametric test. Correlations between the variables were calculated using Spearman rank order correlations. A P value less than .05 (2-tailed) was considered to indicate significance.

Results

Table 1 shows the characteristics of the 14 patients with a remote history of acute self-limited hepatitis B.

Clinical and Virologic Profiles at the Onset of Acute Hepatitis B. The peak alanine aminotransferase activity in the acute phase ranged from 23.0 to 129.3 times the upper limit of normal (median, 65.3), and the peak bilirubin levels ranged from 1.0 to 35.1 times the upper limit of normal (median, 9.8). HBV genotypes and the peak serum HBV DNA levels were determined for 13 patients. Eleven patients were infected with genotype C, whereas the remaining 2 had genotype A. Real-time detection PCR to quantify the HBV genome was performed on serial serum samples in the acute phase. In each case, the peak point determined by the surface primers coincided with that determined by the X primers. A significant correlation was observed between the peak levels of the HBV DNA surface region (median, 2.0×10^6; range, 8.0×10^3 to 3.0×10^8 copies/mL) and those of the X region (median, 3.6×10^5; range, 4.7×10^3 to 1.1×10^8 copies/mL) ($r = 0.989$; $P = .001$). Liver function was normalized within 3 months, and HBsAg was cleared from the serum within 5 months.

Clinical and Serologic Recovery After Follow-up. After 1.8 to 9.5 years (median, 4.2 years) from the onset of acute hepatitis B, the 14 patients were examined for clinical and serologic recovery. None of the patients presented symptoms of liver disease, and all were found to have normal livers on ultrasonography. Liver function data, including serum alanine aminotransferase activity, were normal for all patients. They were all clear of HBsAg, and anti-HBs had developed in 12 patients. All had seroconverted to antibody to hepatitis B e antigen and tested positive for anti-HBc. Using sera diluted 200-fold, anti-HBc was detected in only 1 patient. Overall, all patients showed complete clinical and serologic recovery from acute hepatitis B. When sera collected in the late convalescent phase were subjected to HBV DNA PCR, 3 patients (21%) were found to have low levels of circulating HBV DNA up to 8.9 years after the onset of the disease. The HBV DNA surface region of 7.7×10^2 to 2.4×10^4 copies/mL was detected in the 3 patients, whereas the X region of 7.7×10^3 copies/mL was found in only 1 patient. Neither the surface region nor the X region was amplified in the remaining 11 patients.

Liver Virologic and Histologic Outcomes. Virologic and histologic studies were further performed on liver biopsy specimens obtained from 9 patients at 1.8 to 9.5 years (median, 7.2 years) after the onset of the disease. Although only 2 patients remained positive for circulating HBV DNA, both regions of the HBV genome were found in frozen biopsy specimens from all patients by real-time detection PCR using target-specific fluorogenic probes. The sizes of the PCR amplicons were also confirmed by gel electrophoresis (Fig. 1). The relative amounts of liver HBV DNA measured using the surface primers were significantly correlated to those measured using the X primers ($r = 0.946$; $P = .008$). This figure was similar to that for circulating HBV DNA levels in the acute phase (Fig. 2). Using qualitative PCR to amplify the direct repeat region, PCR products were also observed in all biopsy specimens, thus showing the presence of an intact direct repeat region indicative of the ccc HBV DNA replicative intermediate (Fig. 3). To confirm specific amplification, the PCR amplicons of patients 2 and 3 were subjected to direct sequencing. The amplified sequence showed 94% to 98% homology to the reference sequence of an HBV isolate (GenBank accession no. AY123041) (Fig. 4). None of the serum samples collected in the acute phase or in the late convalescent phase tested positive by the PCR methodology. Based on a serial end-point dilution method, the threshold levels of total hepatic HBV DNA to detect ccc HBV DNA were 10 to 90 copies (median, 35) when assessed by the surface primers and 2 to 20 copies (median, 5) when assessed by the X primers. Thus, the ratio of ccc HBV DNA to total HBV DNA was shown to range within 1 $\log_{10}$.

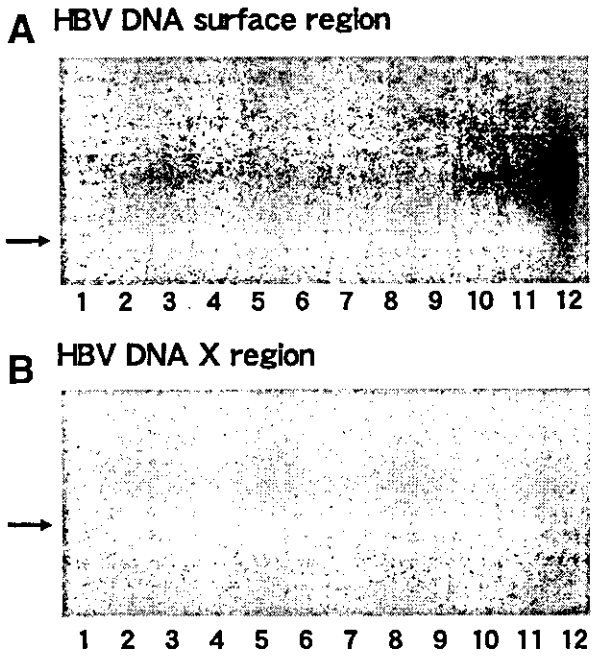

Fig. 1. Detection of the (A) HBV DNA surface and (B) X sequences (222 and 331 base pairs, respectively) in liver tissue from patients with a remote history of acute self-limited hepatitis B. The real-time detection PCR products were run on agarose gels. Lane 1, molecular weight marker (px174 DNA/Hae III [Toyobo]); lanes 2, 3, 4, 5, 6, 7, 8, 9, and 10, liver tissue from patients 1, 2, 3, 4, 9, 11, 12, 14, and 6, respectively; lane 11, positive control (liver tissue from a chronic HBsAg carrier); lane 12, negative control (water).

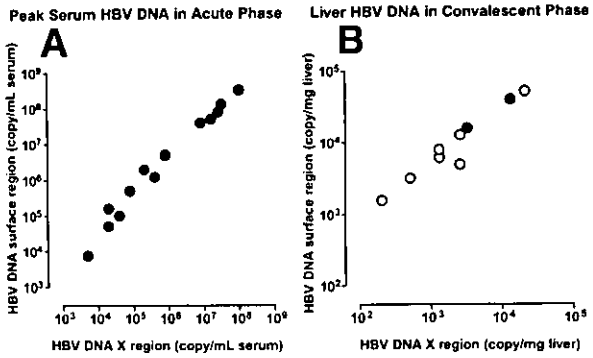

Fig. 2. HBV DNA levels measured by real-time detection PCR using primers derived from the surface region were correlated with those measured by PCR with primers from the X region. (A) A significant correlation was observed for peak serum HBV DNA in the early phase of acute self-limited hepatitis B ($r = 0.989$; $P = .001$). (B) The correlation was also significant for liver HBV DNA, which was persistently detected in the convalescent phase of the disease ($r = 0.946$; $P = .008$). ●, Positive for serum HBV DNA; ○, negative for serum HBV DNA.

Histopathologic examination showed that a low grade of liver inflammation had persisted for a decade. Mild portal inflammation was observed in 7 patients (78%), one of which had mild focal necrosis. None of the patients had piecemeal necrosis or confluent necrosis. Liver fibrosis was frequently observed in the late convalescent phase. The fibrosis score ranged from 0 to 3, and 8 patients (89%) retained liver fibrosis. Six patients showed fibrous expansion of most portal areas accompanied by occasional portal-to-portal bridging, and their fibrosis stage was scored 3. Examples of these histologic alterations encountered are presented in Fig. 5. Overall, only 1 of the 9 patients examined had normal liver histology. The histologic outcomes were unlikely to be affected by an alcoholic factor. The 9 patients with liver biopsies had low levels of alcohol intake (Table 1). Moreover, similar histologic findings were observed for the 5 patients with very low levels of alcohol intake (nondrinker or <5 g/d, if any). Mild portal inflammation persisted in 4 patients, accompanied by mild focal necrosis in 1 patient. Four patients retained liver fibrosis, and the stage was scored 3 in 2 patients. Mild steatosis was seen in patients 1 and 9, but there was no evidence of pericellular/perisinusoidal fibrosis suggestive of alcoholic or nonalcoholic steatohepatitis.

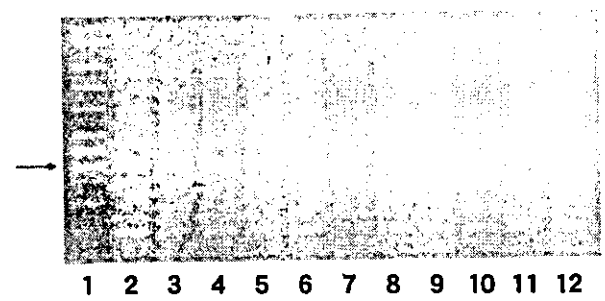

Fig. 3. Detection of a 299-base pair intact direct repeat sequence in liver tissue from patients with a remote history of acute self-limited hepatitis B. Lane 1, molecular weight marker (px174 DNA/Hae III [Toyobo]); lanes 2, 3, 4, 5, 6, 7, 8, 9, and 10, liver tissue from patients 1, 2, 3, 4, 9, 11, 12, 14, and 6, respectively; lane 11, positive control (liver tissue from a chronic HBsAg carrier); lane 12, negative control (liver tissue from a patient without HBV infection).

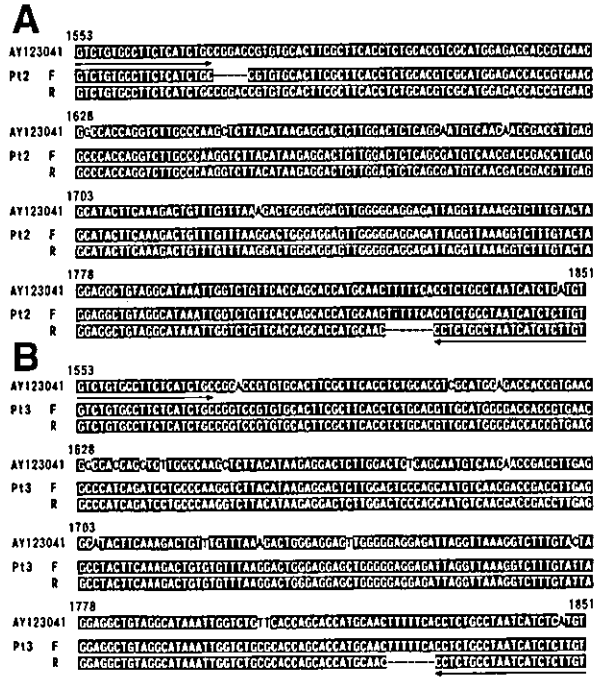

Fig. 4. The direct repeat PCR products recovered from liver tissue samples from patients (A) 2 and (B) 3 were sequenced in both directions. The forward (F) and reverse (R) nucleotide sequences obtained were aligned with the reference sequence of an HBV isolate (GenBank accession no. AY123041). Nucleotide positions are indicated at the **top**. Identical nucleotides are shown by white letters on a black background. **Dashes** denote undetermined nucleotides. Primer sequences used (DRF1/DRR2) are shown by **arrows**.

Liver viral loads and fibrosis stage in the late convalescent phase were further correlated with patient clinical courses. Liver viral loads estimated from the levels of the HBV DNA surface and X regions had no relation to the peak levels of serum alanine aminotransferase activity, bilirubin, and HBV DNA in the acute phase. The relationship was not evident between the liver viral loads and time from the onset of acute hepatitis B. Advanced liver fibrosis stage years after clinical and serologic recovery was associated with high levels of peak serum HBV DNA at the onset ($P = .046$) but not with the liver viral loads in the

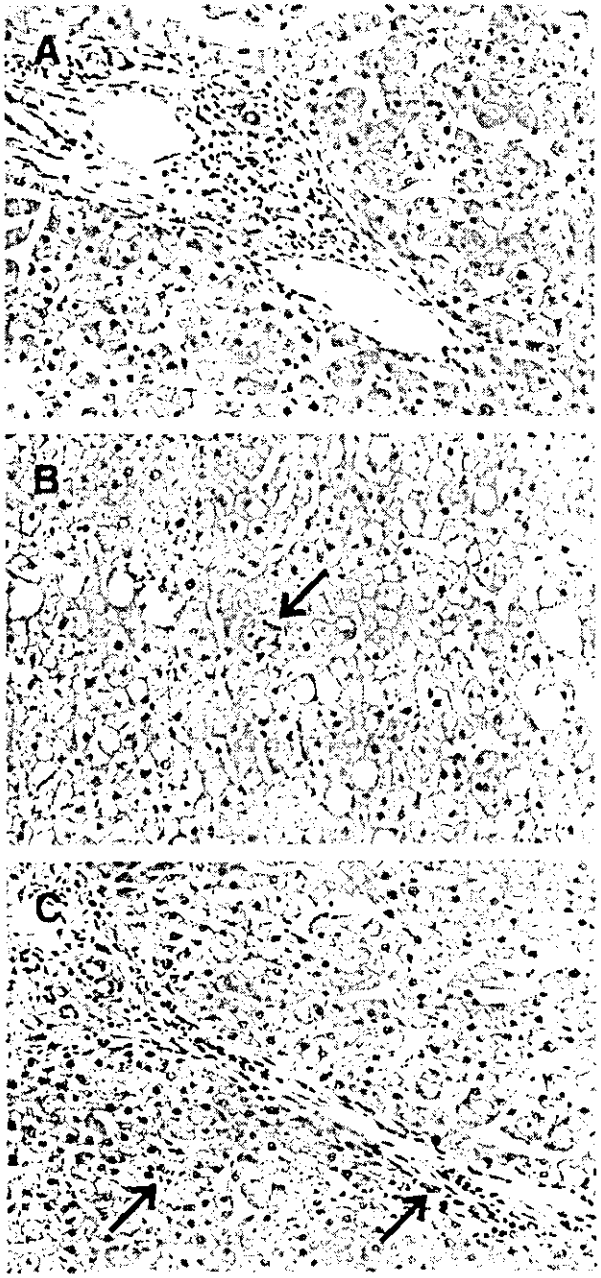

Fig. 5. Representative liver histologic alterations in patients with a remote history of acute self-limited hepatitis B. (A) Mild infiltration of inflammatory cells and fibrous expansion of portal areas were observed in most patients (patient 3). (B) Mild focal necrosis (**arrow**) and mild steatosis were further found in 1 patient (patient 9). (C) Six patients had occasional portal-to-portal bridging of fibrosis. **Arrows** indicate apoptotic cell and apoptotic body seen in this case (patient 11). (Original magnification: A and B, ×400; C, ×200.)

late convalescent phase. No relationship was found between liver fibrosis stage and time from the onset of the disease. The influence of alcohol intake on fibrosis stage was also not evident.

Discussion

At present, the long-term impact of acute self-limited hepatitis B on the liver is unknown in humans. The main aims of the present study were to explore the long-term histologic and virologic outcomes of acute self-limited hepatitis B. The duration and pathologic implications of serologically undetectable HBV persistence in the liver were investigated. Enhanced HBV-specific immune responses are induced following acute exposure to the virus,[4-7] which presents a striking contrast to chronic HBV infection and is believed to lead to termination of the disease. However, there is a report of a chimpanzee that continued to harbor a nonreplicating episomal form of HBV DNA in the liver after resolution of acute hepatitis B by PCR criteria.[20] Similar findings of the persistence of woodchuck hepatitis virus (WHV) DNA in the liver have been described for apparently healthy animals after recovery from acute WHV hepatitis.[21]

All patients studied had demonstrated clinical and serologic recovery from acute hepatitis B. Most (80%) of the patients had developed anti-HBs and were clear of HBV from the serum by PCR criteria. Nevertheless, occult HBV persisted in the livers of all 9 patients studied up to a decade after resolution. One report in the literature showed that HBV DNA was detected in the livers of 2 of 4 patients who had had acute self-limited hepatitis B 30 years previously.[10] Collectively, complete HBV eradication seems to be a very rare event, if it occurs at all, after resolution of acute hepatitis B. In the current study, both of the HBV DNA surface and X sequences derived from 2 distinct virus genomic regions were detected in all liver samples tested. A close correlation was further shown between the levels of the HBV DNA surface sequences and those of the X sequences in the liver, which ensured reliable identification of HBV traces. Moreover, all liver samples tested positive by the PCR methodology to detect an intact direct repeat region, which will amplify the ccc HBV DNA but is unlikely to amplify the incomplete virion HBV DNA and integrated HBV DNA. Although we must stress a possibility that some forms of integrated HBV DNA and other HBV replicative intermediates are amplified, the procedure has been used as a fairly specific assay to detect ongoing occult HBV infection. Collectively, these observations suggest that a replication-competent episomal form of HBV DNA persists in the liver of all patients with a remote history of acute self-limited hepatitis B.

The present study provides direct evidence that liver tissue is a site of virus propagation during convalescence. A previous study showed that HBV infection persisted in the liver but not in peripheral blood mononuclear cells

from patients with acute self-limited hepatitis B 30 years earlier, thus indicating that liver tissue can be the main site of virus propagation in such cases.[10] Unfortunately, occult HBV infections in multiple organs were not compared in the present study, which aimed to explore liver histologic and virologic outcomes. We must stress that further studies are obviously necessary to address this issue. The liver HBV DNA levels were not affected by the severity of liver injury and the viral loads in the acute phase. Interestingly, the levels did not decline with the time elapsed after resolution, and low-grade liver inflammation persisted. Taken together, the presence of ccc HBV DNA may imply low levels of ongoing replication. After infected cells have been massively eliminated by virus-specific immune responses, the HBV replication may be balanced against the host immune pressure for years. Vigorous HBV-specific CTL reactivity has been shown in patients years after clinical and serologic recovery from acute hepatitis B.[7,8] Ample evidence now exists that cytokines (*e.g.*, tumor necrosis factor α and interferon gamma) produced by the CTLs play a major role in the posttranscriptional down-regulation of HBV genome expression without significant liver injury.[22,23] The virus may establish a dynamic equilibrium with the host immune system, where it stimulates the immune response, which in turn keeps HBV replication at an extremely low level. Further quantitative analysis of the liver ccc HBV DNA will shed more light on this issue.

The unresolved problem in the occult HBV infection issue is whether it has any clinical impact. A persistent replication-competent state of HBV infection in the liver raises clinically important problems in the context of liver transplantation and immunosuppressive treatment. In contrast, in an immune-competent state, there is no clear proof that strongly suppressed HBV infection can be involved in the development of advanced chronic liver disease. In the present study, the liver persistence of the replication-competent virus was accompanied by low-grade liver inflammation. A recent study on woodchucks convalescent from acute WHV hepatitis also showed the lifelong persistence of occult infection in the liver, which induces a very mild liver inflammation continuing for life.[21] We must stress that its clinical relevance remains uncertain. Most of our patients retained mild liver fibrosis irrespective of the time elapsed between recovery and liver biopsies. The sample size is not large enough to draw a solid conclusion on factor(s) contributive to liver fibrosis. However, the fibrosis stage was related to the viral loads in the acute phase and seemed to be determined by the nature of the disease at the onset, although the possibility remains that persistent inflammation exerted some influence on the regression of fibrosis. We cannot completely exclude a possibility that low-level alcohol intake in some patients may have affected their fibrosis stage.

The oncogenic potential of occult HBV after resolution of acute hepatitis B is further doubtful. Many studies indicate that occult HBV infection might play a critical role in the development of hepatocellular carcinoma in patients with serologically unidentified pathogenesis of hepatocellular carcinoma.[24,25] Woodchucks that have been infected by the corresponding hepadnavirus (WHV) are at high risk of developing hepatocellular carcinoma after complete resolution of acute WHV hepatitis.[21] In humans, however, long-standing active HBV replication accompanied by significant liver injury is likely to be a very important factor that causes the development of HBV-related hepatocellular carcinoma. Patients with hepatocellular carcinoma with occult HBV may possibly have had chronic HBV infection and lost HBsAg. It is conceivable that hepatocellular carcinoma is unlikely to occur in individuals who recovered from acute hepatitis B.

References

1. Kuhns M, McNamara A, Mason A, Campbell C, Perrillo R. Serum and liver hepatitis B virus DNA in chronic hepatitis B after sustained loss of surface antigen. Gastroenterology 1992;103:1649-1656.
2. Fong T-L, Di Bisceglie AM, Gerber MA, Waggoner JG, Hoofnagle JH. Persistence of hepatitis B virus DNA in the liver after loss of HBsAg in chronic hepatitis B. HEPATOLOGY 1993;18:1313-1318.
3. Komori M, Yuki N, Nagaoka T, Yamashiro M, Mochizuki K, Kaneko A, Yamamoto K, et al. Long-term clinical impact of occult hepatitis B virus infection in chronic hepatitis B patients. J Hepatol 2001;35:798-804.
4. Penna A, Chisari FV, Bertoletti A, Missale G, Fowler P, Giuberti T, Fiaccadori F, et al. Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. J Exp Med 1991;174:1565-1570.
5. Bertoletti A, Ferrari C, Fiaccadori F, Penna A, Margolskee R, Schlicht HJ, Fowler P, et al. HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. Proc Natl Acad Sci U S A 1991;88:10445-10449.
6. Missale G, Redeker A, Person J, Fowler P, Guilhot S, Schlicht HJ, Ferrari C, et al. HLA-A31- and HLA-Aw68-restricted cytotoxic T cell responses to a single hepatitis B virus nucleocapsid epitope during acute viral hepatitis. J Exp Med 1993;177:751-762.
7. Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, Moss B, et al. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 1995;181:1047-1058.
8. Penna A, Artini M, Cavalli A, Levrero M, Bertoletti A, Pilli M, Chisari FV, et al. Long-lasting memory T cell responses following self-limited acute hepatitis B. J Clin Invest 1996;98:1185-1194.
9. Quint WGV, De Bruijn I, Kruining H, Heijtink RA. HBV-DNA detection by gene amplification in acute hepatitis B. HEPATOLOGY 1990;12: 653-656.
10. Bläckberg J, Kidd-Ljunggren K. Occult hepatitis B virus after acute self-limited infection persisting for 30 years without sequence variation. J Hepatol 2000;33:992-997.
11. Michalak TI, Pasquinelli C, Guilhot S, Chisari FV. Hepatitis B virus persistence after recovery from acute viral hepatitis. J Clin Invest 1994;93: 230-239.

12. Rehermann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. Nat Med 1996; 2:1104-1108.
13. Cabrerizo M, Bartolomé J, Caramelo C, Barril G, Carreño V. Molecular analysis of hepatitis B virus DNA in serum and peripheral blood mononuclear cells from hepatitis B surface antigen-negative cases. HEPATOLOGY 2000;32:116-123.
14. Hagiwara H, Hayashi N, Mita E, Takehara T, Kasahara A, Fusamoto H, Kamada T. Quantitative analysis of hepatitis C virus RNA in serum during interferon alfa therapy. Gastroenterology 1993;104:877-883.
15. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, Tanaka S, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;37:2899-2903.
16. Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999;450:66-71.
17. Mason AL, Xu L, Guo L, Kuhns M, Perrillo RP. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. HEPATOLOGY 1998;27:1736-1742.
18. Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1989;339: 237-238.
19. Ishak K, Baptista A, Bianchi L, Callea F, Groote JD, Gudat F, Denk H, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995; 22:696-699.
20. Liang TJ, Blum HE, Wands JR. Characterization and biological properties of a hepatitis B virus isolated from a patient without hepatitis B virus serologic markers. HEPATOLOGY 1990;12:204-212.
21. Michalak TI, Pardoe IU, Coffin CS, Churchill ND, Freake DS, Smith P, Trelegan CL. Occult lifelong persistence of infectious hepadnavirus and residual liver inflammation in woodchucks convalescent from acute viral hepatitis. HEPATOLOGY 1999;29:928-938.
22. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV. Viral clearance without destruction of infected cells during acute HBV infection. Science 1999;284:825-829.
23. Chisari FV. Viruses, immunity, and cancer: lessons from hepatitis B. Am J Pathol 2000;156:1117-1132.
24. Paterlini P, Gerken G, Nakajima E, Terre S, D'Errico A, Grigioni W, Nalpas B, et al. Polymerase chain reaction to detect hepatitis B virus DNA and RNA sequences in primary liver cancers from patients negative for hepatitis B surface antigen. N Engl J Med 1990;323:80-85.
25. Kamito H, Nakata K, Hamasaki K, Daikoku M, Mawatari F, Ueki T, Hayashida Y, et al. Detection of hepatitis B virus genome in hepatocellular carcinoma from both hepatitis B surface antigen- and antibody to hepatitis C virus-negative patients: a study using polymerase chain reaction. Oncol Reports 1996;3:619-623.

Journal of Medical Virology 69:41–49 (2003)

Histological Improvement of Chronic Liver Disease After Spontaneous Serum Hepatitis C Virus Clearance

Yasunori Sugiyasu,[1] Nobukazu Yuki,[1]* Takayuki Nagaoka,[2] Masatoshi Yamashiro,[2] Kunimitsu Kawahara,[3] Kenya Iyoda,[1] Yoshimi Kakiuchi,[1] Akira Kaneko,[1] Keiji Yamamoto,[1] Kazumasa Hikiji,[2] and Michio Kato[1]

[1]*Department of Gastroenterology, Osaka National Hospital, Osaka, Japan*
[2]*SRL Inc., Hino, Japan*
[3]*Department of Pathology, Osaka National Hospital, Osaka, Japan*

The long-term histological and virological outcomes of spontaneous circulating hepatitis C virus (HCV) clearance were studied in chronic liver disease. Between 1979 and 1984, three patients underwent laparoscopy for chronic non-A, non-B liver disease, and two were found to have cirrhosis and one with chronic active hepatitis. After HCV assays became available in 1990, they were positive persistently for HCV antibody without serum HCV RNA. Reductions of antibody levels to HCV core and/or nonstructural proteins were observed, and liver biopsies were undertaken between 1995 and 2000. Liver biopsies at 11–19 years after laparoscopy disclosed marked alleviation of liver inflammation and fibrosis in each case although a low grade of inflammation remained. The two patients with cirrhosis no longer showed histological features of cirrhosis, and the poor liver function in one patient had been ameliorated. Liver specimens from two patients were subjected to polymerase chain reaction to detect positive and negative HCV RNA strands and hepatitis B virus DNA. Only the positive HCV RNA strand was detected for one patient who had previously cirrhosis. Liver specimens were examined from another six nonviremic HCV-seropositive individuals without chronic liver disease. Five patients displayed low-grade liver inflammation without evident fibrosis, but none had any viral genome in the liver. These findings suggest that spontaneous circulating HCV clearance in chronic liver disease confers favorable liver histological outcome, although occult HCV infection persists. ***J. Med. Virol. 69:41–49, 2003.*** © 2003 Wiley-Liss, Inc.

KEY WORDS: HCV antibody; HCV RNA; hepatitis C

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is the major cause of liver cirrhosis and hepatocellular carcinoma worldwide. However, spontaneous circulating HCV clearance is not a very rare event. An unexpectedly high incidence of HCV clearance from the serum has been reported for children [Vogt et al., 1999] and drug users [Beld et al., 1999]. Consequently, there are individuals who have circulating HCV antibodies but are nonviremic, indicating possible recovery from infection. Recently, several groups have reported that low levels of ongoing intrahepatic HCV replication can be present in such individuals [Haydon et al., 1998; Dries et al., 1999]. Thus, the clinical relevance of nonviremic HCV-seropositive states, which occurs in the natural course, has not been fully elucidated. Sustained HCV clearance from sera after interferon (IFN) therapy is usually accompanied by intrahepatic HCV clearance [Marcellin et al., 1997; Lau et al., 1998], decrease in HCV antibody levels [Saracco et al., 1993; Yuki et al., 1993a,b; Diodati et al., 1994], and histological improvement [Marcellin et al., 1997; Lau et al., 1998]. The clinical implications of HCV antibody-positive but serum HCV RNA-negative states in the natural course may differ from those observed after successful IFN therapy. To address these issues, long-term histological and virological outcomes of nonviremic HCV-seropositive states were investigated in patients not given IFN therapy.

*Correspondence to: Nobukazu Yuki, M.D., Department of Gastroenterology, Osaka National Hospital, Hoenzaka 2-1-14, Chuo-ku, Osaka 540-0006, Japan. E-mail: yuki@onh.go.jp

Accepted 19 February 2002
DOI 10.1002/jmv.10250
Published online in Wiley InterScience (www.interscience.wiley.com)

MATERIALS AND METHODS

Patients

Between 1979 and 1984, three patients with chronic non-A, non-B liver disease (2 males and 1 female, age range = 50 to 55 years) underwent diagnostic laparoscopy. They had been negative persistently for hepatitis B surface antigen (HBsAg). They had had no history of administration of hepatotoxic drugs or alcohol abuse (>80 g/day) and showed no evidence of autoimmune liver disease. Thus, there was no apparent cause of hepatocellular injury. Laparoscopic and histological findings of two patients showed the features of liver cirrhosis and chronic active hepatitis, respectively. Laparoscopic findings in another patient showed typical features of established cirrhosis although liver biopsy was not carried out. He also had esophageal varices and other symptoms of liver cirrhosis. After HCV was discovered, these three patients were found to be positive for HCV antibody without viremia between November 1990 and January 1991. They were positive for HCV antibody by a first-generation enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostic Systems Co., Ltd., Tokyo, Japan). These serum samples were retested later with a second-generation assay, and the results were confirmed. Despite the presence of circulating HCV antibody, all three patients were negative for serum HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR). Thereafter, HCV antibody profiles, serum HCV RNA, and routine liver function tests were monitored for 8–9 years. Between 1995 and 2000, these three patients underwent ultrasound-guided liver biopsies again, and the biopsy specimens were reevaluated histologically and virologically. All liver biopsies were performed using 14-gauge Tru-Cut needles to obtain biopsy specimens sufficient for histological evaluation.

Another six nonviremic HCV-seropositive individuals without history of chronic liver disease (3 males and 3 females, age range = 51 to 70 years) underwent liver biopsies in 1999 and were also subjected to histological and virological evaluation. One patient had an episode of acute hepatitis C in February 1998. The diagnosis was based on marked elevation of serum alanine aminotransferase (ALT) activity (peak value = 1,448 U/L) and detection of serum HCV antibody and HCV RNA. The disease resolved spontaneously in one month with disappearance of serum HCV RNA. Thereafter, circulating HCV antibody remained detectable by third-generation ELISA. However, HCV antibody levels decreased, and liver biopsy was done in July 1999. The remaining five patients had had no symptomatic episode of acute hepatitis. Two of these five patients had histories of blood transfusions 46 and 49 years earlier. None had any cause of liver disease other than HCV except that fatty liver was revealed by ultrasonography in one patient. The biopsy study was approved by the local Research Ethics Committee in accordance with the 1975 Declaration of Helsinki, and all patients provided written informed consent.

Laboratory Tests

HCV antibody profiles were tested with a third-generation recombinant immunoblot assay (RIBA-3) (Ortho Diagnostic Systems Co., Ltd.). The accuracy of changes in antibody levels was validated by repeating the assay. Quantitation of the HCV core antibody was also undertaken using a commercially available kit (HCV Core-Ab IRMA, Ortho Diagnostic Systems Co., Ltd.). Serum HCV RNA was detected by RT-PCR. HCV RNA was extracted from 100 μL of serum samples, copied into complementary DNA (cDNA) by RT, and amplified by PCR as described elsewhere [Hagiwara et al., 1993]. Primers were derived from the 5′-noncoding region of the published sequence [Takamizawa et al., 1991]; antisense primer 5′ATGGTGCACGG TCTACGAGACCTCC3′ and sense primer 5′CACTCCCCTGTG AGGAACTACTGTC3′.

Serum samples were tested for HBsAg, antibody to HBsAg (anti-HBs) and antibody to hepatitis B core antigen (anti-HBc) by radioimmunoassay (Abbott Laboratories, North Chicago, IL). To exclude occult hepatitis B virus (HBV) infection, serum HBV DNA was detected by real-time detection PCR based on Taq Man chemistry as previously reported [Abe et al., 1999]. A portion of the HBV surface region was amplified using set 2 primers: antisense primer HBSF2 (5′CTTCATCCTGCTGCTATG CCT3′, nucleotide positions [nt] 406-426) and sense primer HBSR2 (5′AAAGCCCAGG ATGATGGGAT3′, nt 627-646). Another portion of the HBV X region was amplified further using set 3 primers: antisense primer HBXF1 (5′ACGTCCTTTGTTTACGT CCCGT3′, nt 1,414–1,435) and sense primer HBXR1 (5′CCCAACTCCTCCCAGTCCT TAA3′, nt 1,723–1,744).

Liver specimens for PCR testing were obtained by percutaneous needle liver biopsy. Two biopsy samples were obtained from each patient, and one sample was used for routine histological evaluation. The other sample was frozen immediately and then stored at −80°C until PCR testing. Positive and negative HCV RNA strands in the liver were amplified independently by specific RT-seminested PCR as described elsewhere [Tomimatsu et al., 1997]. Briefly, after denaturation of the RNA extracted from the liver, synthesis of the positive and negative HCV cDNA strands was done with an antisense primer YCA (5′ACTCGCAAGCACCCTATCAG3′) or a sense primer YCS13 (5′GAGGAACTACTGTCTTCACG3′) derived from the 5′-noncoding region, respectively. The total cDNA obtained in the RT step was subjected to the first PCR by adding the other primer (YCS13 for the positive strand and YCA for the negative strand amplification). The seminested PCR round was carried out further by adding an inner sense primer YCS2 (5′GAGCCATAGTGGTCTGCCGA3′). The specificity of the detection of each HCV RNA strand was confirmed by the lack of amplification of the synthetic positive and negative strands using primers of the same polarity in the RT step. To test the specificity, several controls were run in parallel to all reactions and included reactions without reverse transcriptase in the