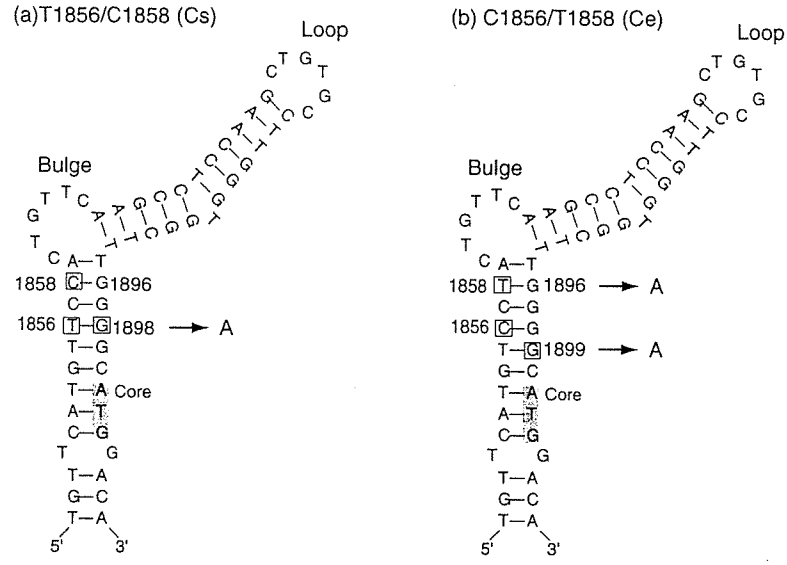

Fig. 4. Conformation of the pregenome encapsidation (ε) signal for (a) HBV/Cs and (b) HBV/Ce. The precore mutation, G1898A accompanied by T1856 forming a base pair with it, was found only in HBV/Cs strains. In contrast, the precore stop mutation, G1896A accompanied by T1858, was found only in HBV/Ce strains. A1899 mutation is significantly predominant in HBV/Ce strains.

is not well known. A previous study among multi-ethnic carriers in Hawaii indicated no significant difference in clinical characteristics between C1858 and T1858 variants [24]. However, as the number of patients was not enough to clarify the significance of this variation, further clinical studies would be required on a case–control study with large-scale cohorts.

A previous study [12] indicated that the amino acid changes specific to HBV/Cs and HBV/Ce were concentrated in the pre-S1, S and P regions, but not in the X and core regions. The pre-S1 region contains the HBV receptor for entering hepatocytes [25] and also has sites for transcriptional factors [26]. Another study [13] showed three amino acids differences in polymerase region. Therefore, the relationship between HBV/Cs and HBV/Ce and their virulence in chronic liver diseases including hepatocellular carcinoma are of great interest, since the prevalence of HBV-related hepatocellular carcinoma is extremely high in Asia compared with other regions.

In conclusion, a new PCR-RFLP method involving 5 SNPs was developed for specifically distinguishing between HBV/Cs and HBV/Ce. The two subtypes (subgenotypes) have distinct geographic distribution and virological characteristics. The novel PCR-RFLP would be useful in evaluating clinical, epidemiological and virological differences between HBV/Cs and HBV/Ce infections in countries where HBV genotype C endemic.

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Effect of Long-Term Postoperative Interferon Therapy on Intrahepatic Recurrence and Survival Rate after Resection of Hepatitis C Virus-Related Hepatocellular Carcinoma

Shuhei Nishiguchi[a] Akihiro Tamori[a] Shoji Kubo[b]

[a]Department of Hepatology, Osaka City University Medical School, and [b]Second Department of Surgery, Osaka City University Graduate School of Medicine, Osaka, Japan

Key Words
Hepatocellular carcinoma · Liver resection · Interferon · Recurrence · Hepatitis C virus · Survival rate

Abstract
Objective: This study was aimed at evaluating the effects of interferon (IFN)-α on survival rate after resection of hepatocellular carcinoma. ***Methods:*** In a randomized, controlled trial by the University Hospital, Medical Center and affiliated hospital in Osaka, Japan, 30 men were after surgery randomly allocated to an IFN-α group (15 patients) and to a control group. Patients in the IFN group received 6 MIU of IFN-α intramuscularly daily for 2 weeks, then three times a week for 14 weeks, and finally twice a week for 88 weeks. The incidence of recurrence and survival rate were then studied. ***Results:*** The response to IFN was sustained viral response (SVR) in 2 patients, biochemical response (BR) in 6, partial response (PR) in 5, and no response (NR) in 2. In the control, 8 of the 15 patients demonstrated continuous abnormally high levels of ALT. At the end point of the study, intrahepatic recurrence was detected in 9 of the IFN group and in 13 of the control (p = 0.065, log-rank test). The cumulative survival rate was higher in the IFN group than in the controls (p = 0.041). ***Conclusion:*** Postoperative IFN therapy improves the outcome after resection of hepatitis C virus-related hepatocellular carcinoma.

Introduction

The outcome after surgical treatment of hepatocellular carcinoma (HCC) related to hepatitis C virus (HCV) is still unsatisfactory because of high rates of the recurrence of HCC and the progress of the underlying liver disease to chronic hepatitis or cirrhosis [1]. We have reported that interferon (IFN)-α helps to prevent the development of HCC in patients with HCV-related cirrhosis in a prospective randomized controlled study [2] and that postoperative IFN therapy demonstrates a decrease in the incidence of recurrence after resection of HCV-related HCC [3]. Recently, IFN has been found to prevent exacerbation of compensated cirrhosis and to inhibit the development of HCC, resulting in higher survival rates [4]. Here we report on the effects of postoperative IFN therapy on the outcome after resection of HCV-related HCC.

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Shuhei Nishiguchi, MD
Department of Hepatology
Osaka City University Medical School
1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585 (Japan)
Tel. +81 6 6645 2292, Fax +81 6 6646 1433, E-Mail snishiguch@med.osaka-cu.ac.jp

Table 1. Clinical characteristics in each group

Characteristics	IFN group	Control group	p
Mean age ± SD, years	61.9 ± 5.8	60.0 ± 4.8	0.33
Men:women	15:0	15:0	>0.99
Albumin, g/dl	3.6 (3.3, 4.3)	3.6 (3.3, 4.1)	0.77
ALT activity, IU/l	105 (45, 131)	86 (58, 151)	0.49
Platelets $\times 10^4$ cells/l	14.7 (7.5, 23.1)	11.2 (6.1, 20.9)	0.14
AFP > 100 ng/l	4	4	>0.99
Child-Pugh score A:B	11:4	12:3	>0.99
Tumor size, cm	2.5 (1.9, 3.5)	2.6 (2.4, 3.5)	0.68
Differentiation of tumor			
Well/moderate/poor	1/11/3	2/11/2	0.77
Histological findings			
Grade, 1/2/3	4/6/5	1/7/7	0.33
Stage(s), 1–3/4	8/7	7/8	>0.99

ALT = Alanine aminotransferase; AFP = alpha-fetoprotein; IFN = interferon. Results of laboratory tests are given as medians, with 10th and 90th percentiles in parentheses.

Methods

Patients and Trial Profile

The protocol of this study was as described [3] and comprised the same 30 patients. Thirty male patients who underwent HCC resection were randomly allocated to an IFN-α group (15 patients) and a control group. The criteria for eligibility were (1) single tumor less than 5 cm in maximum diameter disclosed by preoperative imaging; (2) detectable HCV RNA without hepatitis B surface (HBs) antigen or HIV antibodies; (3) chronic hepatitis or a Child-Pugh score of A or B [5] for compensated cirrhosis, and (4) no severe thrombocytopenia. Clinical characteristics and laboratory test results were similar in the two groups, as were the surgical procedures (table 1). Most of the variables in the enrolled patients were similar to those of eligible patients who declined participation in the study. In the IFN group, patients received 6 MIU of IFN-α (human lymphoblastoid interferon; Sumiferon, Sumitomo Pharmaceuticals, Osaka, Japan) intramuscularly every day for 2 weeks, then 3 times weekly for 14 weeks, and finally twice weekly for 88 weeks (total dose, 1,572 MIU). None of the patients in the control group received treatment for carcinoma or liver disease after the resection until the detection of recurrence; none of the patients in the IFN group received chemotherapy or any treatment other than IFN administration before the detection of recurrence. Methods for the treatment of the recurrence were determined on the basis of the number and location of the recurrent tumor(s) and on the results of liver function tests. One or two recurrent tumors were treated with ablation therapy such as resection or microwave coagulation therapy. For multiple recurrent tumors or in cases where ablation therapy was considered difficult because of the location of the recurrent tumor(s) or because of liver function, transarterial therapy (transcatheter arterial embolization and hepatic arterial infusion chemotherapy) was applied.

This study was conducted in accordance with the Helsinki Declaration and was approved by the Ethics Committee of our institutions. Written informed consent was obtained from each patient.

Statistical Analysis

For all cases of recurrence, laboratory data before surgery were compared with those upon detection of recurrence; the latter were also compared between the two groups. The Mann-Whitney test was used to evaluate differences among the laboratory test results. The length of time until death was considered the interval between resection and death. The cumulative survival rates (which included data on patients who did not undergo or complete IFN therapy in the IFN group) were calculated by the Kaplan-Meier method, and significant differences between the groups were assessed by the log-rank test. All data were analyzed with SAS statistical software (version 6.12, SAS Institute, Cary, N.C., USA).

Results

The median follow-up period (from the resection to death or to the end point of the study) was 1,817 days (25th and 75th percentiles, 1,579 and 2,008 days) for patients receiving IFN-α and 1,487 days (1,194 and 2,055 days) for the 15 controls. As previously reported [3], IFN therapy was not administered to 1 patient because of premature ventricular contractions, was not completed by 3 patients because of adverse events, and was stopped prematurely in 4 patients with recurrence so that the tumor(s) could be treated. The response to IFN was sustained viral response (SVR) in 2 patients, biochemical response (BR) in 6 patients, partial response (PR) in 5 patients, and no response (NR) in 2 patients (fig. 1). In the controls, 8 of the 15 patients showed continuous abnormally high ALT activity. Intrahepatic recurrence was detected in 9 patients in the IFN group and in 13 controls at the end point of the study (fig. 2, p = 0.065). One or two

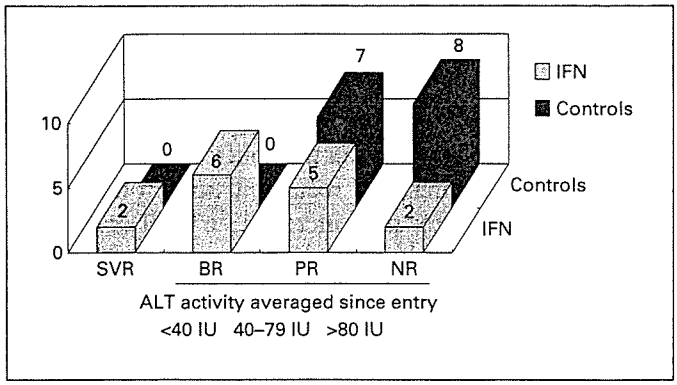

Fig. 1. Effects of IFN on viral clearance and on alanine aminotransferase (ALT) activity averaged since the end of treatment. The response to IFN was a SVR in 2 patients, a BR in 6 patients, a PR in 5 patients, and no response (NR) in 2 patients. In the controls, 8 of the 15 patients continued to have abnormally high ALT activities.

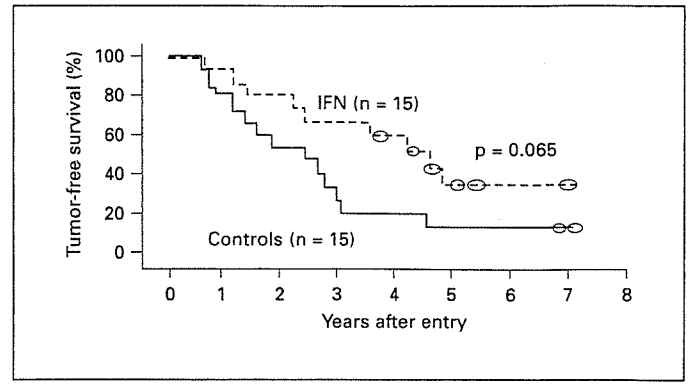

Fig. 2. Nonrecurrence rates after resection in patients given IFN or not. The median follow-up has been 5.0 years for patients receiving IFN-α and 4.1 years for the controls. Recurrences had been detected in 9 patients in the IFN group and in 13 control patients.

recurrent tumors were detected in 8 patients of the IFN group and in 7 of the control; three or more recurrent tumors were found in one of the former group and in six of the latter. Ablation therapy was carried out on 6 patients of each group. Transarterial therapy was used on 3 patients in the IFN group and on 6 control patients. One patient in the control group could not undergo any treatment for recurrent tumors because of decompensation. IFN therapy was completed after treatment of the recurrent tumors at the request of 3 of the 4 patients in whom recurrence was detected during IFN therapy.

In the IFN group, the serum concentration of total bilirubin and ALT activity had decreased by the time recurrence was detected (p = 0.062 and 0.066, respectively). In the control group, the serum concentration of total bilirubin had increased (p = 0.042).

At recurrence, the median serum concentration of albumin was 40 g/l in the IFN group and 37 g/l in the control (table 2, p = 0.052). The total bilirubin concentration was significantly lower in the IFN group than in the control (p = 0.044).

All of the patients who died (3 in the IFN group and 9 in the control) had recurrences of HCC. The cumulative survival rate was significantly higher in the IFN group than in the control (fig. 3, p = 0.041, log-rank test).

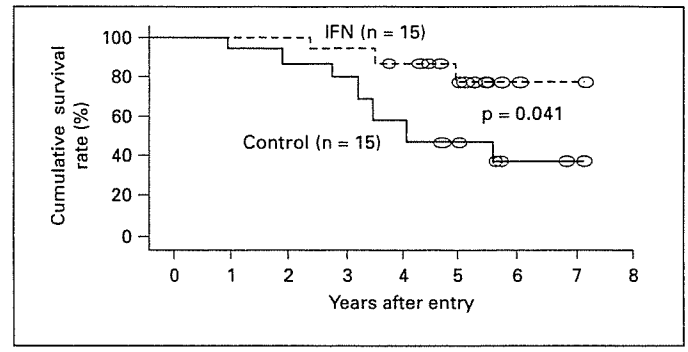

Fig. 3. Cumulative survival rates. Three patients in IFN group and 9 patients in the control group have died. All patients who died had recurrence of HCC.

Table 2. Results of laboratory tests at the detection of recurrent tumor(s)

	IFN group (n = 9)	Control group (n =13)	p
Albumin, g/l	40 (36, 42)	37 (28, 39)	0.052
Total bilirubin, mol/l	12 (10, 17)	18 (10, 35)	0.044
ALT, IU/l	57 (26, 110)	70 (59, 160)	0.12
Platelet count, $\times 10^9$/l	115 (79, 202)	123 (68, 215)	0.85

ALT = Alanine aminotransferase; IFN = interferon. Results of laboratory tests are given as medians, with 10th and 90th percentiles in parentheses.

Discussion

In this study, the cumulative survival rate after resection of HCC was significantly higher in the IFN group than in the control: 1 of the 9 patients in the former group

and 6 of the 13 in the latter had multiple recurrent tumors. Some recurrent tumors were intrahepatic metastases from primary tumors that were not detected at the resection, and other tumors were new carcinomas that developed after the resection. The prevalence of multicentric carcinogenesis increases during progression to active hepatitis and hepatic fibrosis [6, 7]. IFN suppresses the development of HCC by inducing remission of active hepatitis and by improving hepatic fibrosis in patients infected with HCV [2, 4, 8, 9]. IFN may by such effects suppress multiple recurrences arising from multicentric carcinogenesis after surgery. IFN-α has shown antiproliferative effects on a human hepatoma cell line [10]. IFN can enhance natural-killer cell activity and demonstrates antiangiogenic properties [11, 12]. Ikeda et al. [13] have reported that IFN-β prevents recurrence of HCC after treatment, suggesting that it acts as an antitumor agent. A combination of anticancer agents and IFN-α has shown improvement in the survival rate of patients with advanced HCC [14]. Thus, the anticancer effects of IFN may be another reason for the small number of recurrent tumors in our IFN group, which may have contributed to the length of survival time, especially that the occurrence of multiple recurrent tumors is a risk factor for the short survival time reported in other studies [15, 16].

Previous studies on survival after treatment for recurrence have shown that a low serum concentration of albumin and a high one of total bilirubin are risk factors for short survival [15, 16]. Risk factors for short survival in patients with HCV-related cirrhosis are low albumin concentration, high concentration of total bilirubin, and absence of IFN therapy [17]. IFN-α prevents exacerbation of compensated cirrhosis [4]. In this study, laboratory test results showed that liver function improved in the IFN group, but did not change, or even worsened in the control. At the detection of recurrence, the serum concentration of total bilirubin was significantly lower in the IFN group. Moreover, 1 patient in the control group could not undergo any treatment for recurrent tumors because of decompensation. IFN may increase survival in patients given IFN by improving both liver function and indications for radical treatment of recurrences.

In this study, a low platelet count ($<100 \times 10^9$/l) and a high histologic activity index of grade 3 or 4 were not significant factors by the log-rank test ($p = 0.66$ and 0.53, respectively). In a study of 3-year survival, a low platelet count has been shown as a possible risk factor after the detection of recurrence [15]. Although the platelet count at recurrence was not different between our groups, it may have been a contributing factor to the short survival in the control group; the number of patients was too small for a clear conclusion.

This study suggests that postoperative IFN therapy improves the outcome after resection of HCV-related HCC not only because of suppression of recurrence but also because of improvement in liver function. Patients with early-stage HCC and with liver function good enough to undergo liver resection and IFN therapy would be candidates for postoperative IFN therapy.

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Journal of Medical Virology 77:77–82 (2005)

Chemiluminescence Enzyme Immunoassay for Monitoring Hepatitis C Virus Core Protein During Interferon-α2b and Ribavirin Therapy in Patients With Genotype 1 and High Viral Loads

Masaru Enomoto,[1] Shuhei Nishiguchi,[2] Akihiro Tamori,[1]* Modoka Kohmoto,[1] Daiki Habu,[1] Hiroki Sakaguchi,[1] Tadashi Takeda,[1] Norifumi Kawada,[1] Shuichi Seki,[1] and Susumu Shiomi[3]

[1]*Department of Hepatology, Osaka City University Medical School, Osaka, Japan*
[2]*Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan*
[3]*Department of Nuclear Medicine, Osaka City University Medical School, Osaka, Japan*

This study evaluated an updated chemiluminescence enzyme immunoassay (CLEIA) for hepatitis C virus (HCV) core protein for monitoring viral kinetics during treatment with interferon (IFN)-α and ribavirin. Using the CLEIA, serum levels of HCV core protein were measured in 17 patients with genotype 1 and high baseline viral loads during the first 4 weeks of combination therapy. HCV RNA was measured by the Amplicor Monitor test for comparison. At the start of therapy, the median HCV level (interquartile range) was 700 (540–940) kIU/ml of viral RNA and 11,310 (5,528–14,238) fmol/L of core protein. HCV RNA was above the upper limit of the linear range of the Amplicor Monitor test in 13 of the 17 patients, while the core protein level was within the linear range of the CLEIA in all patients. During therapy, the proportion of patients with HCV levels below the cutoff values at each time point was less with the Amplicor Monitor test than with CLEIA. Serum HCV core protein level decreased rapidly during the first 24 hr of therapy and more slowly thereafter, with median exponential decays of 1.08 and 0.046 log10/day, respectively. In the second phase, between day 1 and 28, the median decrease in HCV core protein level was higher in four patients with sustained virologic response (0.13 log10/day) than in 13 patients with no response (0.028 log10/day, $P=0.042$). The wide linear range of the HCV core protein assay is appropriate for measuring viral loads during therapy with IFN-α and ribavirin. ***J. Med. Virol. 77:77–82, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: core protein; enzyme immunoassay; hepatitis C; interferon; ribavirin; viral kinetics

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease worldwide [Lauer and Walker, 2001]. Persistent infection with HCV often progresses to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma over the course of several decades. Since the report by Hoofnagle et al. [1986], describing the effects of interferon (IFN) therapy on chronic hepatitis C, this drug has been approved for the eradication of HCV, and may reduce the incidence of hepatocellular carcinoma [Nishiguchi et al., 1995; Yoshida et al., 1999; Kubo et al., 2001]. Ribavirin is a synthetic guanosine nucleoside analog that inhibits replication of various RNA and DNA viruses. In patients with chronic hepatitis C, the combination of IFN-α and ribavirin yields a higher rate of sustained virologic response than IFN-α alone [Davis et al., 1998; McHutchison et al., 1998; Poynard et al., 1998; Reichard et al., 1998]. However, the rate of sustained eradication of HCV achieved by combination therapy remains unsatisfactory for patients with HCV genotype 1 and high baseline viral loads.

Analysis of the dynamics of HCV during the early phase of IFN-based therapy is important for monitoring the response to therapy, and sometimes for modifying treatment regimens. Beginning 7–10 hr after initiation of IFN administration, the serum level of HCV declines

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*Correspondence to: Akihiro Tamori, Department of Hepatology, Osaka City University Medical School, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan.
E-mail: atamori@med.osaka-cu.ac.jp

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rapidly, with an estimated half-life of 5.0–7.2 hr during the first 1 or 2 days of therapy, and then declines more slowly [Neumann et al., 1998; Yasui et al., 1998]. This second-phase decrease in HCV level is especially important when analyzing viral kinetics for prediction of long-term response to therapy [Enomoto et al., 2002a,b, 2004].

A simple and inexpensive method with a wide linear range of quantitation is needed for frequent measurement of serum HCV levels to assess responses to antiviral therapy. Several assays for measurement of serum HCV levels are available commercially, including reverse-transcription polymerase chain reaction (PCR) for HCV RNA [Zeuzem et al., 1994; Lee et al., 2000] and enzyme immunoassay (EIA) for HCV core protein [Tanaka et al., 1995, 1996; Nishiguchi et al., 2002]. Although the sensitivity of the conventional EIA for HCV core protein was previously inferior to that of PCR, EIA has been improved in its analytical sensitivity [Aoyagi et al., 1999; Tanaka et al., 2000; Zanetti et al., 2003]. In particular, a chemiluminescence enzyme immunoassay (CLEIA) has been developed recently utilizing a partially automated system with specialized equipment that is both simple to perform and can yield results rapidly. However, the usefulness of this assay for monitoring serum HCV levels during IFN-α and ribavirin therapy has not been assessed clinically.

The aim of this study was to evaluate whether the CLEIA for HCV core protein can be used to examine viral kinetics in the early phase of treatment with IFN-α and ribavirin. CLEIA was used to monitor serum levels of HCV core protein in patients with genotype 1 and high viral loads during the first 4 weeks of combination therapy. HCV RNA was measured by the PCR-based Amplicor Monitor test for comparison.

MATERIALS AND METHODS

Patients

There were 17 patients with chronic hepatitis C (10 men and 7 women; mean age, 54 ± 10 years) who began combination therapy with IFN-α2b and ribavirin at our hospital between March 1999 and December 2002. The inclusion criteria were as follows: persistent elevation of serum alanine aminotransferase for at least 6 months before therapy; presence of genotype 1 of HCV in serum; presence of serum HCV RNA at levels above 200 kIU/ml as determined by the Amplicor Monitor test; absence of serum hepatitis B surface antigen and of signs of other likely causes of chronic liver disease; histological features of chronic hepatitis in liver biopsy specimens obtained within 6 months before the start of therapy; absence of anemia (hemoglobin concentration less than 12 g/dl in women and less than 13 g/dl in men); and no evidence of hepatocellular carcinoma on ultrasonographic or computed tomographic examinations. Serum samples were obtained from the patients before administration of the drug(s) on the first day of therapy (day 0) and on day 1, 7, 14, and 28. The samples were stored at −80°C before being tested. The procedures of the study were in accord with the Declaration of Helsinki of 1975 (1983 revision) and were approved by the ethics committee of our hospital.

Treatment

Patients received recombinant IFN-α2b (Intron A, Schering-Plough, Kenilworth, NJ) by intramuscular injection at a dosage of 6 MU every day for 2 weeks, followed by 6 MU three times a week for 46 weeks. Ribavirin (Rebetol, Schering-Plough) was given orally twice a day for the first 24 weeks at a total daily dose of 600 mg in the nine patients who weighed 60 kg or less and 800 mg in the remaining eight patients, who weighed more than 60 kg. This protocol was commonly used in this country at the time of this study. The response to therapy was assessed virologically by repeated PCR assays for serum HCV RNA. A sustained virologic response was defined as one in which serum HCV RNA was not found more than 6 months after the end of therapy. Patients who did not meet these criteria were considered to have no response to therapy.

Assays

Routine hematological and biochemical tests were performed using standard procedures. Serum HCV RNA was measured by the Amplicor Monitor test (Roche Diagnostics, Branchburg, NJ) [Zeuzem et al., 1994; Lee et al., 2000], which exhibits good linearity between 0.5 and 500 kIU/ml. When HCV RNA was not detected by this method, the serum was tested again using the more sensitive, qualitative Amplicor test [Lee et al., 2000]. Genotypes of HCV were identified by direct sequencing of the amplification products generated during the Amplicor Monitor test with an ABI 3700 DNA sequencer (Perkin Elmer Corp./Applied Biosystems, Foster City, CA) [Kuboki et al., 2000].

Serum HCV core protein was measured by CLEIA (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. In brief, 200 μl of serum sample was mixed with 100 μl of a pretreatment solution containing 0.3% Triton X-100 and 15% sodium dodecyl sulfate. After incubation at 56°C for 30 min, 100 μl of the pretreatment solution was added to a well, coated with monoclonal antibodies to the HCV core antigen, and filled with 160 μl of reaction buffer. The mixture was incubated with agitation for 10 min at 37°C and then washed with buffer. Alkaline phosphatase-conjugated monoclonal antibodies to HCV core antigen were then added to the well, which was then incubated for 10 min at 37°C. After washing, 200 μl of a substrate buffer was added, and the mixture was incubated for 5 min at 37°C. The reactive chemiluminescence unit was measured, and the concentration of HCV core antigen was determined according to a standard curve generated using the recombinant HCV core antigen. All steps of this assay after the first incubation (at 56°C for 30 min) were performed on a fully automated chemiluminescence analyzer system (Lumipulse-f, Fuji Rebio, Tokyo,

Japan). The total assay time was 30 min. The linear range of the assay was 15–50,000 fmol/L.

Histological Evaluation

Liver biopsy was performed for each patient within 6 months before the start of therapy. Histopathological findings were assessed by grading inflammatory activity and staging fibrosis according to the classification of Desmet et al. [1994]. All evaluations were done by an experienced pathologist blinded to the clinical data.

Statistical Analysis

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

RESULTS

Baseline Characteristics of Patients

Of the 17 patients, 9 had a history of IFN monotherapy. All patients were infected with genotype 1b of HCV, which is the most common kind in Japan. At the start of treatment with IFN-α2b and ribavirin, the median alanine aminotransferase activity (interquartile range) was 80 (46–114) IU/L. The median HCV level (interquartile range) was 700 (540–940) kIU/ml of viral RNA and 11,310 (5,528–14,238) fmol/L of core protein. The baseline HCV RNA level was above the upper limit of linear range of the Amplicor Monitor test in 13 of the 17 patients, but the core protein level of all patients was within the linear range of the CLEIA. The grade of inflammatory activity was mild in nine patients and moderate in seven. The stage of fibrosis was mild in eight patients, moderate in six, severe in one, and cirrhotic in one. In one patient, the biopsy sample was too small to evaluate.

Relationship Between HCV RNA Level and Core Protein Concentration

The relationship between the results of measuring HCV RNA by the Amplicor Monitor test and those of measuring HCV core protein by CLEIA is shown in Figure 1. In three samples taken from one patient during therapy, HCV core protein was above the cutoff value for the CLEIA (534, 221, and 55.4 fmol/L, respectively), while HCV RNA was below the cutoff value for the Amplicor Monitor test. In eight samples taken from five patients, HCV RNA was above the cutoff value for the Amplicor Monitor test (median, 2.7 kIU/ml; range, 0.6–20 kIU/ml), while HCV core protein was below the cutoff value for CLEIA. Samples below the cutoff value of each assay were assigned the viral load of the cutoff value for calculation. There was a significant correlation between the results obtained with the two techniques at the start of therapy ($r = 0.589$, $P = 0.011$).

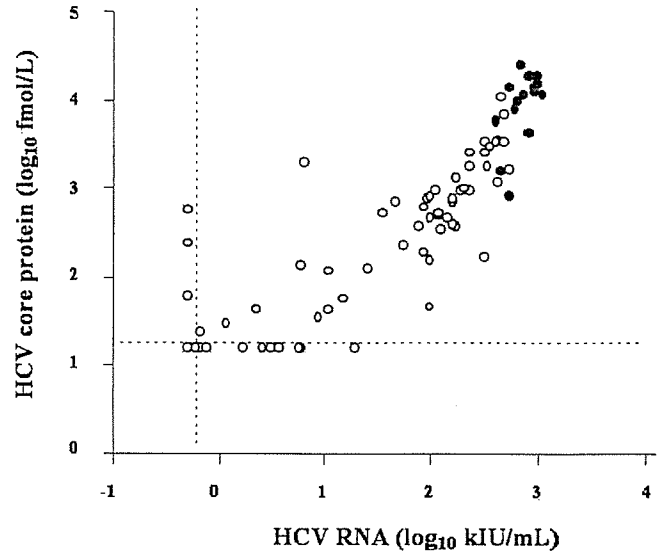

Fig. 1. Correlation between serum HCV RNA level measured by the Amplicor Monitor test and HCV core protein level measured by the CLEIA. Broken lines show the cutoff value for each assay. ●, samples taken at the start of therapy with IFN-α2b and ribavirin. ○, samples taken during therapy.

The correlation between results was also significant when all serum samples taken during therapy were included in analysis ($r = 0.859$, $P < 0.0001$).

Changes in HCV Levels During the First 4 Weeks of Treatment

On day 1, 7, 14, and 28 of treatment with IFN-α2b and ribavirin, HCV RNA was below the cutoff value by the Amplicor Monitor test in zero (0%), one (6%), four (24%), and four (24%) patients, respectively, while HCV core protein was below the cutoff value by CLEIA in 0 (0%), 1 (6%), 5 (29%), and 8 (47%) of the 17 patients. The proportion of patients with HCV levels below the respective cutoff values during therapy was lower with the Amplicor Monitor test than with CLEIA.

Changes in serum HCV core protein in all patients monitored by the CLEIA are shown in Figure 2. Changes in HCV RNA as measured by the Amplicor Monitor test paralleled those in core protein (data not shown). As reported previously, HCV core protein decreased rapidly during the first 24 hr of therapy and more slowly thereafter. We defined the period between 0 and 24 hr of therapy (day 0) as "the first phase," and the period from day 1 to 28 (day 14 if core protein was below the cutoff value on day 28, or day 7 if core protein was below the cutoff value on day 14) as "the second phase." The median rate of exponential decay of serum HCV core protein (interquartile range) in the first and in second phases were 1.08 (0.69–1.34) and 0.046 (0.016–0.11) log10/day, respectively.

Of the 17 patients treated with combination therapy, 4 had a sustained virologic response and 13 had no response. The median rate of decay in the first phase (interquartile range) was 1.06 (0.93–1.24) log10/day in

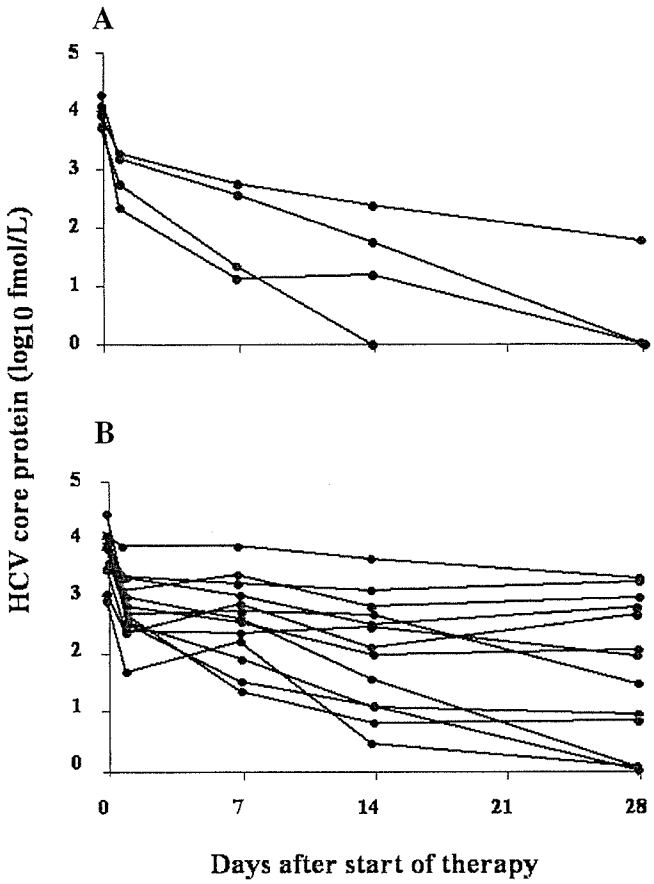

Fig. 2. Time course of serum HCV core protein level monitored by CLEIA during the first 4 weeks of IFN-α2b and ribavirin treatment (**A**) in the four patients with sustained virologic response, and (**B**) in the 13 patients with no response. In the second phase between day 1 and 28, the median decrease in HCV core protein was larger in patients with sustained virologic response (0.13 log10/day) than in patients with no response (0.028 log10/day, $P = 0.042$).

patients with sustained virologic response and 1.11 (0.69–1.34) log10/day in patients with no response; the differences in first-phase viral decline were not significant ($P = 0.73$). The median rate of decay in the second phase (interquartile range) was 0.13 (0.045–0.21) log10/day in patients with sustained virologic response and 0.028 (0.0048–0.11) log10/day in patients with no response; the differences in second-phase viral decline between the sustained-response and no-response groups were significant ($P = 0.042$).

DISCUSSION

Among commercial assays for the measurement of serum HCV levels, the Amplicor Monitor test is used widely, as a sensitive PCR-based method [Zeuzem et al., 1994; Lee et al., 2000]. However, it yields results with limited reproducibility and requires expensive equipment. The cumbersome procedures make testing of many samples difficult and increase the risk of contamination. High levels of HCV RNA may be underestimated with it, and, in addition, its analytical sensitivities may be affected by the genotype, of the resulting in underestimation of viral loads of genotype 2 or 3.

The serum concentration of HCV core protein is correlated with HCV RNA titer [Tanaka et al., 1995, 1996]. Viral protein is more resistant to multiple freezing and thawing than viral RNA. In the new version of the EIA, HCV core antigens are released from the virion and antibodies to HCV core are inactivated by pretreatment with sodium dodecyl sulfate. This pretreatment step increases sensitivity by approximately 100-fold. Aoyagi et al. [1999] demonstrated high intra-assay precision and inter-assay reproducibility of the EIA over a wide range of values. This method, which uses specific monoclonal antibodies specific to the conserved region of core protein, can evaluate the viral loads of different genotypes with equal sensitivity. In particular, the partially automated CLEIA assay requires less than 1 hr to yield results. Clinically, rapid availability of results allows viral loads to be accurately monitored and treatment regimens to be modified as required. The low cost of the assay enables serum samples to be frequently tested to evaluate viral kinetics.

The cutoff value of the CLEIA was set at 15 fmol/L of HCV core protein, based on the results of measurement of serially diluted standard samples. This value is equivalent to 1–2 kIU/ml of viral RNA (unpublished observations). Although the proportions of patients with HCV levels below the cutoff values during therapy were smaller with the Amplicor Monitor test than with the CLEIA, the analytical sensitivity of the CLEIA is nearly the same as that of the Amplicor Monitor test. In addition, the baseline HCV core protein level of all patients with genotype 1 and high viral loads was within the linear range of the CLEIA, whereas in a majority of the patients the viral RNA was above the upper limit of the linear range of the Amplicor Monitor test. The wide linear range permitted by the HCV core protein assay is appropriate for accurate measurement of the high viral loads present before and during the first few days after the start of therapy.

Davis [2002] showed that a decrease in serum HCV level by 2 log10 units within the first 12 weeks of therapy with IFN-α and ribavirin can be used as the optimal definition of an early virologic response which, if not achieved, is associated with a low likelihood of sustained virologic response. Discontinuation of therapy should be recommended for patients with genotype 1 who do not achieve an early virologic response. This type of prospective assessment also requires methods with wide linear ranges to precisely evaluate viral loads before and during antiviral therapy.

Previous studies of the dynamics of HCV in the first few weeks of IFN treatment have revealed biphasic viral decline [Neumann et al., 1998; Yasui et al., 1998], as we also observed. Neumann et al. [1998] suggested that the rapid viral decrease in the first phase reflects the dose-dependent effects of IFN on HCV production, and that the slower decrease in the second phase arises from the

death of hepatocytes infected with HCV. It was found previously that the second-phase decrease in HCV RNA monitored by quantitative PCR was correlated with the long-term effects of IFN therapy [Enomoto et al., 2002a,b]. In the present study, CLEIA also exhibited significant correlation between the second-phase decrease in HCV core protein and the sustained virologic response to treatment with IFN-α and ribavirin. The changes in serum HCV core protein monitored by the CLEIA early during IFN-α and ribavirin treatment can be used to predict long-term therapeutic response.

Randomized controlled trials have shown that weekly treatment with pegylated IFN-α plus ribavirin yields higher rates of sustained virologic response than treatment with unmodified IFN-α plus ribavirin [Manns et al., 2001; Fried et al., 2002]. Owing to advances in treatment, IFN is now indicated even for difficult-to-treat patients with HCV genotype 1 and high baseline viral loads. Evaluation of HCV levels by assays with wide linear ranges will most likely become more important in the future.

In summary, the CLEIA is a simple, sensitive, specific, reproducible, and inexpensive method for the measurement of HCV core protein. The wide linear range of the HCV core protein assay is appropriate for monitoring viral loads in patients with genotype 1 and high viral loads during therapy with IFN-α and ribavirin.

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Distinct Geographic Distributions of Hepatitis B Virus Genotypes in Patients With Acute Infection in Japan

Hiroshi Yotsuyanagi,[1]* Chiaki Okuse,[1] Kiyomi Yasuda,[2] Etsuro Orito,[3] Shuhei Nishiguchi,[4] Joji Toyoda,[5] Eiichi Tomita,[6] Keisuke Hino,[7] Kiwamu Okita,[7] Shiro Murashima,[8] Michio Sata,[8] Hiromi Hoshino,[9] Yuzo Miyakawa,[10] Shiro Iino,[1,2] and for the Japanese Acute Hepatitis B Group

[1]*Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University School of Medicine, Kawasaki, Japan*
[2]*Center for Liver Diseases, Kiyokawa Hospital, Tokyo, Japan*
[3]*Department of Clinical Molecular Informative Medicine, Nagoya City University Graduate School of Medicine, Nagoya, Japan*
[4]*Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka, Japan*
[5]*Department of Hepatology, Sapporo Kosei General Hospital, Sapporo, Japan*
[6]*Department of Gastroenterology, Gifu Municipal Hospital, Gifu, Japan*
[7]*Department of Gastroenterology and Hepatology, Yamaguchi University School of Medicine, Ube, Japan*
[8]*Second Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan*
[9]*Hepatitis Research Institute, Tokorozawa, Japan*
[10]*Miyakawa Memorial Research Foundation, Tokyo, Japan*

Genotypes of hepatitis B virus (HBV) were determined in 145 patients with acute hepatitis B from various districts in Japan to establish their geographic distribution and evaluating the influence on the clinical illness and outcome. Genotypes were A in 27 (19%) patients, B in 8 (5%), C in 109 (75%) and mixed with B and C in the remaining one (1%). Genotype A was more frequent in metropolitan than the other areas (21/69 (30%) vs. 6/76 (8%), $P < 0.001$). On phylogenetic analysis, seven of the nine (78%) HBV/A isolates selected at random clustered with those from Europe and the United States, while the remaining two with those of subgroup A′ prevalent in Asia and Africa. Maximum ALT levels were lower (2069 ± 1075 vs. 2889 ± 1867 IU/L, $P = 0.03$) and baseline HBV DNA titers were higher (5.90 ± 1.45 vs. 5.13 ± 1.36 log genome equivalents (LGE)/ml, $P = 0.002$) in patients infected with genotype A than C. Hepatitis B surface antigen persisted longer in patients infected with genotype A than C (1.95 ± 1.09 vs. 1.28 ± 1.42 months, $P = 0.02$). HBV infection became chronic in one (4%) patient with genotype A and one (1%) with genotype C infection. Fulminant hepatic failure developed in none of the patients with genotype A, one (13%) with genotype B and five (5%) with genotype C. The point mutation in the precore region (A1896) or the double mutations in the basic core promoter (BCP) region (T1762/A1764) were detected in none of the patients with genotype A, two (25%) with genotype B and 27 (26%) with genotype C. In conclusion, genotype A is frequent in patients with acute hepatitis B in metropolitan areas of Japan, probably reflecting particular transmission routes, and associated with longer and milder clinical course than genotype C. ***J. Med. Virol. 77:39–46, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: acute hepatitis; genotypes; epidemiology; hepatitis B virus; hepatitis B e antigen; sexuality; Japan

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Dr. Hiroshi Yotsuyanagi's present address is Department of Infectious Diseases, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.

*Correspondence to: Dr. Hiroshi Yotsuyanagi, Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University School of Medicine, 2-16-1, Sugao, Miyamae-ku, Kawasaki, 216-8511, Japan.
E-mail: hyotsu@marianna-u.ac.jp

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INTRODUCTION

The clinical outcome in patients with acute hepatitis B varies widely. Although hepatitis is self-limited in most patients, the clinical features range from asymptomatic to fulminant hepatic failure, while some patients become carriers of hepatitis B virus (HBV) [Chan HL and Lok, 1999; Chan HLY, 1999]. Factors that determine the clinical outcome remain unknown.

Viral nucleotide (nt) mutations have been shown to influence the clinical outcome of acute hepatitis B. Mutations in the precore region (A1896) and the basic core promoter (BCP) region (T1762/A1764) are common in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996]. Viral factors other than these mutations may influence the clinical outcome of acute hepatitis B.

Eight genotypes of HBV have been identified by sequence divergence greater than 8% in the entire genome, and they are designated by capital alphabet letters from A to H [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. Furthermore, recombinant HBV strains consisting of two different genotypes have been reported [Bollyky et al., 1996; Morozov et al., 2000]. Genotype distribution is different in different countries and even in distinct areas of the same country [Orito et al., 2001a; Kao, 2002; Kato et al., 2002; Miyakawa and Mizokami, 2003]. Therefore, surveys on genotype distribution may be helpful in identifying transmission routes and evaluating clinical relevance.

It has been shown that the clinical outcome of chronic hepatitis B is influenced by HBV genotypes. In Asian patients with chronic hepatitis B, genotype C is associated with later seroconversion of hepatitis B e antigen (HBeAg) and more severe liver damage than genotype B [Kao et al., 2000; Orito et al., 2001b; Chu et al., 2002; Ding et al., 2002; Sugauchi et al., 2002a]. Likewise, a study from India has shown that genotype D is associated with more severe liver disease than genotype A [Thakur et al., 2002]. Genotype A is peculiar in that A1896 in the precore region occurs infrequently, because it causes instability of the stem-loop structures of the pregenome encapsidation signal [Li et al., 1993; Lok et al., 1994]. These reports suggest that HBV genotypes also influence the clinical characteristics of acute hepatitis. Recent studies on small numbers of patients with acute hepatitis B suggest that the clinical course may differ among infections with distinct HBV genotypes [Mayerat et al., 1999; Kobayashi et al., 2002; Ogawa et al., 2002]. However, the association between viral genotype and severity of liver disease remains uncertain in acute HBV infection.

To evaluate the effect of HBV genotypes on the clinical characteristics of acute hepatitis B, a multi-center study on 145 patients was conducted in Japan.

MATERIALS AND METHODS

Patients

During 1992 through 2001, serum samples were collected from 147 patients diagnosed with acute hepatitis B in our institutions. Only patients from whom sera at the onset of hepatitis were stored were included in this study. Sixty-nine (47%) patients lived in metropolitan areas (Kawasaki, Tokyo and Tokorozawa), while the others in Kurume, Ube, Osaka, Gifu, Nagoya and Sapporo. Criteria for the diagnosis of acute hepatitis B were: (1) Acute onset of liver injury without a history of liver dysfunction and detection of hepatitis B surface antigen (HBsAg) in serum; and (2) IgM antibody to HBV core (anti-HBc) in high titer. Co-infection with hepatitis A virus or hepatitis C virus was excluded by serological tests.

Among the 147 patients, acute hepatitis B in six (4%) was complicated by hepatic encephalopathy and prolonged prothrombin time for the diagnosis of fulminant hepatic failure. Other two (1%) patients remained positive for HBsAg for longer than 6 months, and they were considered to have acquired chronic infection.

Sera from the 147 patients with acute hepatitis B were examined virologically, and the results were correlated with clinical and demographic characteristics. Informed consent was obtained from each patient for the purpose of this study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Ethics Committees of our institutions.

Determination of HBV DNA

Levels of HBV DNA were determined using transcription-mediated amplification (TMA) and hybridization-protection assay (Chugai Diagnostics Science Co., Ltd., Tokyo, Japan) after the protocol as reported [Kamisango et al., 1999]. The range of detection by TMA was from 3.7 log genome equivalents (LGE)/ml ($10^{3.7}$ copies/ml corresponding to 5,000 copies/ml) to 8.7 LGE/ml ($10^{8.7}$ copies/ml). In 16 of 86 studied sera, levels of HBV DNA were under 3.7 LGE/ml and categorized in 3.7 LGE/ml.

Genotyping HBV

HBV genotypes in most samples were determined with commercial enzyme immunoassay kits (HBV Genotype EIA, Institute of Immunology Co. Ltd., Tokyo, Japan) involving monoclonal antibodies to genotype-specific epitopes in the preS2-region, as reported previously [Usuda et al., 1999, 2000; Kato et al., 2001]. Genotypes in 18 (12%) samples were determined by genotype-specific probe assay (Smitest HBV Genotyping Kit, Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by the polymerase chain reaction (PCR) with three sense primers (s1: 5′-ACC AAC CCT CTG GGA TTC TTT CC-3′, s2: 5′-ACC AAT CCT CTG GGA TTC TTC CC-3′ and s3: 5′-AGC AAT CCT CTA GGA TTC CTT CC-3′ [nt 2902–2924]) and an antisense primer (as1: 5′-GAG CCT GAG GGC TCC ACC C-3′ [nt 3091–3073]) biotinated at the 5′-end;

they were deduced from conserved sequences in the preS1 region of HBV. The biotin-labeled and amplified HBV DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one or other of the seven genotypes (A–G) immobilized on wells of a 96-well microplate. Thereafter, hybiridization was detected by staining with the streptavidine-horseradish peroxidase (HRP) conjugate [Kato et al., 2003].

Subtypes of genotype B, in terms of Ba with the recombination with genotype C and Bj without it were determined by direct sequencing of precore and core regions by the method reported previously [Sugauchi et al., 2002b].

Amplifying and Sequencing HBV DNA of Genotype A Isolates

A subgroup of genotype A is reported with the designation of A' from South Africa, Philippines, Malawi, and Belgium [Bowyer et al., 1997; Kramvis et al., 2002; Sugauchi et al., 2004]. Randomly selected HBV/A samples were classified into genotype A and subtype A' by sequencing the S region. For amplification and sequencing, the entire S region was divided into two fragments, spanning nt 3130–478 and nt 378–878, respectively, and they were amplified by two-stage PCR. The outer primers for amplification of the 1st fragment were: 5'-ACC AAT CGG CAG TCA GGA AG-3' (sense: nt 3121–3140) and 5'-CTG GAA TTA GAG GAC AAA CG-3' (antisense: nt 488–469) and the inner primers were: 5'-CAG TCA GGA AGG CAG CCT ACT-3' (sense: nt 3130–3150) and 5-AGG ACA AAC GGG CAA CAT AC-3' (antisense: nt 478–459). The outer primers for amplification of the 2nd fragment were: 5'-TGT CCT GGT TAT CGC TGG AT-3' (sense: nt 359–378) and 5'-CAA CGT ACC CCA ACT TCC AA-3' (antisense: nt 909–890) and the inner primers were: 5'-TGT GTC TGC GGC GTT TTA TC-3' (sense: nt 378–397) and 5'-ATG AAG TTT AGG GAA TAA CC-3' (antisense: nt 878–859).

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 μl of the reaction mixture containing 200 μM dNTPs, 1.0 μM each of primers and 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% (wt/vol) gelatin) and 2 U of Ampli-Taq polymerase (Perkin Elmer Cetus Corp., Connecticut). PCR products (2 μl) were subjected to the second stage of amplification under the same conditions as the first stage. Standard precautions to avoid contamination were exercised during PCR, with a negative control serum included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Wisconsin), and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, California) using the PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377: PE Applied Biosystems).

The nucleotide sequences of HBV/A isolates from patients were compared with those of 25 reference HBV/A strains including subtype A' retrieved from the DDBJ/EMBL/GenBank database, as well as representatives of the other six major genotypes (B–G). Phylogenetic trees were constructed with the mega program version 2.1 using the Kimura two-parameter matrix and the neighbor-joining method [Sugita et al., 1991]. To confirm the reliability of phylogenetic tree analysis, bootstrap resampling, and reconstruction were carried out 500 times.

Detection of Point Mutations in the Precore and BCP Regions of HBV

Mutation in the precore region for A1896 was detected by enzyme-linked minisequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostics, Tokyo, Japan) and mutations in the BCP region for T1762/A1764 were detected by enzyme-linked specific probe assay (Smitest HBV Core Promoter Mutation Detection Kit; Genome Science Laboratory, Tokyo, Japan) according to the manufacturer's instructions, after the principles described previously [Orito et al., 2001b]. The results were recorded as "the wild-type" and "the mutant-type" expressed dominantly by HBV isolates.

Statistical Analysis

Data were analyzed by chi-square test or Fisher's exact test for categorical data and Student's *t*-test or Mann–Whitney *U*-test for continuous variables. *P*-values less than 0.05 were regarded as statistically significant. Logistic regression (backward logistic regression) was used in the multivariate analysis to evaluate the factors associated with differences between genotypes A and C.

RESULTS

Distribution of HBV Genotypes

HBV genotypes were determined in 145 of the 147 (99%) patients with acute hepatitis B; they were untypeable in the remaining two patients (Table I). Genotype A was detected in 27 (19%) patients, B in 8 (5%), C in 109 (75%), and mixed genotypes with B and C in the remaining one (1%). In the 69 patients with acute hepatitis B from metropolitan areas (Tokyo, Kawasaki, and Tokorozawa), genotype A was found in 21 (30%), B in 5 (7%), and C in 43 (63%). In the 76 patients from the other areas in the mainland, by contrast, genotype A occurred in 6 (8%), B in 3 (4%), C in 66 (87%), and mixed genotypes with B and C in one (1%). Thus, genotype A was significantly more frequent in patients with acute hepatitis B from the metropolitan than the other areas (30% vs. 8%, $P < 0.001$).

Demographic and Clinical Differences Among Patients Infected With HBV of Distinct Genotypes

Clinical and demographic backgrounds in patients with acute hepatitis B who were infected with HBV of

TABLE I. Demographic and Clinical Differences Among Patients With Acute Hepatitis Who Were Infected With HBV of Distinct Genotypes

Features	Genotypes of HBV				Differences (A vs. C)	
	A (n = 27)	B (n = 8)	C (n = 109)	B/C (n = 1)	Univariate (*P*-value)	Multivariate logistic regression (*P*-value)
Areas					<0.001	0.03
Metropolitan (n = 69)	21 (30%)	5 (7%)	43 (63%)	0		
Others (n = 76)	6 (8%)	3 (4%)	66 (87%)	1 (1%)		
Age (years)	29.3 ± 8.0	35.7 ± 10.1	36.6 ± 13.6	51	0.016	0.152
Male	25 (93%)	7 (88%)	69 (57%)	1 (100%)	0.003	0.018
Transmission routes						
Heterosexual	15 (56%)	3 (37%)	52 (48%)	0	0.197	
Homosexual	5 (19%)	1 (13%)	2 (2%)	0	<0.001	0.133
IV drugs	0	0	8 (7%)	0	0.280	
Unknown	7 (25%)	4 (50%)	47 (43%)	1 (100%)	0.102	
Fulminant hepatic failure	0	1 (13%)	5 (5%)	0	0.582	
ALT (IU/L)[a]	2069 ± 1075	2952 ± 1106	2889 ± 1867	646	0.030	0.084
Bilirubin (mg/dl)[a]	10.7 ± 14.1	10.3 ± 4.9	7.8 ± 6.7	4.8	0.533	
ALP (IU/L)[a]	476 ± 161	501 ± 94	432 ± 116	No data	0.542	
HBeAg	24/26 (92%)	4/8 (50%)	57/93 (61%)	1/1 (100%)	0.357	
Precore and BCP mutations						
Precore (1896A)	0/27	1/8 (13%)	20/102 (20%)	No data	0.250	
BCP (1762T/1764A)	0/27	1/6 (17%)	14/75 (19%)	No data	0.357	
Precore or BCP	0/27	2/8 (25%)	27/102 (26%)	No data	0.096	

[a]Maximum data are shown for alanine aminotransferase (ALT), bilirubin and alkaline phosphatase (ALP).

different genotypes are compared in Table I. Patients with genotype A were younger than those with genotype C (29.3 ± 8.0 vs. 36.6 ± 13.6 years, $P = 0.016$). The proportion of male patients was higher in genotype A than C infection (93% vs. 57%, $P = 0.003$). The main route of transmission identified in the patients with acute hepatitis B was extramarital heterosexual contacts. Homosexual activity was more frequent in patients with genotype A than C (5/27 (19%) vs. 2/109 (1.8%), $P < 0.001$).

The maximum ALT levels were lower in patients with genotype A than B or C infection (2069 ± 1075, 2952 ± 1106 and 2889 ± 1867 IU/L, respectively: A vs. B, $P = 0.02$; A vs. C, $P = 0.03$). The maximum bilirubin and alkaline phosphatase levels were no different among patients infected with HBV of different genotypes. Fulminant hepatic failure developed in one (13%) patient with genotype B and five (5%) with genotype C; no patients with genotype A came down with it. Evolution into chronic infection occurred in two patients (one with genotype A and one with genotype C). The remaining 137 (96%) patients ran a non-fulminant and self-limited disease.

HBeAg was found in 24 of the 26 (92%) patients with genotype A, 4 of the 8 (50%) with genotype B and 57 of the 93 (61%) with genotype C; it was no different between genotype A than genotype C infection ($P = 0.357$). Of the six patients with fulminant hepatic failure, only one (17%) had HBeAg.

With logistic multivariate regression analysis, the variables for differences between genotypes A and C were sex (odds ratio (OR), 6.45; 95% confidence interval (CI), 1.378–30.213; $P = 0.0018$) and area (OR, 0.25; 95% CI, 0.076–0.830; $P = 0.0024$).

Routes of transmission were compared between genotypes A and C in patients with acute hepatitis B from metropolitan areas. Although the mean age was no different, frequently the proportion of male patients was higher in genotype A than C infection (20/21 (95%) vs. 28/43 (65%), $P = 0.012$). Homosexual patients had more frequently genotype A than C infection (5/21 (24%) vs. 1/44 (2%), $P = 0.012$). Additionally heterosexuals with multiple unspecific partners had in genotype A more frequently than C infection (7/12 (58%) vs. 6/26 (23%), $P = 0.035$, respectively). However, with logistic multivariate regression analysis, none of these variables differed between genotype A and C infections.

Figure 1 compares serum HBV DNA levels on admission among patients infected with different genotypes. HBV DNA levels were higher in patients with genotype A than C (5.90 ± 1.45 vs. 5.13 ± 1.36 LGE/ml, $P = 0.002$).

Among the 145 patients whose HBV genotypes could be determined, 54 (A: 15, B: 4, and C: 35) were followed for HBsAg in serum every 2–4 weeks until it disappeared. The time between the first and last detection of HBsAg was defined as the duration of HBsAg, and compared between patients infected with HBV of genotypes A and C (Fig. 2a). The duration of HBsAg was longer in patients with genotype A than C infection (1.95 ± 1.09 (n = 15) vs. 1.28 ± 1.42 months (n = 35), $P = 0.02$). When patients with fulminant hepatic failure were excluded, the mean duration of HBsAg in patients with genotype C became longer, but it was still shorter

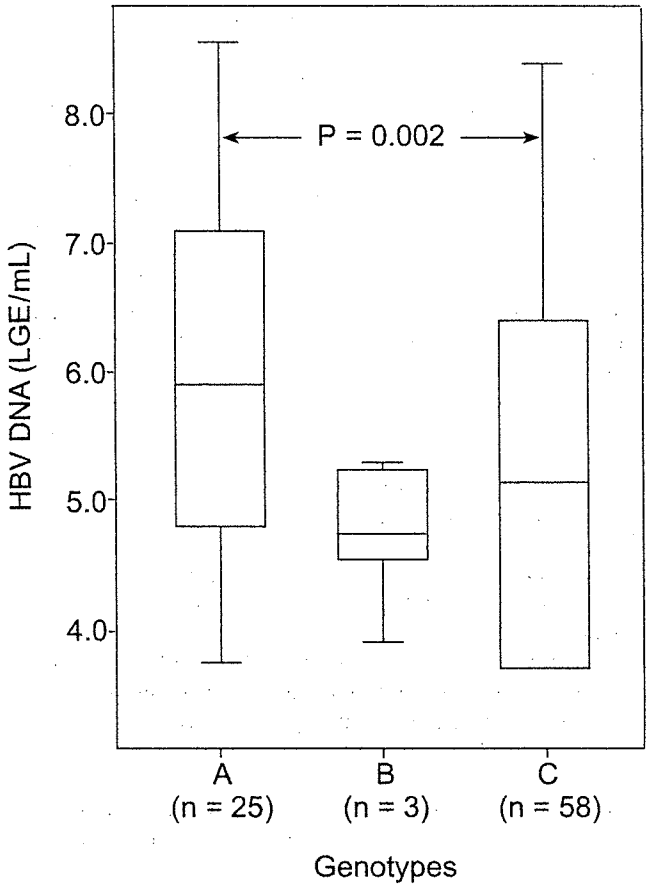

Fig. 1. HBV-DNA levels in patients with acute hepatitis B with genotypes A, B, or C at the presentation. Box plots are given with horizontal lines for the medians, upper and lower edges indicating the 25th and 75th centiles, respectively, and bars represent the extremes without including outliers. Shaded areas are outside the range of detection by the TMA method.

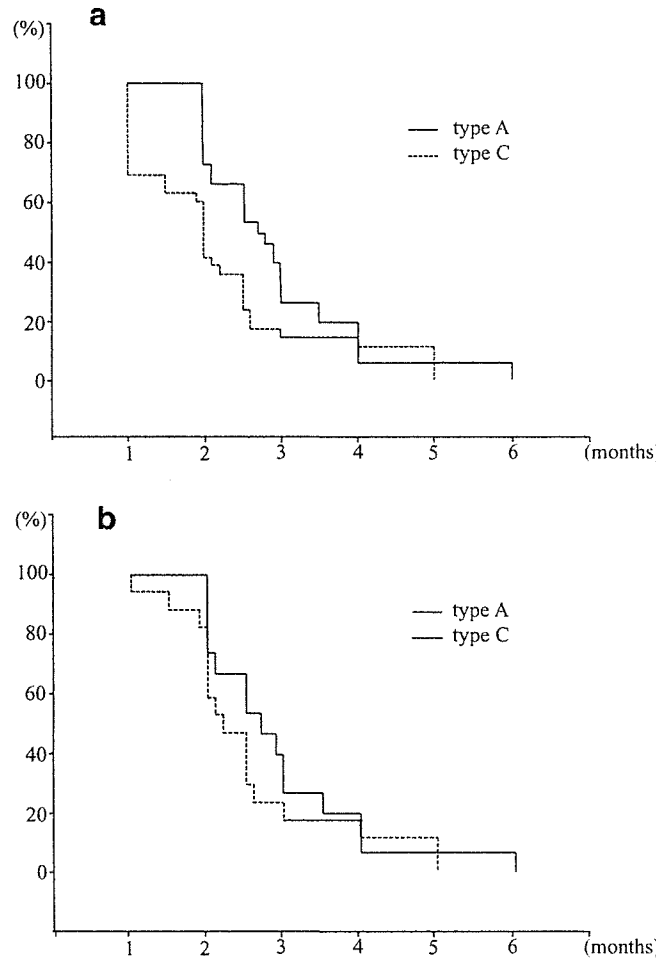

Fig. 2. The duration of HBsAg in patients with acute hepatitis B with genotypes A or C. The results are shown for (**a**) all patients, and (**b**) patients with the wild-type sequences both in precore and BCP regions of HBV.

than that in those with genotype A (1.95 ± 1.09 (n = 15) vs. 1.41 ± 1.42 (n = 31) months, $P = 0.03$).

Subtypes of Genotypes A and B

Among the 27 HBV/A isolates, 9 were selected at random and the entire S region was amplified and sequenced for them. Seven of them were classified into genotype A and the remaining 2 into subgroup A′. The sequence divergence within the seven genotype A isolates ranged from 0.12% to 2.01% in pair-wise comparison, while that between two subgroup A′ and seven genotype A isolates spanned from 5.70% to 6.53%.

A phylogenetic tree was constructed on the entire *S*-gene sequences from these nine sequences along with those from 31 HBV isolates retrieved from the database (Fig. 3). The seven (78%) HBV isolates classified into genotype A clustered with reported HBV/A isolates, while the remaining two isolates classified into subgroup A′ (cases 3 and 4) joined the branch of subgroup A′.

Six of the eight HBV/B isolates were available for analysis of subtypes. Two (both from the metropolitan area) were classified as Ba and the remaining four, including two from Tokyo and two from the other areas, as Bj. One of the four patients infected with subtype Bj developed fulminant hepatic failure, while the remaining three with subtype Bj as well as the two with subtype Ba ran a non-fulminant course.

Point Mutations in the Precore and Basic Core Promoter Regions of HBV

All the 27 HBV isolates of genotype A in which mutations were sought had the wild-type sequences both in the precore and BCP regions. In contrast, of the 102 genotype C isolates whose precore and BCP sequences were examined, 27 (26%) had mutations in the precore or BCP regions ($P = 0.096$). Furthermore, of the four genotype C isolates from patients with fulminant hepatic failure whose genetic mutations could be determined, three had mutations in the BCP region (T1762/A1764) and two had a mutation in the precore region (A1896). Only one isolate had the wild-type sequences both in the precore and BCP regions. Of

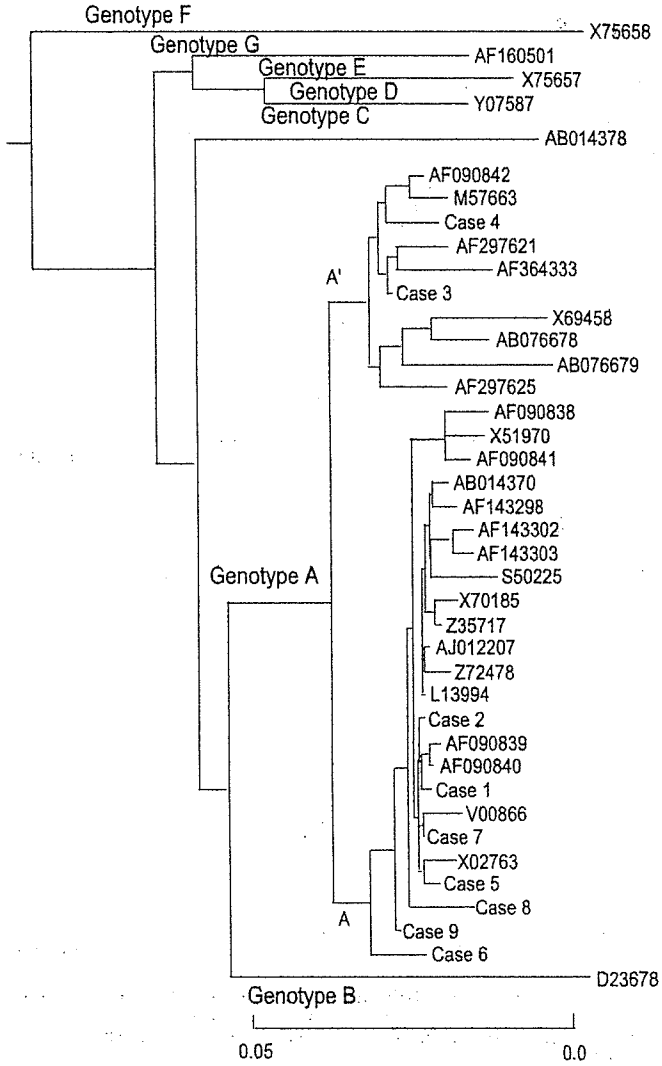

Fig. 3. A phylogenetic tree constructed on HBV DNA sequences spanning the major *S*-gene of all known HBV genomes, including the nine of genotype A. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates, which have been deposited in the DDBJ/EMBL/GenBank databases. HBV sequences in cases 1–9 were determined in the present study. The HBV/A sequences from cases 1, 2, and 5–9 clustered with the European-American genotype A, while those from cases 3 and 4 clustered with genotype A' that is the African subgroup of genotype A.

the eight genotype B isolates, two (25%) had mutations in the precore or BCP region (Table I).

To examine further differences between genotype A and C infections, patients infected with HBV strains with the wild-type sequences both in precore and BCP regions were compared. The maximum ALT levels were still lower in patients with genotype A than C infection (2069 ± 1075 and 2594 ± 1015 IU/L, respectively, $P = 0.02$), but the maximum bilirubin and alkaline phosphatase levels were no different amongst patients infected with HBV of distinct genotypes. There were no differences in the duration of serum HBsAg between patients with genotype A and C infections (1.95 ± 1.09 vs. 1.58 ± 1.24 months, $P = 0.35$) (Fig. 2b).

DISCUSSION

The salient finding in this study is that infection with HBV genotype A is frequent in patients with acute hepatitis in Japan, lending support to previous studies [Kobayashi et al., 2002; Ogawa et al., 2002]. Substantial portion of patients with acute hepatitis were infected with genotype A, which is detected rarely among patients with chronic hepatitis in Japan [Orito et al., 2001a; Kobayashi et al., 2002]. Genotype A prevails in North-Western Europe, United States, Central Africa, and India [Kao, 2002; Miyakawa and Mizokami, 2003]. This genotype may be prevalent in countries elsewhere, since the distribution of HBV genotypes has not been examined in many districts of the world. Phylogenetic analysis has shown that seven (78%) HBV/A strains of the nine patients examined with acute hepatitis B were of the European-American type. Although the HBV/A sequences from four, (cases 1, 2, 5, and 7) clustered with those reported previously, those from three (cases 6, 8, and 9) were separated genetically (Fig. 3), which suggests their distinct geographic origin.

Notably, the genotype distribution differed between patients with acute hepatitis B from metropolitan areas and the others including many large cities. As genotype A is seen rarely in patients with chronic hepatitis [Orito et al., 2001a; Kobayashi et al., 2002], it is suspected that genotype A in metropolitan areas has a distinct geographic origin. Many patients with genotype A infection in these areas had a history of extramarital sexual contacts with plural unspecified partners. Such sexual behavior may increase the risk of infection with genotype A. In support of this view, most homosexual people in Tokyo who have human immunodeficiency virus type I are coinfected with HBV genotype A [Koibuchi et al., 2001]. Taken together, homosexual activity would increase the risk of genotype A infection in metropolitan areas. Further molecular analysis on HBV isolates from transmitters and recipients will verify this hypothesis. With respect to genotype B, both Ba, and Bj subtypes [Sugauchi et al., 2002b] were detected. Although the number of studied patients was small, patients with subtype Ba were found in the Tokyo metropolitan area exclusively. Whether subtype Ba intrinsic to the metropolitan area has a peculiar geographic origin is currently unknown and awaits further analyses.

Another point made in this study is that HBV genotypes influence clinical features and the outcome of acute hepatitis B. It has been shown that the proportion of patients who develop chronic HBV infection is close to 10% in European and American countries [Sherlock S, 1997] but rare in Japan [Kobayashi et al., 2002]. Recent studies suggest that chances for evolution into chronicity may differ among patients acutely infected with HBV of distinct genotypes [Mayerat et al., 1999; Ogawa et al., 2002]. Our study has shown that patients with genotype A had higher HBV DNA and lower ALT levels, as well as a longer duration of HBsAg in serum. Development of chronic hepatitis was seen in one of the 27 (4%) patients with genotype A as against one of the 109 (1%)

with genotype C infection. Although the number of patients studied was not large enough for statistical evaluation, the transition to chronic infection may be more frequent in infection with genotype A than the other genotypes, insofar as higher viral loads can predict chronic infection [Fong et al., 1994]. Further studies on more patients are required to evaluate whether or not viral persistence occurs more often after HBV infection with genotype A than the other genotypes.

Patients with fulminant hepatic failure in the present study were infected with either genotypes B or C; no patient with genotype A developed hepatic failure. As mutations at nt 1896 in the precore and nt 1762/1764 in the BCP regions, which are found frequently in patients with fulminant hepatic failure [Carman et al., 1991; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Hawkins et al., 1994; Sato et al., 1995; Baumert et al., 1996; Chu et al., 1996], were not detected in patients with genotype A, low frequency of fulminant hepatic failure associated with genotype A infection may be attributed to the lack of these mutations. The high frequency of HBeAg in genotype A infection may also be related to low frequency of fulminant hepatic failure. However, interpretation on this data should be made carefully, because the number of patients studied was small. Further research is necessary to determine if the genotype itself affects the clinical course of acute hepatitis B.

In summary, (1) infection with HBV genotype A is common in patients with acute hepatitis in Japan; (2) patients with genotype A are more frequent in metropolitan areas and may be associated with particular sexual behavior; (3) patients with genotype A have a milder but longer course of infection, which may lead to increased risk of progression to chronic disease.

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