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Journal of Viral Hepatitis, 2004, 11, 1–7

Effects of ribavirin combined with interferon-α2b on viral kinetics during first 12 weeks of treatment in patients with hepatitis C virus genotype 1 and high baseline viral loads

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Received ????? 2003; accepted for publication November 2003

SUMMARY. This study aimed to find how ribavirin increases viral disappearance in patients with hepatitis C virus (HCV) of genotype 1 and high baseline viral loads (>5.0×10^5 copies/mL) when given with interferon (IFN). Using the real-time quantitative polymerase chain reaction, we measured serum HCV in 20 patients during the first 12 weeks of therapy with IFN-α2b and ribavirin. Controls were 10 similar patients given IFN-α2b alone. IFN-α2b was given at 6 MU daily for 2 weeks, and then three times weekly. Ribavirin was given at 600 or 800 mg daily. Serum HCV RNA decreased rapidly in the first phase, during the first 24 h of therapy (day 0), and more slowly in the early second phase (days 1–14). The median decrease was by 1.41 and 0.078 log 10/day in these two phases in the combination therapy group, and 0.90 and 0.081 log 10/day in the monotherapy group. The difference between groups in the first phase was not significant ($P = 0.24$), nor was that in the next phase ($P = 0.68$). Later in the second phase, between days 14 and 84, the median decrease was larger in the combination therapy group (0.030 log 10/day) than in the monotherapy group (0.015 log 10/day, $P = 0.035$). In patients with HCV genotype 1 and high viral loads, the effects of ribavirin with IFN-α appeared slowly, after the earliest days of treatment. A long-term favourable outcome of combination therapy may be associated with a rapid viral decline in this later phase of therapy.

Keywords: hepatitis C, interferon, polymerase chain reaction, ribavirin, viral kinetics.

INTRODUCTION

Hepatitis C virus (HCV) infects some 170 million people worldwide and is an important health care problem [1]. Persistent infection with HCV often progresses to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma over the course of several decades. As the report by Hoofnagle *et al.* [2] in 1986 on the effects of interferon (IFN) therapy on chronic hepatitis C, this drug has been the only approved agent for eradication of HCV and perhaps reduction of the incidence of hepatocellular carcinoma [3–5]. Several factors identifiable before therapy are independent predictors of the response to IFN, including the baseline level of serum HCV [6,7] and the HCV genotype [8]. In addition, analysis of the changes in HCV titres in the early part of IFN treatment is useful for prediction of the therapeutic response. After a delay of 7–10 h after IFN administration starts, the amount of viral RNA declines rapidly, with an estimated half-life of 5.0–7.2 h, during the first 1 or 2 days of therapy, and then declines more slowly [9,10]. In patients with HCV genotype 1, viral decline in the early phase of IFN treatment is slower than that in patients with genotype 2 [11], which may explain in part their lower rate of sustained virological response. We reported earlier [12] that the changes in serum levels of HCV genotype 1 during the first 2 weeks of IFN-α treatment can be used for prediction of the long-term outcome of therapy.

Ribavirin is a synthetic guanosine nucleoside analogue that inhibits the replication of various RNA and DNA viruses. In patients with chronic hepatitis C, the combination of IFN-α and ribavirin gives a higher rate of sustained virological response than IFN-α alone [13–17]. The combination has become the standard therapy, especially for patients with HCV genotype 1 and high baseline viral loads. However, the

Abbreviations: HCV, hepatitis C virus; IFN, interferon; TaqMan PCR, real-time quantitative polymerase chain reaction. PEG, polyethyleneglycol.

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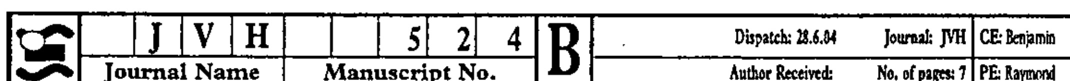

synergistic effects of ribavirin when combined with IFN on the changes in HCV levels have not been fully identified. Zeuzem *et al.* reported that viral kinetics do not differ between patients treated with IFN-α alone and those given ribavirin also [18]. One possible reason for this unexpected finding may be that viral kinetics could be analysed in that study only in the first few weeks of therapy. That is, patients with genotype 2 or with low viral loads were included, and the analytical sensitivity of the assay used to measure HCV RNA was limited, so viral RNA became undetectable in most subjects within the first 4 weeks of therapy. Pharmacokinetic studies showed that serum ribavirin concentrations take 4–8 weeks to reach a plateau [19,20]; the synergistic effects of this drug with IFN might become evident slowly, after the earliest phase of treatment. To test this hypothesis, a study of the kinetics of HCV in patients with genotype 1 and high viral loads monitored by a more sensitive quantitative method during a longer term of therapy with IFN alone and in combination with ribavirin is needed.

The aim of this study was to find how ribavirin increases viral decline in patients with chronic HCV infection when given with IFN-α. Using a sensitive real-time quantitative polymerase chain reaction (TaqMan PCR), we monitored serum HCV levels in patients with genotype 1 and high viral loads during the first 12 weeks of combination therapy with IFN-α2b and ribavirin. The results were compared with those in patients with similar baseline characteristics treated with IFN-α2b alone.

MATERIALS AND METHODS

Patients

The subjects were 20 patients with chronic hepatitis C (13 men and seven women; mean age, 55 ± 11 years) who started combination therapy with IFN-α2b and ribavirin at our hospital between March 1999 and December 2002. Ten patients with chronic hepatitis C (six men and four women; mean age, 56 ± 9 years) who were treated with IFN-α2b alone between October 1998 and July 2001 were used as historic controls. The inclusion criteria were as follows: persistent elevation of serum alanine aminotransferase activity for at least 6 months before therapy, presence of genotype 1 of HCV in serum, presence of serum HCV RNA of more than 5.0×10^5 copies/mL by TaqMan PCR, absence of serum hepatitis B surface antigen and signs of other likely causes of chronic liver disease, histological features of chronic hepatitis found in liver biopsy specimens taken within 6 months before the start of therapy, and no evidence of hepatocellular carcinoma on ultrasonography or computed tomography. Serum samples were obtained from the patients before the administration of the drug(s) on the first day of therapy (day 0) and on days 1, 7, 14, 28, and 84, and were stored at −80 °C before being tested. Procedures of the study were in accord with the Helsinki Declaration of 1975 (1983 revision) and were approved by our hospital ethics committee.

Treatment

Patients were treated with recombinant IFN-α2b (Intron A, Schering-Plough, Kenilworth, NJ, USA) by intramuscular injection at the dosage of 6 MU every day for 2 weeks, followed by 6 MU three times a week for 22–46 weeks. Ribavirin (Rebetol; Schering-Plough) was given orally twice a day at a total daily dose of 600 mg for the 10 patients who weighed 60 kg or less and 800 mg for the remaining 10 patients who weighed more than 60 kg for 24 weeks. The most common adverse effect of ribavirin is haemolysis. The dose of ribavirin was reduced by 200 mg per day in patients whose haemoglobin concentrations fell below 10 g/dL.

Assays

Routine haematological and biochemical tests were performed by the standard procedures. Genotypes of HCV were identified by direct sequencing of the amplification products generated during the Amplicor Monitor test (Roche Diagnostics, Branchburg, NJ, USA) [21] with an ABI 3700 DNA sequencer (Perkin Elmer Corp./Applied Biosystems, Foster City, CA, USA) [22]. Serum HCV RNA was measured by TaqMan PCR as described by Takeuchi *et al.* [23]. In brief, HCV RNA was extracted from 250 mL of a serum sample, converted to complementary DNA with reverse transcriptase, and amplified by PCR with a TaqMan EZ RT-PCR kit and the ABI Prism 7700 sequence detection system (Perkin Elmer). A TaqMan probe, labelled with fluorescent reporter and quencher dyes, annealed specifically to the template between the primers and then was digested during the PCR, resulting in the emission of fluorescence. Successive PCR cycles exponentially amplified the PCR product and increased the fluorescence intensity. The detection range of the assay was between 2.0×10^2 and 1.0×10^9 copies/mL of HCV RNA. For comparison, a second generation version of the Amplicor Monitor test [21] was used also to measure HCV RNA in serum. The detection range of the assay was between 0.5 and 500 kIU/mL (a standard sample containing 10^5 copies/mL of HCV was assigned a titre of 10^5 IU/mL).

Histology

Liver biopsy was performed for each patient within 6 months before the start of therapy. The histopathological findings were assessed by grading of inflammatory activity and staging of fibrosis by the classification of Desmet *et al.* [24] 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, USA). Distributions of continuous variables were ana-

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Table 1 Baseline characteristics of patients with chronic hepatitis C treated with IFN-α2b with or without ribavirin

Characteristics	Combination therapy group (n= 20)	Monotherapy group (n= 10)	P-value
Age (years)	55 ± 11	56 ± 9	0.96
Sex (M/F)	13/7	6/4	0.93
Previous IFN treatment (+/–)	13/7	6/4	0.93
Haemoglobin (g/dL)	14.3 ± 1.2	14.2 ± 1.5	0.86
ALT (IU/L)	102 (74–135)	94 (73–109)	0.64
HCV RNA (log 10 copies/mL)	7.07 ± 0.35	6.78 ± 0.51	0.11
Grade of inflammation			
Mild	11	7	0.52
Moderate	8	3	
Severe	0	0	
Stage of fibrosis			
Mild	8	6	0.42
Moderate	5	3	
Severe	6	1	

Values represented as mean ± SD for normally distributed variables, and medians (with the interquartile range) for non-normally distributed variables.
Serum HCV RNA was measured by TaqMan PCR.
IFN, interferon; ALT, alanine amino transferase; HCV, hepatitis C virus.

lysed by the Mann–Whitney U-test. Differences in proportions were tested by Fisher's exact test. The significance of correlation was evaluated by Spearman's rank analysis. A two-tailed P-value of <0.05 was taken to indicate statistical significance.

RESULTS

Baseline characteristics of patients

The baseline characteristics of patients in the two groups were similar (Table 1). All patients were infected with genotype 1b of HCV, which is the most common kind in this country. In one patient in the combination therapy group, the biopsy sample was too small for evaluation, except for the finding of chronic hepatitis.

Changes in HCV RNA in first 12 weeks of treatment

In the first 12 weeks of treatment, no patient needed reduction in the dose of IFN-α2b. The dose of ribavirin was reduced in one patient at week 10, because the haemoglobin concentration was <10 g/dL. The proportions of patients without HCV RNA detectable by the Amplicor Monitor test and by TaqMan PCR at different times during therapy are shown in Tables 2 and 3, respectively. Of the patients in whom serum HCV RNA decreased to under the detection limit, none had relapse of viraemia during the 12 weeks. Changes in serum HCV RNA as monitored by TaqMan PCR during the first 12 weeks of therapy are shown in Fig. 1. As previously reported, serum HCV levels decreased rapidly during the first 24 h of therapy and more slowly thereafter. For the analysis here, we tentatively defined the period between 0 and 24 h of therapy (day 0) as the first phase, the period between days 1 and 14 as the early second phase, and the period between days 14 and 84 as the late second phase (see Discussion).

Decline of HCV RNA in different phases of treatment

The rate of decrease in serum HCV RNA as monitored by TaqMan PCR in each phase of treatment with IFN-α2b alone

Table 2 Patients without detectable serum HCV RNA by Amplicor Monitor test four times during treatment

Group	n	Numbers (%) on Day 7	Day 14	Day 28	Day 84
Combination therapy	20	0 (0)	3 (15)	8 (40)	13 (65)
Monotherapy	10	0 (0)	0 (0)	0 (0)	6 (60)

Table 3 Patients without detectable serum HCV RNA by TaqMan PCR four times during treatment

Group	n	Numbers (%) on Day 7	Day 14	Day 28	Day 84
Combination therapy	20	0 (0)	1 (5)	3 (15)	12 (60)
Monotherapy	10	0 (0)	0 (0)	0 (0)	4 (40)

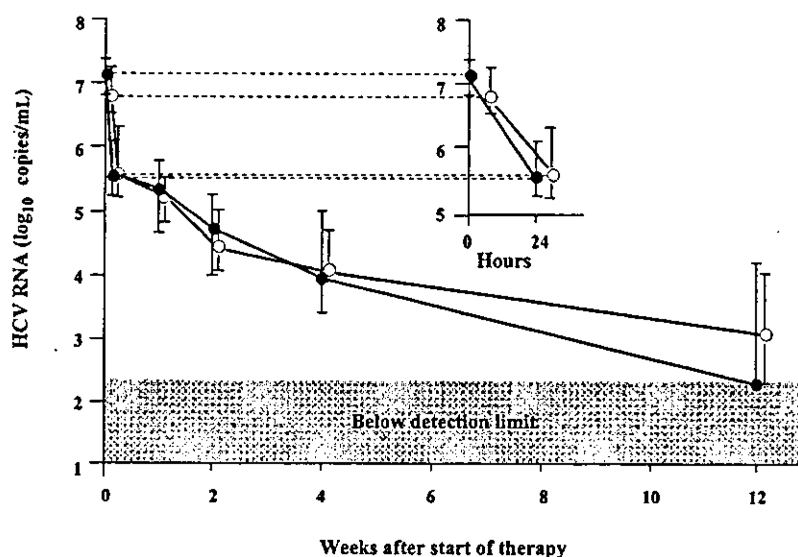

Fig. 1 Changes in serum hepatitis C virus (HCV) RNA during the first 12 weeks of therapy: (●) in 20 patients treated with interferon (IFN)-α2b in combination with 600 or 800 mg of ribavirin daily depending on body weight, and (○) in 10 patients treated with IFN-α2b alone. IFN-α2b was given at the dosage of 6 MU every day for 2 weeks, followed by 6 MU three times a week. Serum HCV RNA was measured by TaqMan PCR. Values are medians, with bars showing the interquartile range. The lower detection limit of TaqMan PCR was 2.0×10^2 copies/mL.

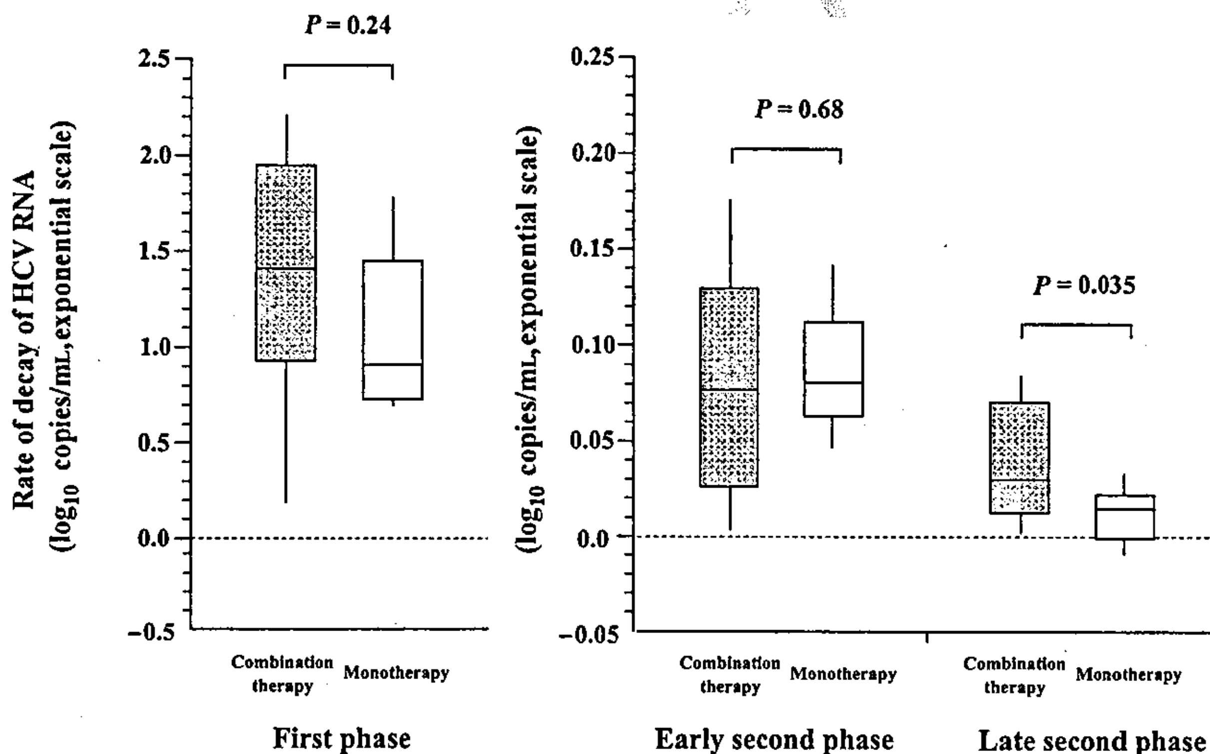

Fig. 2 Rate of decrease in serum hepatitis C virus (HCV) RNA per day in patients treated with interferon (IFN)-α2b alone or in combination with ribavirin: in the first phase between 0 and 24 h of therapy (day 0), in the early second phase between days 1 and 14, and in the late second phase between days 14 and 84. Serum HCV RNA was measured by TaqMan PCR. The boxes show medians and interquartile ranges; the vertical bars show the ranges. In the late second phase between days 14 and 84, the rate of decrease in the combination therapy group was significantly higher than that in the monotherapy group (P = 0.035).

or in combination with ribavirin is shown in Fig. 2. The differences in the first 14 days between the two groups were not significant (first phase $P = 0.24$, and second phase 0.68). In the late second phase (between days 14 and 84, or between days 14 and 28, if HCV RNA was not detected on day 84), the decrease in the combination therapy group was

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larger than that in the monotherapy group ($P = 0.035$), when samples below the detection limit of the assay were assigned the viral load of the detection limit and one patient without detectable HCV RNA at day 14 was excluded from analysis.

In patients given both drugs, correlation was not significant between the first phase and early second phase decreases ($r = -0.084$, $P = 0.73$), between the early second phase and late second phase decreases ($r = -0.12$, $P = 0.63$), nor between the late second phase and first phase decreases ($r = 0.29$, $P = 0.23$). In patients given IFN-α2b only, correlation was not significant in any of these comparisons ($r = -0.27$, $P = 0.54$; $r = 0.36$, $P = 0.39$; $r = -0.29$, $P = 0.51$, respectively).

DISCUSSION

Earlier [25], we compared the analytical sensitivity and validity of TaqMan PCR for measurement of HCV RNA with those of other widely used quantitative methods. The sensitivity of TaqMan PCR was the highest, and the method gave accurate results throughout the wide detection range. In this study, the baseline HCV level of all patients was within the detection range of TaqMan PCR, but was more than the upper detection limit (500 kIU/mL) of the Amplicor Monitor test in 18 of the 30 patients. The proportion of patients without detectable HCV RNA during therapy was smaller with TaqMan PCR than with the Amplicor Monitor test. TaqMan PCR was more suitable for the monitoring of changes in HCV RNA levels during antiviral therapy, because of its wide detection range.

We found that the second phase decrease in HCV monitored by TaqMan PCR is correlated with the sustained virological response to IFN-α monotherapy [12]. Neumann *et al.* [9] 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. The rate of HCV-infected cell death may depend on cellular immunity involving cytotoxic T-lymphocytes. Therefore, the results of our previous study may mean that a strong cellular immune response is needed for sustained loss of HCV by treatment with IFN alone.

For two reasons, we divided the second phase into the early second phase (first 2 weeks) and the late second phase (after the first 2 weeks) and evaluated changes in HCV RNA. First, our protocol of IFN administration, which is common in this country, consisted of daily induction therapy for the first 2 weeks and then thrice weekly maintenance therapy. As expected, in both groups, viral decrease in the early second phase (still induction therapy) was larger than that in the late second phase (maintenance therapy). Secondly, it takes several weeks for serum ribavirin concentrations to reach a plateau [19,20]. The exact mechanism of action of ribavirin when combined with IFN is not known [26]. Ribavirin may inhibit HCV RNA-dependent RNA polymerase, the capping structure of viral messenger RNA, and inosine monophosphate dehydrogenase. Other immunomodulatory actions also may contribute to the effects of this drug [27,28]. No matter which action is most important, these pharmacokinetic results suggested that the synergistic effects of ribavirin given with IFN might appear slowly after the earliest phase of treatment. The most striking finding in our study was that in the late second phase, the rate of decrease in HCV in the combination therapy group was larger than that in the monotherapy group. Because the observation period in this study was 12 weeks, we do not know which phase(s) of viral decrease is associated with sustained virological response to combination therapy. However, faster viral decline in the late second phase would contribute to the long-term outcome of treatment.

Changes in serum HCV RNA during the first few weeks of IFN monotherapy are useful in prediction of the outcome [29,30]. Reliable prediction of the response to therapy early would be useful, because IFN is expensive and sometimes has serious side-effects. In this study, however, the viral decrease in the first 2 weeks was similar in the two groups, and was not correlated with the viral decrease afterwards. Perhaps viral decline in the first few days of treatment cannot be used for prediction of the long-term response to combination therapy with IFN-α and ribavirin.

The attachment of a polyethyleneglycol (PEG) moiety to IFN-α produces a biologically active molecule, PEG-IFN-α, with a long half-life and favourable pharmacokinetics. Randomized controlled trials have shown that weekly treatment with PEG-IFN-α plus ribavirin is well tolerated, and gives higher rates of sustained virological response than treatment with unmodified IFN-α plus ribavirin [31,32]. Changes in HCV levels during combination therapy with PEG-IFN-α and ribavirin should be further evaluated in large clinical studies.

In summary, in a group of patients with HCV genotype 1 and high viral loads treated with IFN-α2b plus ribavirin and such a group treated with IFN-α2b alone, differences in the rate of decrease in viral RNA in the first phase, during the first 24 h of therapy, and in the early second phase, between days 1 and 14, were not significant. Later in the second phase, between days 14 and 84, the rate of decrease in the combination therapy group was greater than that in the monotherapy group. Our results suggest that a long-term favourable response to therapy with IFN-α2b and ribavirin for chronic hepatitis C is associated with rapid viral disappearance in this later phase.

ACKNOWLEDGMENTS

We thank Ms Caroline Latta for critical reading of the manuscript, and Ms Megumi Kimura, Ms Ikuyo Nakamachi, and Ms Seiko Harima for technical assistance. We are

grateful to all participants in the trial and to others who contributed in various ways. This work was supported in part by a grant from the Ministry of Health, Labour and Welfare, Japan.

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2003. 12. 24
Am. J. Gastroenterology

Adefovir Dipivoxil to Prevent Exacerbation of Lamivudine-resistant Hepatitis B Infection during Chemotherapy for Non-Hodgkin's Lymphoma

TO THE EDITOR: Lamivudine is a potent inhibitor of hepatitis B virus (HBV) replication and is widely used for the treatment of chronic hepatitis B (1). In HBV carriers receiving cytotoxic or immunosuppressive chemotherapy for hematological malignancies, acute exacerbation of hepatitis following viral reactivation is well documented; therefore, prophylactic or therapeutic use of lamivudine is highly recommended (2, 3). However, prolonged treatment may induce the emergence of lamivudine-resistant variants with mutations in the reverse transcriptase (rt) domain of the HBV polymerase gene. There is currently no consensus on the management of exacerbations of chronic HBV infection in patients with lamivudine-resistant variants during chemotherapy. Adefovir dipivoxil, another nucleotide analogue, has recently demonstrated potency against HBV, including lamivudine-resistant strains (4–6). We hereby report a patient with lamivudine-resistant HBV who was treated with adefovir dipivoxil during chemotherapy for non-Hodgkin's lymphoma.

A 44-year-old woman with malaise and generalized lymphadenopathy was admitted to our hospital in December 2002. About 5 years previously, chronic hepatitis B had been diagnosed. Her mother and all 4 siblings were also carriers of HBV. Treatment with lamivudine was started at a daily dose of 100 mg in June 2001. The subsequent clinical course is shown in Figure 1. Shortly after the start of therapy, the serum HBV DNA level declined, and transaminase activity normalized by week 20. At week 40 of therapy, viral DNA became undetectable by transcription-mediated amplification assay (<3.7 $\log_{10}$ copies/ml). At week 64, however, lamivudine-resistant HBV variants emerged, and viral DNA became detectable again in serum. On admission (at week 70), alanine aminotransferase (ALT) activity was 21 IU/L, aspartate aminotransferase (AST) activity 45 IU/L, lactate dehydrogenase activity 872 IU/L, and γ-glutamyltransferase activity 29 IU/L. The hepatitis B e (HBe) antigen was positive

and the anti-HBe negative, with an HBV DNA level of 6.7 $\log_{10}$ copies/ml. The genotype of the HBV was type C. The stop codon mutation at nucleotide (nt) 1896 in the precore region of HBV DNA was not found, but mutations were found at nt 1762 and nt 1764 in the basal core promoter. Mutations related to lamivudine resistance were detected from leucine to methionine at amino acid rt180 and from methionine to valine at rt204.

On the basis of the findings of lymph node biopsy, ultrasonography, computed tomography, and gallium scintigraphy, diffuse large-B-cell non-Hodgkin's lymphoma (stage III) was diagnosed. Five courses of chemotherapy with adriamycin, cyclophosphamide, and vincristine were administered between December 2002 and April 2003. After three courses of chemotherapy, lymph nodes regressed considerably in size. However, lymphoma recurred after the fifth course; involvement of the liver and spleen were found on gallium scintigraphy. The patient did not respond to subsequent intensive chemotherapy with a combination of cytarabine and carboplatin in May 2003, two courses of rituximab plus etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin between June and July 2003, or two courses of fludarabine, mitoxantrone, and dexamethasone in August 2003. On September 25, the patient died suddenly of rupture of splenic lesions.

At the start of antineoplastic chemotherapy, the ALT activity was 58 IU/L, and the AST activity 71 IU/L with an HBV DNA level of 8.3 $\log_{10}$ copies/ml. To prevent chemotherapy-induced exacerbation of lamivudine-resistant HBV infection, treatment with adefovir dipivoxil at 10 mg daily was added to the lamivudine. The serum HBV DNA level fell immediately after the commencement of adefovir dipivoxil and became undetectable at week 20 of therapy. HBe antigen did not seroconvert to anti-HBe. ALT activity was 170 IU/L at week 8 of therapy, decreased thereafter, and normalized at week 16 of therapy. During 36 weeks of therapy with adefovir dipivoxil, suppression of HBV replication was sustained, allowing chemotherapy to proceed without delay or modification, albeit the response to chemotherapy was not favorable. There were no

clinically significant adverse events related to adefovir dipivoxil, such as nephrotoxicity, which has been reported with higher doses (≥30 mg daily).

Ohmoto *et al.* described a case of lamivudine-resistant HBV reactivation that was successfully treated with a combination of lamivudine and interferon-α during chemotherapy for non-Hodgkin's lymphoma (7). However, randomized controlled trials have not shown long-term beneficial effects of this combination (8, 9). Interferon-α sometimes causes serious adverse effects, including hemopoietic toxicity. Adefovir dipivoxil is generally well tolerated. Resistance to adefovir dipivoxil was not found in large, placebo-controlled 48-week studies (5, 6), although a novel mutation associated with resistance has more recently been identified in the HBV polymerase gene (10).

There remain two unsolved issues related to the treatment schedule. First, should adefovir dipivoxil be initiated as prophylaxis in patients with lamivudine-resistant HBV at the start of chemotherapy? Second, how long should lamivudine be continued after the initiation of adefovir dipivoxil? Delayed treatment due to acute exacerbation of HBV may preclude the subsequent completion of chemotherapy protocols. Withdrawal of lamivudine can cause breakthrough of wild-type HBV strains. We believe that prophylactic use of adefovir dipivoxil with continued use of lamivudine (at least for the first few weeks) is indicated in the management of hematological malignancies by chemotherapy.

In conclusion, adefovir dipivoxil should be considered to prevent viral reactivation in patients who carry lamivudine-resistant HBV variants during intensive chemotherapy. Further studies are needed to establish optimal treatment regimens.

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LEGEND

Figure 1. ALT (●) and HBV DNA (▲) levels during the clinical course of the patient from the time of initiation of lamivudine treatment. CHO, adriamycin, cyclophosphamide, and vincristine; AraC, cytarabine; CBDCA, carboplatin; EPOCH-R, rituximab plus etoposide, prednisone, vincristine, cyclophosphamide, and doxorubicin; FND, fludarabine, mitoxantrone, and dexamethasone. Broken line shows the detection limit of the transcription-mediated amplification assay (3.7 $\log_{10}$ copies/ml of HBV DNA).

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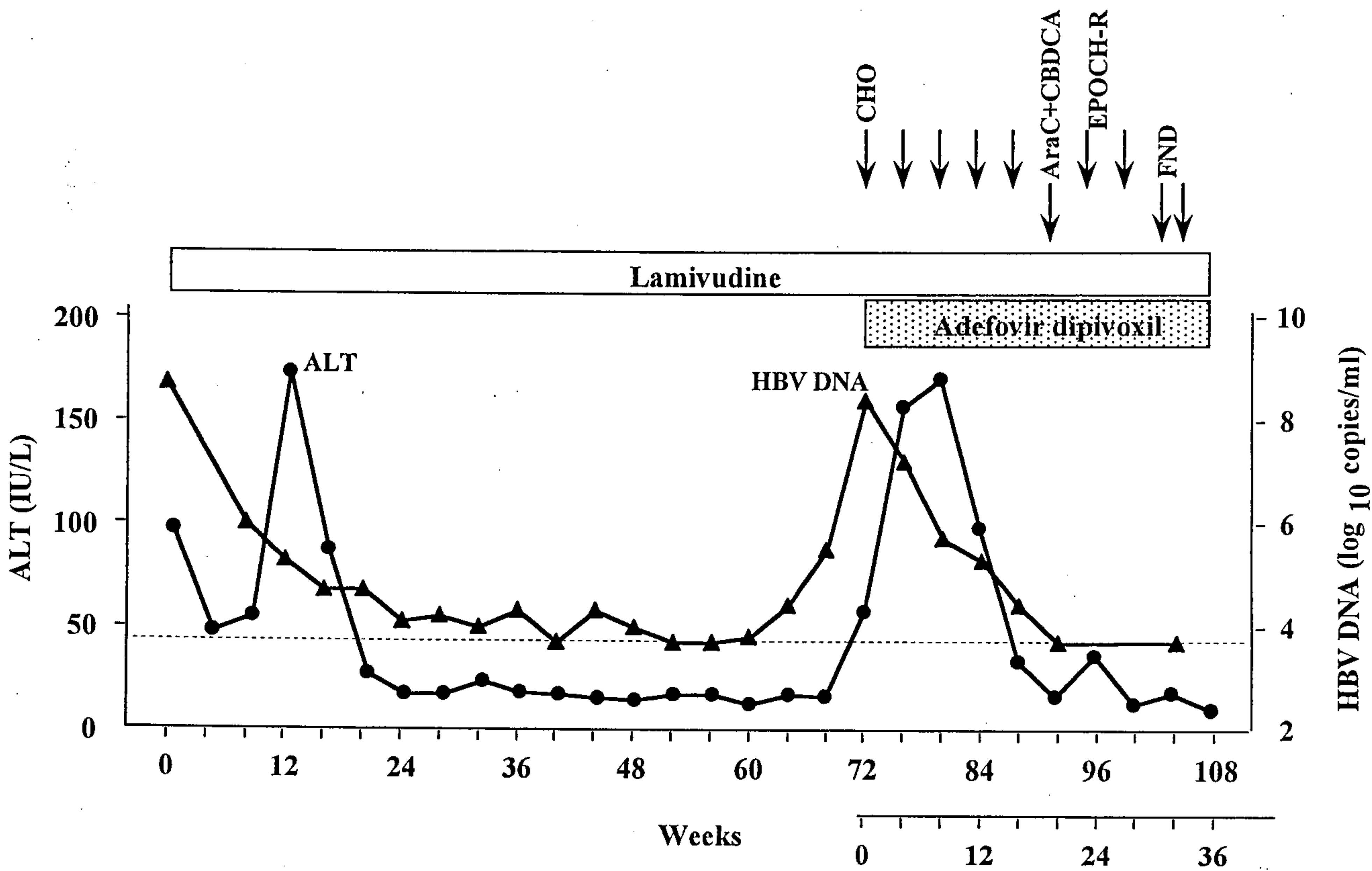
CHO
AraC+CBDCA
EPOCH-R
FND
Lamivudine
Adefovir dipivoxil
ALT
HBV DNA
ALT (IU/L)
200
150
100
50
0
HBV DNA (log 10 copies/ml)
10
8
6
4
2
0
12
24
36
48
60
72
84
96
108
Weeks
0
12
24
36

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Hepatology Research 29 (2004) 9–12

Hepatology Research

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A randomized controlled trial of consensus interferon with or without lactoferrin for chronic hepatitis C patients with genotype 1b and high viral load

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Received 14 October 2003; received in revised form 22 December 2003; accepted 13 January 2004

Abstract

Recently, lactoferrin has been reported to have anti-HCV effects. The aim of this study was to investigate the effect of combination therapy using consensus interferon (CIFN) and lactoferrin in patients with chronic hepatitis C. Twenty-one patients with chronic HCV infection, who were positive for HCV-RNA genotype 1b with serum viral loads from 100 to 700 KIU/ml, were randomly assigned to two groups; the CIFN + Lac group received CIFN with lactoferrin and the CIFN group received CIFN alone. Nine patients in each group completed this trial; the other patients dropped out because of side effects. Three, two and four patients were categorized as complete responders, relapsers and non-responders, respectively, in the CIFN + Lac group, and four, one and four in the CIFN group, respectively. There was no statistically significant difference in virologic response between the two groups. During the follow up after CIFN therapy with continued lactoferrin, there were two relapsers in the CIFN + Lac group and their HCV-RNA titers before treatment were over 400 KIU/ml. In conclusion, the combination therapy of CIFN and lactoferrin did not increase the response rate or prevent relapse after discontinuation of IFN.
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Keywords: Chronic hepatitis C; Consensus interferon; Lactoferrin

1. Introduction

Interferon-α (IFN) treatment has been shown to be effective in a proportion of patients with chronic hepatitis C virus (HCV) infection. However, the overall rate of HCV eradication is at most 30–40% of patients treated with IFN [1–3], and patients without HCV eradication are at risk for liver cirrhosis and hepatocellular carcinoma [4,5]. Consensus interferon (CIFN), a recombinant type-1 interferon, has been shown to be more effective in the treatment of HCV patients than other IFNs [6,7].

Bovine lactoferrin, a milk protein in the iron transporter family, has been reported to effectively prevent HCV infection in cultured human hepatocytes [8–10]. Lactoferrin is a potential candidate as an anti-HCV reagent that may be effective for the treatment of patients with chronic hepatitis. Therefore, the aims of this study were to evaluate the efficacy of CIFN combination therapy with lactoferrin in patients with chronic hepatitis C who had never undergone IFN therapy and to investigate whether this combination therapy prevents relapse after the discontinuation of treatment.

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doi:10.1016/j.hepres.2004.01.002

2. Patients and methods

2.1. Patients

Patients with chronic HCV infection who had never received IFN therapy were eligible for this study. The patients were continuously positive for HCV-RNA genotype 1b with serum titers from 100 to 700 KIU/ml using the amplicor HCV monitor assay and were diagnosed with chronic active hepatitis from a liver biopsy. Pathological diagnosis was made using the Inuyama criteria renewed in 1996 [11]. Patients with decompensated liver cirrhosis were not enrolled in this trial.

2.2. Study design

This study was a randomized controlled trial. Twenty-one patients gave written informed consent and were then randomly assigned to two groups: the CIFN + Lac group received CIFN with lactoferrin, and the CIFN group received CIFN alone. All patients were given CIFN subcutaneously every day for 14 days and then three times a week for 24 weeks at a dose of 18 mega units (MU). The 10 patients in the CIFN + Lac group were given 3 g lactoferrin orally three times a day with CIFN for 26 weeks, and lactoferrin administration was continued for 24 weeks after the discontinuation of CIFN, for a total of 50 weeks. The 11 patients in the CIFN group were given CIFN according to the protocol for 26 weeks. Serum HCV-RNA was measured before treatment and every month for one year during and after CIFN therapy. Based on the virologic response, patients were classified into three groups: complete responders, who had undetectable serum HCV-RNA at the end of treatment and sixth months after IFN therapy; relapsers, who had undetectable HCV-RNA at the end of treatment but HCV-RNA relapse during follow up; and non-responders, who had detectable HCV-RNA during and after treatment.

2.3. Statistical analyses

The group means of age, ALT level, HCV RNA titer, leucocyte count and platelet count were evaluated by Fisher's exact test. Proportions were also compared between the groups by Fisher's exact test. Differences with a P value <0.05 were considered significant.

3. Results

Table 1 shows the biochemical, virologic and histological features of the patients who were randomly assigned to the CIFN + Lac group or the CIFN group. One patient in each group needed a CIFN dose reduction from 18 to 12 MU because of malaise. One patient in the CIFN + Lac group and two in the CIFN group discontinued the therapy because of severe general malaise. Leucocyte, neutrophil and platelet counts decreased in both groups during CIFN administration but no patients discontinued treatment. No differences were seen in any features between the two groups.

There were three, two and four complete responders, relapsers and non-responders, respectively, in the CIFN + Lac group and four, one and four in the CIFN group, respectively. There was no significant difference in virologic response between the two groups (Table 1). The ALT levels during CIFN administration increased even in the complete responders, but decreased after completion of the therapy. The HCV-RNA titer before treatment in the complete responders was below 400 KIU/ml in both groups. Even in the CIFN + Lac group, virologic relapse could not be prevented after discontinuation of CIFN (Fig. 1).

Table 1
Characteristics of the patients

Characteristics	CIFN + Lac	CIFN	P
Number of entry	10	11	
Sex (male:female)	7:3	8:3	NS
Age[a] (years)	56.1 ± 11.3	56.0 ± 12.8	NS
Histological findings (staging)			
F1	2	2	
F2	2	3	NS
F3	5	5	
F4	1	1	
Pretreatment ALT[a] (IU/l)	78 ± 47	71 ± 36	NS
Pretreatment HCV RNA titer[a] (KIU/ml)	357 ± 190	326 ± 160	NS
Pretreatment leucocyte counts[a] ($\times 10^3$ mm^{-3})	4.5 ± 0.8	4.7 ± 0.9	NS
Pretreatment platelet counts[a] ($\times 10^3$ mm^{-3})	14.0 ± 5.9	15.6 ± 6.4	NS
Discontinuation for malaise	1	2	
CIFN dose reduction (18 MU → 12 MU)	1	1	
Patients who completed this trial	9	9	
Virologic response			
100 < pretreatment HCV-RNA < 400	CR = 3, Rel = 1, NR = 0	CR = 4, Rel = 0, NR = 1	NS
400 ≤ pretreatment HCV-RNA < 700	CR = 0, Rel = 1, NR = 4	CR = 0, Rel = 1, NR = 3	

[a] Mean ± S.D.; NS: not significant; CR: complete response, Rel: relapse, NR: no response.

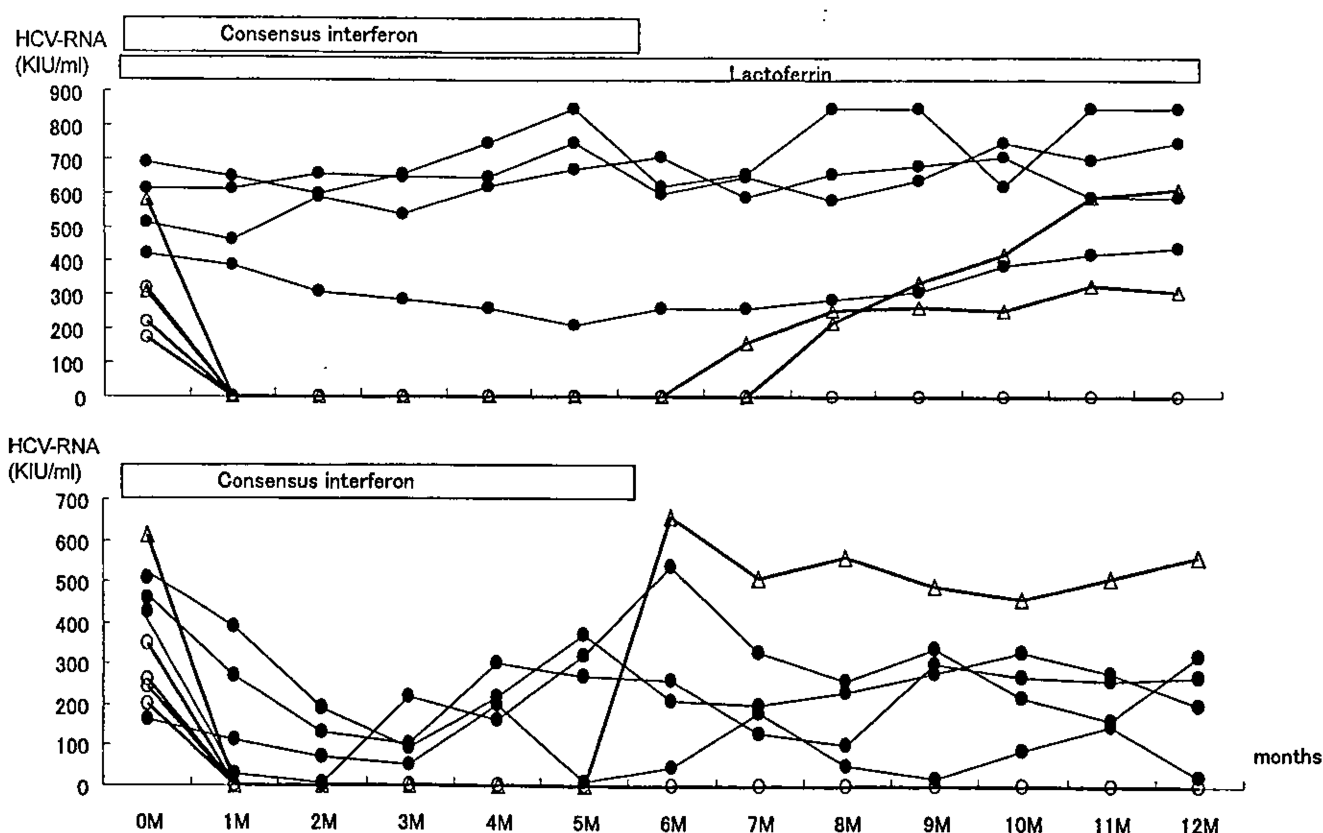

Fig. 1. Serum HCV-RNA levels during and after therapy in the CIFN + Lac group and the CIFN group. There was no significant difference in HCV-RNA levels during and after therapy in either group. In addition, relapsers were also observed in both groups. The open circle, open triangle and closed circle indicate complete virologic responders, relapsers and non-responders, respectively.

4. Discussion

The sustained complete virologic response rate to IFN therapy for 6 months in patients with chronic hepatitis C genotype 1b and high HCV-RNA titers is reported to be very poor, less than 10%. By extending the duration of IFN treatment up to one year, a significant increase of the response rate to about 25% was observed [12,13]. The suppression of HCV replication with longer IFN administration is considered to lead to better virologic response. One of the ways to prevent relapse after the discontinuation of IFN and increase the response rate would, to some extent, depend on the duration or combination of IFN treatment.

CIFN is a genetically engineered molecule derived from commonly observed amino acids of several natural IFN subtypes to develop a novel type I IFN [6,7,14]. CIFN has been shown to be effective in naive patients, in patients who have either not responded to previous IFN therapy or relapsed after discontinuation of IFN therapy [6,7,15]. It was reported that the virologic response rate was higher than IFN-α 2b in the treatment of naive patients infected with HCV genotype 1b (24% versus 15%). However, the patients infected with HCV genotype 1b in these reports had low viral loads. The real response rate to CIFN in patients infected with HCV genotype 1b in high titer is therefore not known.

Lactoferrin, which is an 80 kDa, iron-binding glycoprotein, has several biological activities, including anti-viral activity. Lactoferrin has been reported to inhibit HCV infection in cultured human hepatocytes [9]and to inhibit HCV viremia in low pretreatment HCV-RNA titers of patients with chronic hepatitis C [8]. We hypothesized that CIFN and lactoferrin in combination may yield higher response rates during the first 26 weeks of use and that 24 weeks of continued lactoferrin use after the discontinuation of CIFN may prevent virologic relapse. In this study, however, no significant difference in virologic response was observed between the two groups. The HCV-RNA titers before the treatment of responders in both groups were below 400 KIU/ml. Two relapsers were found in the CIFN + Lac group and their titers of HCV-RNA before treatment was over 400 KIU/ml. Discontinuation of therapy for adverse events did not occur in both groups with and without lactoferrin.

In conclusion, CIFN and lactoferrin in combination did not improve the response rate or prevent relapse after discontinuation of CIFN. High viral loads of over 400 KIU/ml may indicate a low probability of complete virologic response even with the combined treatment of CIFN and lactoferrin.

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Journal of Gastroenterology and Hepatology (2004) **19**, 1343–1347 DOI: 10.1111/j.1400-1746.2004.03439.x

HEPATOLOGY

Lack of association between occult hepatitis B virus DNA viral load and aminotransferase levels in patients with hepatitis C virus-related chronic liver disease

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Abstract

Background and Aim: Occult hepatitis B virus (HBV) infection in hepatitis C virus (HCV)-infected patients might enhance the severity of chronic liver disease (CLD). To elucidate the correlation between occult HBV infection and the clinical course of HCV-related CLD, we evaluated whether the fluctuation of occult HBV-DNA directly affects the serum alanine aminotransferase (ALT) level.

Methods: Forty-one patients with HCV-related CLD who received regular outpatient treatment and 42 age-, sex-, and antibody to hepatitis B core antigen positivity-matched healthy volunteers were enrolled. Serum HBV-DNA was quantitatively detected using real-time detection polymerase chain reaction (RTD–PCR). Serial serum samples in three patients were measured for HBV-DNA, ALT and HCV core antigen.

Results: Hepatitis B virus DNA was amplified in eight of the HCV-related CLD patients (19.5%), which was significantly higher than that of healthy volunteers (2.4%). No significant difference between the genotype 1 HCV-related CLD group and the genotype 2 group was found. Based on the analyses using serial serum samples, the elevation of HBV-DNA did not occur before the ALT flares, but occurred at the same time or after the ALT flares.

Conclusions: The prevalence of occult HBV infection of HCV-related CLD is significantly higher than that of control. Occult HBV infection has no influence on ALT flares among patients with HCV-related CLD.

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Key words: aminotransferase, hepatitis C virus, occult hepatitis B virus infection, real-time detection polymerase chain reaction.

INTRODUCTION

Hepatitis B virus (HBV) infection is usually diagnosed when circulating hepatitis B surface antigen (HBsAg) is identified, and the disappearance of this antigen indicates the clearance of HBV. However, HBV-DNA has been identified in the sera and liver tissues of HBsAg-negative patients with chronic liver disease (CLD)[1] who recovered from self-limited acute hepatitis B,[2] resolved chronic HBV infection[3] or chronic hepatitis C.[4–6] HBV-DNA has also been reported in HBsAg-negative volunteer blood donors[7] and healthy individuals.[8] In particular, the prevalence of HBV-DNA detection in patients with chronic HCV infection is high.[4–6,9–12] The HBV-DNA persistence in HBsAg-negative subjects is defined as occult HBV infection.[12]

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Accepted for publication 3 December 2003.

The clinical impacts of occult HBV infection have been reported, namely, the transmission of HBV from HBsAg-negative donors to recipients by liver graft occurs.[13,14] HBV reactivation in patients without HBsAg occurred by immunosuppressive treatment.[15] Although there has been a report that occult HBV infection in anti-HCV-positive chronic hepatitis enhances the severity of the liver disease,[4] the significance of occult HBV infection has still not been clearly elucidated.

In chronic hepatitis C, HCV RNA loads are not correlated to alanine aminotransferase (ALT) fluctuation.[16] In HCV-related CLD patients with occult HBV infection, fluctuation of HBV-DNA might directly affect the ALT level, and such an accumulation might increase the severity of liver disease in occult HBV-infected patients.

To elucidate the correlation between occult HBV infection and the clinical course of HCV-related CLD, we evaluated whether the fluctuation of occult HBV-DNA directly affects the serum ALT levels. First we analyzed the prevalence of occult HBV infection in patients with HCV-related CLD compared with healthy volunteers in a matched cross-sectional study, and then evaluated whether fluctuation of occult HBV-DNA directly affects the clinical course of HCV-related CLD by using serial serum samples.

METHODS

Patients

Forty-one patients with HCV-related CLD who visited Nagoya City University Hospital from August 2001 to September 2001 were consecutively selected. All were positive for antibody to HCV (anti-HCV), but negative for HBsAg. All patients had undergone diagnostic liver biopsy, apart from six patients with clinically evident cirrhosis. For HCV genotypes, 28 (68.3%) were genotype 1b, 11 (26.8%) were genotype 2, and two were not detectable. The background of the 41 patients is listed in Table 1.

For controls, 230 healthy volunteers were enrolled from a local medical examination center in Nagoya, Japan. HBsAg was found in one. Anti-HCV was found in one. Antibody to hepatitis B core antigen (anti-HBc) was found in 52 (22.6%). Forty-two subjects matched by age, sex, and anti-HBc-positivity to the 41 CLD patients were randomly selected for the control study. All patients and volunteers provided written informed consent.

Serological testing

Anti-HCV was determined using a commercially available second-generation enzyme immunoassay (EIA; EIA II, Ortho, Raritan, NJ, USA). Reverse transcription-polymerase chain reaction (RT-PCR) for the detection of the HCV-genome was carried out using a qualitative commercial RT-PCR assay (AMPLICOR HCV test; Nippon Roche, Tokyo, Japan). HCV core antigen was measured in serum using an EIA method (Ortho Diagnostics, Tokyo, Japan). Serological markers of HBV infection such as HBsAg, antibody to hepatitis B core antigen (anti-HBc), and antibody to HBsAg (anti-HBs) were determined by radioimmunoassay (RIA) using commercially available kits (Dinabot, Tokyo, Japan). The serum was considered anti-HBc positive when the percentage inhibition in the assay was

Table 1 Background of 41 patients with hepatitis C virus (HCV)-related chronic liver disease

Characteristics	
Sex (male : female)	19:22
Age (years)	60.0 ± 8.5†
Disease (CH:LC)	34:7
HCV genotype (1:2)‡	28:11
HCV-RNA level (high : low)§	33:8
ALT (IU/L)	61.4 ± 43.5†
Positive for anti-HBc	18 (43.9%)

†Values are means ± SD. ‡HCV genotype was not detected in two. §HCV RNA level: high indicates an HCV RNA level of ≥100 KIU/mL; low indicates an HCV RNA level of <100 KIU/mL. ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; CH, chronic hepatitis; LC, liver cirrhosis.

Table 2 Prevalence of occult hepatitis B virus (HBV)-DNA in hepatitis C virus (HCV)-related chronic liver disease (CLD) patients and sex-, age-, and antibody to hepatitis B core antigen (anti-HBc)-positivity-matched healthy volunteers

Characteristics	HCV-related CLD patients (n = 41)	Healthy volunteers (n = 42)	P-value
Sex (male : female)	21:20	20:22	NS
Age (years)†	60.0 ± 8.5	60.0 ± 15.1	NS
Positive for HBsAg	0	0	NS
Positive for anti-HBc (%)	18 (43.9)	20 (47.6)	NS
ALT (IU/L)†	61.4 ± 43.5	19.3 ± 10.9	<0.0001
Positive for HBV-DNA (%)	8 (19.5)	1 (2.4)	<0.05

†Values are means ± SD. ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; NS, not significant.

>30%. Anti-HBc of the volunteers was measured by a PHA kit (recombinant HBcAg, Institute of Immunology, Tokyo, Japan). The HCV genotypes were determined using RTD–PCR using type-specific primers for the core region of the viral genome.[17]

Hepatitis B virus DNA in serum

Serum HBV-DNA was quantitatively detected using real-time detection polymerase chain reaction (RTD–PCR) based on Taqman chemistry as reported previously.[18] In brief, total DNA was extracted from 100 μL of serum using microspin columns (QIAamp Blood kit, Qiagen KK Tokyo, Japan). Purified DNA was resuspended in 50 μL of distilled water and a 25 μL aliquot of DNA solution (50 μL serum equivalent) was used for RTD-PCR. Amplification was carried out using primers corresponding to conserved sequences of the surface region. A portion of the HBV surface region was amplified using primers: forward primer HBSF2 (5′CTTCATCCTGCTGCTATGCCT3′, nucleotide position (nt) 406–426) and reverse primer HBSR2 (5′AAAGCCCAGGATGGGAT3′, nt 646–627), and HBSP2 (5′ATGTTGCCCGTTTGTCCTCTAAT TCCA3′, nt 461–488). In preliminary experiments, the efficacy of RTD–PCR was evaluated by quantitatively measuring the sequential levels of synthetic standard HBV-DNA. The detection limit of this system was as low as five DNA copies/assay, and a linear standard curve was obtained between five and 10^6 DNA copies/assay.

Statistical analysis

Statistical analysis was carried out using Fisher's exact test, χ^2 test, and Student's *t*-test as appropriate. A *P*-value <0.05 was considered significant.

RESULTS

With the RTD–PCR method, HBV-DNA was amplified in eight of the HCV-related CLD patients (19.5%) and one of the 42 healthy volunteers (2.4%). The prevalence of occult HBV infection of HCV-related CLD was significantly higher than that of age-, sex-, and anti-HBc-positivity-matched healthy volunteers. This difference was significant ($P < 0.05$; Table 2). Interestingly, all subjects with HBV-DNA were positive for anti-HBc. The comparison of the genotype 1 HCV-related CLD group and the genotype 2 HCV-related CLD group indicated that no significant difference between the two groups in terms of occult HBV-DNA positivity was found (Table 3).

To investigate whether the fluctuation of occult HBV-DNA directly affected the clinical course of HCV-related CLD, the correlation between ALT, HCV RNA levels, and HBV-DNA levels was examined. Of eight HBV-DNA-positive CLD patients, three patients who had elevated serum ALT levels of more than three-fold the upper normal limit during the clinical course were studied. The characteristics are shown in Table 4.

For patient 1, there were four spikes of ALT elevation; two were post-interferon (post-IFN) relapses and

Table 3 Prevalence of occult hepatitis B virus (HBV)-DNA in genotype 1 and genotype 2 hepatitis C virus (HCV)-related chronic liver disease patients

Characteristics	Genotype 1 (*n* = 28)	Genotype 2 (*n* = 11)	*P*-value
Sex (male : female)	13:15	6:5	NS
Age (years)†	56.9 ± 16.7	62.1 ± 9.46	NS
Disease	22:6	10:1	NS
HCV-RNA level (high : low)‡	25:3	5:6	<0.05
Positive for anti-HBc (%)	11 (39.3)	5 (45.5)	NS
ALT (IU/L)†	66.6 ± 48.2	52.7 ± 32.4	NS
Positive for HBV-DNA (%)	5 (17.9)	2 (18.2)	NS

†Mean ± SD. ‡HCV-RNA level (high : low): high indicates an HCV RNA level of ≥100 KIU/mL; low indicates an HCV RNA level of <100 KIU/mL. ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; NS, not significant.

Table 4 Clinical characteristics of three patients

	Patient no. 1	Patient no. 2	Patient no. 3
Sex	M	M	F
Age (years)	23	26	24
Liver disease	CH	CH	CH
Hepatitis C virus genotype	1b	1b	1b
Follow-up period (years)	7.5	7.5	6.5
Blood transfusion	–	–	–
Maximum ALT (IU/L)	227.0	270.0	487.0
(means ± SD)	(87.7 ± 44.1)	(66.5 ± 50.9)	(92.0 ± 93.2)

ALT, alanine aminotransferase; CH, chronic hepatitis; F, female; M, male.