a-glucosidase inhibitor, voglibose, in nondiabetic hyperinsulinemic subjects. *Metabolism* 1996; **45**: 731–7.
32 Zillikens MC, Swart GR, van den Berg JW *et al.* Effects of the glucosidase inhibitor acarbose in patiens with liver cirrhosis. *Aliment Pharmacol Ther* 1989; **3**: 453–9.
33 Blackburn GL, Bistrian BR, Maini BS *et al.* Nutrition and metabolic assessment of the hospitalized patients. *JPEN* 1977; **1**: 11–22.
34 Kato M, Miwa Y, Tajika M *et al.* Preferential use of branched-chain amino acids as an energy substrate in patients with liver cirrhosis. *Intern Med* 1998; **37**: 429–34.
35 Yoshitsugu M, Hiyoshi T, Akasu F. Effect of an a-glucosidase inhibitor, voglibose, in diabetic patients combined with liver cirrhosis. *Med Cons and New -Remed* 1999; **36**: 218–22.
36 Holt PR, Atillasoy E, Lindenbaum J *et al.* Effect of acarbose on fecal nutrients, colonic pH, and short chain fatty acids and rectal proliferative indices. *Metabolism* 1996; **45**: 1179–87.

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# Splenectomy and antiviral treatment for thrombocytopenic patients with chronic hepatitis C virus infection

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SUMMARY. Thrombocytopenic patients with chronic hepatitis C virus (HCV) infection are poor candidates for antiviral treatment with interferon (IFN), but no standard treatment for thrombocytopenia has yet been established. We evaluated the safety of splenectomy and its efficacy for the initiation and continuation of antiviral therapy. From March 2003 to April 2006, 10 patients (mean age 62.5 years) with HCV-related cirrhosis, low platelet count ($\leqq$ 106 000/mm$^3$) and splenomegaly (spleen size $\geqq$ 10 cm) underwent splenectomy. Platelet counts significantly increased at 4–8 weeks after splenectomy [pre: 64 200 ± 6900/mm$^3$ *vs* post 209 000 ± 40 600/mm$^3$ ($P$ = 0.004)]. No severe operative complications were observed. All patients subsequently received antiviral therapy. Of the eight patients who were infected with HCV genotype 1 and had a high viral load ($\geqq$ 100 KIU/mL), four received combination therapy with pegylated IFNα-2b plus ribavirin, and the other four received standard IFNα-2b plus ribavirin. One patient infected with HCV genotype 2 and another with HCV genotype 1 and a low viral load (<100 KIU/mL) were treated with pegylated IFNα-2a. Six patients achieved sustained virologic response (SVR). Among four patients who failed to achieve SVR, one was given retreatment with pegylated IFN plus ribavirin, and the other three received low-dose long-term IFN therapy. Although this study was small, the treatment results were similar to those for patients without thrombocytopenia and suggested that splenectomy would not reduce the antiviral efficacy of IFNα-based treatment.

*Keywords*: antiviral treatment, hepatitis C virus, interferon, splenectomy, splenomegaly, thrombocytopenia.

## INTRODUCTION

Patients with hepatitis C virus (HCV) infection usually show evidence of chronic hepatitis [1], a slowly progressive liver disease. Cirrhosis occurs in at least 20% of chronically infected patients within 20 years [2], some of whom develop hepatocellular carcinoma (HCC). In Japan, HCC occurs at an annual rate of 7–8% in cirrhotic patients with HCV [3].

Interferon (IFN) is among the most frequently used agents in the treatment of chronic liver disease with HCV [4]. A number of studies have demonstrated that sustained virologic response (SVR: undetectable HCV-RNA levels in the blood at 6 months after the end of therapy) to IFN improves prognosis in terms of reduction of cirrhosis and HCC development [5,6].

Abbreviations: HALS, hand-assisted laparoscopic splenectomy; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PSE, partial splenic embolization; SVR, sustained virologic response.

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Thrombocytopenia, a frequent complication of chronic liver disease, is considered to be an indicator of advanced disease and makes it difficult to initiate antiviral treatment with IFN [7,8]. Patients with low platelet counts often receive an inadequate or incomplete course of IFN therapy because of necessary dose reduction or discontinuation of treatment [9,10]. Therefore, management of thrombocytopenia before antiviral treatment should be carefully considered.

Splenectomy [11–13], partial splenic embolization (PSE) [14,15] and administration of a thrombopoietin receptor agonist [16] are considered to be useful treatment options for thrombocytopenia with chronic liver disease or that associated with hypersplenism [17], decreased thrombopoietin production [18] and virus-induced bone marrow suppression [7,19]. However, a standard treatment for thrombocytopenia in patients with chronic liver disease is yet to be established.

Splenectomy is the definitive treatment for hypersplenism, but the operation may be hazardous in patients with poor liver function because of postoperative complications such as

portal vein thrombosis [20–22]. In this study, we evaluated the safety of splenectomy and assessed whether it can increase platelet counts and make IFN-based antiviral therapy possible. We also examined the efficacy of IFN treatment after splenectomy.

## METHODS

### *Patients*

At our hospital, from March 2003 to April 2006, 10 thrombocytopenic patients with chronic HCV infection (four men, six women) underwent splenectomy (Table 1). The eligibility criteria for this study were adequate liver function (Child-Pugh class A or B), thrombocytopenia (platelet count of $<110\ 000/mm^3$), an enlarged spleen size ($\geqq 10$ cm, measured with computed tomography and/or ultrasound) and no severe comorbid disease such as HCC. Before surgery, we suggested three treatment options together with their risks and benefits: antiviral therapy after splenectomy, antiviral therapy after PSE, and low-dose long-term IFN therapy.

The mean age was 62.5 years (range 51–69), and mean platelet count was $64\ 200/mm^3$ (range, 26 000–106 000). Nine patients were rated as Child-Pugh class A at baseline, and one as class B. Two patients failed prior IFN therapy because of dose reduction based on thrombocytopenia and breakthrough (a transient undetectable serum HCV-RNA value that returns to detectable levels during continued IFN treatment). One patient received treatment for HCC (transcatheter arterial embolization and operation) and was free of recurrence at splenectomy.

### *Splenectomy*

Six patients who had not undergone any abdominal surgery had hand-assisted laparoscopic splenectomy (HALS). The other four patients had open abdominal surgery. Two required concurrent surgery on other abdominal organs (splenic artery aneurysm and gastric cancer). The other two had undergone abdominal surgery in the past.

**Table 1** Baseline characteristics of the patients ($n$ = 10)

| | |
|---|---|
| Age (year; mean, range) | 62.5 (51–69) |
| Sex (male/female) | 4/6 |
| Platelet count (per $mm^3$; mean, range) | 64 200 (26 000–106 000) |
| Child-Pugh class (A/B) | 9/1 |
| HCV genotype (type 1/2) | 9/1 |
| Serum level of HCV RNA 100/<100 KIU/mL | 8/2 |
| Spleen size (cm; mean, range) | 12.1(10.0–16.0) |

Patients undergoing HALS were placed semilaterally with the left flank elevated at 45°. The hand-assisted device was inserted through a 7-cm midline incision at the level of the umbilicus. Ports for the laparoscope and the surgeon's right-hand instrument were positioned just below the tip of the spleen. The short gastric vessels were divided using LigaSure™ (Valleylab, Boulder, CO, USA), and the stomach was retracted to the right. Splenic attachments were divided with the ultrasonic dissector. Hilar dissection progressed from an anteroinferior approach taking care to carefully divide and dissect any varices with the ultrasonic dissector. The splenic artery and vein were meticulously divided and clipped and/or ligated. The remaining posterior attachments were then divided with the ultrasonic dissector, and the spleen was removed via the midline incision.

### *Antiviral treatment*

Antiviral treatment was started when platelet counts were increased, and no severe surgical complications developed after splenectomy. The treatment options were based on HCV genotype and HCV viral load. One patient infected with HCV genotype 2 and having a low viral load (≤100 KIU/mL) was treated by pegylated IFNα-2a for 24 weeks [administered as a subcutaneous injection once weekly (180 μg/week)] and another patient with HCV genotype 1 and low viral load, for 48 weeks. Among eight patients infected with HCV genotype 1 and high viral load ($\geqq$ 100 KIU/mL), four patients received combination therapy with pegylated IFNα-2b (subcutaneously administered once a week) plus ribavirin (orally administered daily) for 48 weeks, and the other four patients received standard IFNα-2b (subcutaneously administered three times a week) plus ribavirin for 48 weeks. Dosage was determined based on a weight-based regimen. Dose reduction and discontinuation measures were taken in the event of cytopenia, other complications, and ineffectiveness of the treatment.

Retreatment to achieve SVR or low-dose long-term IFN therapy, which is supposed to be beneficial for prevention of HCC [23,24], was considered for the patients who failed to show a favourable response to the initial treatment.

### *Statistical analysis*

The paired $t$-test was used for comparison of perioperative laboratory data. A $P$-value of $< 0.05$ was considered significant.

## RESULTS

### *Splenectomy*

Significant increases in platelet counts and leukocyte counts were observed after splenectomy (Table 2). The mean platelet count at 4–8 weeks after splenectomy was

209 000/mm$^3$ (range 97 000–530 000). The increased amount from baseline platelet count differed greatly among patients (range 36 000–320 000). The Child-Pugh class did not change in any patients although albumin significantly decreased. There was also no change in the serum level of HCV RNA.

No patient had severe complications such as portal vein thrombosis intra- and postoperatively although one patient developed a wound infection, which was subsequently resolved with antibiotics. Median operative blood loss was 300 mL (range 100–1200 mL). The patient with gastric cancer needed red blood cell transfusion because of a large amount of blood loss (1200 mL).

### *Antiviral treatment*

All 10 patients received antiviral treatment after surgery. One patient infected with HCV genotype 2 and another with HCV genotype 1 and having a low viral load achieved SVR. Among eight patients infected with HCV genotype 1 and having a high viral load, three achieved SVR, but the other five did not because of no response (detectable serum HCV RNA levels by the end of treatment) ($n$ = 3), breakthrough ($n$ = 1) and treatment discontinuation owing to angina ($n$ = 1) (Fig. 1).

Because of reduced platelet count during treatment, dose reduction of IFN and ribavirin was required in two patients, and discontinuation in one. Because of reduced leukocyte count, no dose reduction or discontinuation of antiviral therapy was required. Other mild adverse effects were observed with two patients (anorexia and eruption).

### *Retreatment*

All the five patients who did not achieve SVR received retreatment (Fig. 1). One patient infected with HCV genotype 1 and high viral load received combination therapy with IFNα-2b plus ribavirin, which resulted in breakthrough. This patient was retreated (combination pegylated IFN-ribavirin therapy) and achieved SVR. One patient who took a treatment break owing to angina restarted the same combination of pegylated IFN-ribavirin therapy after completion of the treatment for angina. The other three patients whose initial treatment resulted in nonresponse received low-dose long-term IFN therapy [IFN-α (3MU, administered three times a week subcutaneously)].

## DISCUSSION

Thrombocytopenia with chronic liver disease caused by HCV limits IFN-based antiviral treatment [9,10], but there is no established standard therapy. Splenectomy is the definitive treatment for hypersplenism, one of the major causes of thrombocytopenia with chronic liver disease. In our study, platelet counts significantly increased after splenectomy, and these results are consistent with those obtained in other studies [11–13]. Steady improvement of platelet count is beneficial for the subsequent antiviral treatment. Significant

**Table 2** Perioperative laboratory data

| | Preoperative data | Postoperative data | *P*-value |
|---|---|---|---|
| Platelet count (per mm$^3$) | | | |
| Mean | 64 200 ± 6 900 | 209 000 ± 40 600 | 0.004* |
| Range | 26 000–106 000 | 97 000–530 000 | |
| Leucocyte count (per mm$^3$) | | | |
| Mean | 3840 ± 360 | 5530 ± 640 | 0.0005* |
| Range | 1600–5900 | 3800–7100 | |
| Alanine aminotransferase (IU/L) | | | |
| Mean | 56.9 ± 7.0 | 68.0 ± 9.2 | 0.73 |
| Range | 29–87 | 26–134 | |
| Aspartate aminotransferase (IU/L) | | | |
| Mean | 61.3 ± 6.6 | 54.7 ± 9.9 | 0.44 |
| Range | 28–90 | 20–114 | |
| Total bilirubin (μmol/L; median, range) | | | |
| Mean | 0.89 ± 0.10 | 0.74 ± 0.10 | 0.21 |
| Range | 0.6–1.8 | 0.3–1.4 | |
| Albumin (g/L; median, range) | | | |
| Mean | 3.88 ± 0.10 | 3.68 ± 0.10 | 0.04* |
| Range | 3.4–4.3 | 2.9–4.2 | |
| HCV RNA (KIU/mL) | | | |
| Mean | 1056 | 1053 | 0.98 |

Postoperative data was measured 4–8 weeks after splenectomy.
*Statiscally significant

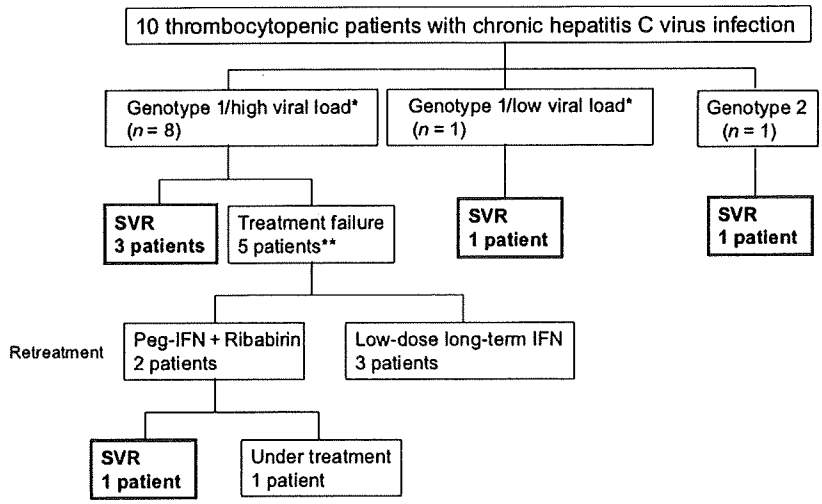


Fig. 1 Flowchart of study patients. *High viral load, ≥ 100 KIU/mL. ** Treatment failure includes NR ($n$ = 3), breakthrough ($n$ = 1) and treatment break owing to angina ($n$ = 1).

increases in leukocytes after splenectomy was also observed and was beneficial for the continuation of antiviral therapy, because there was no discontinuation or dose reduction as a result of leukocytepenia.

Portal vein thrombosis, one of the main complications after splenectomy, has been reported in 8–10% of the patients [20–22]. CT with intravenous contrast and Doppler ultrasound are useful for early detection. Overwhelming postsplenectomy infection (fulminant bacterial sepsis in asplenic patients) is a relatively rare but critical complication [25]. The clinical features are cryptic infection (no obvious focus), septic shock with disseminated intravascular coagulation, marked virulence (50–70% mortality) and death within 24–48 h. Pneumococcal vaccination is reported to provide effective prophylaxis. Although careful postoperative follow-up with CT or ultrasound and prevention of postsplenectomy infection should be considered to increase the safety of splenectomy, our study showed this procedure was safe and efficacious.

Partial splenic embolization by the injection of microspheres via a catheter comprising 30–80% of the splenic parenchyma can be an effective treatment for thrombocytopenia with hypersplenism. Miyake *et al.* [14] showed that in 10 patients, PSE was successfully performed without serious adverse events, and two of six patients infected with genotype 1, and all four patients infected with genotype 2 achieved SVR. Recently, serious complications such as bacterial peritonitis and splenic abscesses have decreased as a result of technical advances [26,27] but should be kept in mind. Both splenectomy and PSE could be useful treatments for thrombocytopenia, but further investigation is needed to identify which treatment is better in terms of safety and for a successful outcome after antiviral therapy.

Eltrombopag, an orally active thrombopoietin receptor agonist that stimulates thrombopoiesis and permits the initiation of antiviral therapy, is a promising agent for thrombocytopenia [16]. Although it might be a less invasive treatment option than splenectomy or PSE, the results of antiviral therapy with Eltrombopag are still under investigation.

In two earlier studies of splenectomy for thrombocytopenic patients with chronic HCV infection, the outcome of antiviral treatment was evaluated only for five out of seven patients [11] and four out of eleven patients [12]. In the study of Morihara *et al.* [13], 16 patients received splenectomy and subsequent antiviral therapy with IFN, but no patient among the eleven patients infected with genotype 1 achieved SVR, because all but one received IFN monotherapy for the maintenance of a low ALT. In our study, the outcome of the initial treatment for the eradication of HCV was evaluated for all 10 patients, and retreatment was given to all patients who did not achieve good results after the initial treatment. Four out of eight patients infected with genotype 1 and having a high viral load achieved SVR with the initial treatment and retreatment. The other two patients (one infected with genotype 1 and having a low viral load and the other with genotype 2) achieved SVR in the initial treatment. Although the number of eligible cases was small, the treatment results are similar to the results for patients without thrombocytopenia [28,29] and suggest that splenectomy does not reduce the antiviral efficacy of IFN-based treatment.

Three patients received low-dose long-term IFN therapy which is supposed to be beneficial for prevention of HCC [23,24]. These patients infected with genotype 1 had a high viral load and did not show good results with the initial treatment. Sustained platelet count increases after splenectomy would be a great advantage for patients infected with genotype 1 and a high viral load, because about half of them are refractory to combination therapy with pegylated IFN plus ribavirin [28,29].

We conclude that splenectomy could play a beneficial role in the treatment for thrombocytopenic patients with chronic HCV infection and that splenectomy would not influence the antiviral efficacy of IFN-based treatment.

## DISCLOSURE

The authors have no commercial associations that might be a conflict of interest in relation to this article.

## REFERENCES


1 Alter MJ, Margolis HS, Krawczynski K *et al.* The natural history of community-acquired hepatitis C in the United States. The Sentinel Counties Chronic non-A, non-B Hepatitis Study Team. *N Engl J Med* 1992; 327: 1899–1905.

2 Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002; 36: S35–S46.

3 Kiyosawa K, Umemura T, Ichijo T *et al.* Hepatocellular carcinoma: recent trends in Japan. *Gastroenterology* 2004; 127: S17–S26.

4 National Institutes of Health National Institutes of Health Consensus Development Conference Statement Management of hepatitis C: 2002 – June 10–12, 2002. *Hepatology* 2002; 36:S3–S20.

5 Yoshida H, Arakawa Y, Sata M *et al.* Interferon therapy prolonged life expectancy among chronic hepatitis C patients. *Gastroenterology* 2002; 123: 483–491.

6 Yu ML, Lin SM, Chuang WL *et al.* A sustained virological response to interferon or interferon/ribavirin reduces hepatocellular carcinoma and improves survival in chronic hepatitis C: a nationwide, multicentre study in Taiwan. *Antivir Ther* 2006; 11: 985–994.

7 Giannini EG. Review article: thrombocytopenia in chronic liver disease and pharmacologic treatment options. *Aliment Pharmacol Ther* 2006; 23: 1055–1065.

8 Bashour FN, Teran JC, Mullen KD. Prevalence of peripheral blood cytopenias (hypersplenism) in patients with nonalcoholic chronic liver disease. *Am J Gastroenterol* 2000; 95: 2936–2939.

9 Shiffman ML, Ghany MG, Morgan TR *et al.* Impact of reducing peginterferon alfa-2a and ribavirin dose during retreatment in patients with chronic hepatitis C. *Gastroenterology* 2007; 132: 103–112.

10 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; 123: 1061–1069.

11 Hayashi PH, Mehia C, Joachim Reimers H, Solomon HS, Bacon BR. Splenectomy for thrombocytopenia in patients with hepatitis C cirrhosis. *J Clin Gastroenterol* 2006; 40: 740–744.

12 Kercher KW, Carbonell AM, Heniford BT, Matthews BD, Cunningham DM, Reindollar RW. Laparoscopic splenectomy reverses thrombocytopenia in patients with hepatitis C cirrhosis and portal hypertension. *J Gastrointest Surg* 2004; 8: 120–126.

13 Morihara D, Kobayashi M, Ikeda K *et al.* Effectiveness of combination therapy of splenectomy and long-term interferon in patients with hepatitis C virus-related cirrhosis and thrombocytopenia. *Hepatol Res* 2009; 39: 439–447.

14 Miyake Y, Ando M, Kaji E, Toyokawa T, Nakatsu M, Hirohata M. Partial splenic embolization prior to combination therapy of interferon and ribavirin in chronic hepatitis C patients with thrombocytopenia. *Hepatol Res* 2008; 38: 980–986.

15 Lee CM, Leung TK, Wang HJ *et al.* Evaluation of the effect of partial splenic embolization on platelet values for liver cirrhosis patients with thrombocytopenia. *World J Gastroenterol* 2007; 13: 619–622.

16 McHutchison JG, Dusheiko G, Shiffman ML *et al.* Eltrombopag for thrombocytopenia in patients with cirrhosis associated with hepatitis C. *N Engl J Med* 2007; 357: 2227–2236.

17 McCormick PA, Murphy KM. Splenomegaly, hypersplenism and coagulation abnormalities in liver disease. *Baillieres Best Pract Res Clin Gastroenterol* 2000; 14: 1009–1031.

18 Rios R, Sangro B, Herrero I, Quiroga J, Prieto J. The role of thrombopoietin in the thrombocytopenia of patients with liver cirrhosis. *Am J Gastroenterol* 2005; 100: 1311–1316.

19 Koike Y, Yoneyama A, Shirai J *et al.* Evaluation of thrombopoiesis in thrombocytopenic disorders by simultaneous measurement of reticulated platelets of whole blood and serum thrombopoietin concentrations. *Thromb Haemost* 1998; 79: 1106–1110.

20 Winslow ER, Brunt LM, Drebin JA, Soper NJ, Klingensmith ME. Portal vein thrombosis after splenectomy. *Am J Surg* 2002; 184: 631–635; discussion 5–6.

21 Hassn AM, Al-Fallouji MA, Ouf TI, Saad R. Portal vein thrombosis following splenectomy. *Br J Surg* 2000; 87: 362–373.

22 Chaffanjon PC, Brichon PY, Ranchoup Y, Gressin R, Sotto JJ. Portal vein thrombosis following splenectomy for hematologic disease: prospective study with Doppler color flow imaging. *World J Surg* 1998; 22: 1082–1086.

23 Kudo M, Sakaguchi Y, Chung H *et al.* Long-term interferon maintenance therapy improves survival in patients with HCV-related hepatocellular carcinoma after curative radiofrequency ablation. A matched case-control study. *Oncology* 2007; 72 (Suppl. 1): 132–138.

24 Arase Y, Ikeda K, Tsubota A *et al.* Interferon therapy for 2 years or longer reduces the incidence of hepatocarcinogenesis in patients with chronic hepatitis C viral infection. *Intervirology* 2004; 47: 355–361.

25 Okabayashi T, Hanazaki K. Overwhelming postsplenectomy infection syndrome in adults – a clinically preventable disease. *World J Gastroenterol* 2008; 14: 176–179.

26 N'Kontchou G, Seror O, Bourcier V *et al.* Partial splenic embolization in patients with cirrhosis: efficacy, tolerance and long-term outcome in 32 patients. *Eur J Gastroenterol Hepatol* 2005; 17: 179–184.

27 Sangro B, Bilbao I, Herrero I *et al.* Partial splenic embolization for the treatment of hypersplenism in cirrhosis. *Hepatology* 1993; 18: 309–314.

28 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–982.

29 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358: 958–965.

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# Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin

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SUMMARY. The impact of ribavirin exposure on virologic relapse remains controversial in combination therapy with pegylated interferon (Peg-IFN) and ribavirin for patients with chronic hepatitis C (CH-C) genotype 1. The present study was conducted to investigate this. Nine hundred and eighty-four patients with CH-C genotype 1 were enrolled. The drug exposure of each medication was calculated by averaging the dose actually taken. For the 472 patients who were HCV RNA negative at week 24 and week 48, multivariate logistic regression analysis showed that the degree of fibrosis ($P = 0.002$), the timing of HCV RNA negativiation ($P < 0.001$) and the mean doses of ribavirin ($P < 0.001$) were significantly associated with relapse, but those of Peg-IFN were not. Stepwise reduction of the ribavirin dose was associated with a stepwise increase in relapse rate from 11% to 60%. For patients with complete early virologic response (c-EVR) defined as HCV RNA negativity at week 12, only 4% relapse was found in patients given ≥12 mg/kg/day of ribavirin and ribavirin exposure affected the relapse even after treatment week 12, while Peg-IFN could be reduced to 0.6 μg/kg/week after week 12 without the increase of relapse rate. Ribavirin showed dose-dependent correlation with the relapse. Maintaining as high a ribavirin dose as possible (≥12 mg/kg/day) during the full treatment period can lead to suppression of the relapse in HCV genotype 1 patients responding to Peg-IFN alpha-2b plus ribavirin, especially in c-EVR patients.

*Keywords*: chronic hepatitis C, drug exposure, pegylated interferon plus ribavirin, virologic relapse.

## INTRODUCTION

Combination therapy of pegylated interferon (Peg-IFN) plus ribavirin is very effective for patients with chronic hepatitis C (CH-C). However, sustained virologic response (SVR) in current therapy occurs in only 40–50% of patients with hepatitis C virus (HCV) genotype 1 [1–4]. Also, SVR is reduced in patients with genotype 1 who require reduction of either Peg-IFN or ribavirin, although dose reduction has little influence on SVR in those with genotype 2 or 3 [1–3,5,6]. Therefore, it is important to clarify the degree to which these medications can be reduced without adversely affecting SVR in patients with CH-C genotype 1.

In an early report on the relationship between drug exposure and antiviral effect in patients with CH-C genotype 1, patients who received ≥80% of their total planned cumulative doses of Peg-IFN and ribavirin for ≥80% of the scheduled duration of therapy had an SVR of 51% compared with only 34% for patients who received lesser amounts of one or both

Abbreviations: CH-C, chronic hepatitis C; c-EVR, complete early virologic response; ETR, end-of-treatment virologic response; Hb, haemoglobin; HCV, hepatitis C virus; IFN, interferon; LVR, late virologic response; Peg-IFN, pegylated interferon; PP, per protocol; Plt, platelet; RVR, rapid virologic response; SVR, sustained virologic response; VR, virologic response; WBC, white blood cell.

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medications [7]. On the other hand, Shiffman *et al.* [8] recently reported that reducing ribavirin did not affect SVR as long as the dose of Peg-IFN was maintained, while reducing the Peg-IFN dose significantly reduced SVR. The results of these observations are consistent with respect to the effect of Peg-IFN on SVR. However, what is controversial is whether or not reducing the ribavirin dose affects the antiviral effect.

Adding ribavirin to either interferon (IFN) or Peg-IFN monotherapy for patients with CH-C genotype 1 has been shown to reduce the relapse rate in large randomized trials [1,2,9–11]. In detail, adding ribavirin to the usual IFN monotherapy (3MIU, three-times-weekly) in 48-week treatment raised the end-of-treatment virologic response (ETR) rate from approximately 30% to 50% and also lowered the relapse rate from mid-40% to approximately 20% [9–11]. Lindsay *et al.* [12] reported that Peg-IFN alpha-2b (Peg-IFN α-2b) monotherapy (1.5 μg/kg, once-weekly), as compared with IFN alpha-2b (IFNα-2b) monotherapy (3MIU, three-times-weekly), improved ETR (49% vs. 24%), but not the relapse rate (53% vs. 50%). In the trial of Peg-IFN alpha-2a (Peg-IFN α-2a) plus ribavirin *vs* IFN α-2b plus ribavirin or Peg-IFN α-2a alone, the ETR rates were 69%, 52% and 59%, and the relapse rates were 19%, 15% and 52%, respectively [2]. These findings from large-scale trials indicate that the main role of ribavirin is to reduce relapse in the combination therapy with Peg-IFN, although ribavirin affects both ETR and relapse in combination therapy with the usual IFN.

In the present study, we tried to determine whether or not dose reduction of ribavirin (or Peg-IFN) has an effect on virologic relapse in Peg-IFN plus ribavirin treatment for patients with CH-C genotype 1.

## PATIENTS AND METHODS

### *Patients*

This study was a multicentre trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 984 patients with CH-C were enrolled in this study between December 2004 and September 2006, and treated with a combination of Peg-IFN α-2b plus ribavirin. The baseline characteristics of the patients are shown in Table 1. All patients were Japanese infected with HCV genotype 1 and a viral load of more than $10^5$ IU/mL. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcohol liver disease, autoimmune hepatitis), coinfection with hepatitis B or anti-human immunodeficiency virus. This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

### *Treatment*

All patients received Peg-IFN α-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough) for the duration of the study of 48 weeks. As a starting dose, Peg-IFN α-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg) according to the manufacturer's drug information available in Japan.

**Table 1** Baseline characteristics of patients and drug doses at start of treatment

| Factor | Mean ± SD or *n* |
|---|---|
| *n* | 984 |
| Age (years) | 56.3 ± 10.1 |
| Sex (male/female) | 555/429 |
| Body weight (kg) | 61.8 ± 11.5 |
| History of IFN treatment Naïve/experienced (relapser/nonresponder)* | 575/409 (160/182) |
| White blood cells (/mm$^3$) | 5052 ± 1550 |
| Neutrophils (/mm$^3$) | 2577 ± 1092 |
| Red blood cells (×10$^4$/mm$^3$) | 442 ± 47 |
| Haemoglobin (g/dL) | 14.1 ± 1.4 |
| Platelets (×10$^4$/mm$^3$) | 15.9 ± 5.5 |
| AST (IU/L) | 66 ± 45 |
| ALT (IU/L) | 79 ± 61 |
| Serum HCV RNA (kIU/mL)† | 1600 |
| Histology (METAVIR)‡ | |
| Fibrosis; 0/1/2/3/4 | 49/314/197/105/18 |
| Activity; 0/1/2/3 | 23/329/304/27 |
| Peg-IFN dose (μg/kg/week) | 1.45 ± 0.17 |
| Ribavirin dose (mg/kg/day) | 11.4 ± 1.6 |

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus. *Viral response to previous treatment was unknown in 57 patients, and 10 patients had discontinued treatment. †Data shown are median values. ‡301 missing.

### *Dose reduction and discontinuance*

Dose modification also followed, as a rule, the manufacturer's drug information according to the intensity of the haematologic adverse effects. The dose of Peg-IFN α-2b was reduced to 50% of the assigned dose when the white blood cell (WBC) count was below 1500/mm$^3$, the neutrophil count below 750/mm$^3$ or the platelet (Plt) count below $8 \times 10^4$/mm$^3$, and was discontinued when the WBC count was below 1000/mm$^3$, the neutrophil count below 500/mm$^3$ or the Plt count below $5 \times 10^4$/mm$^3$. Ribavirin was also reduced from 1000 mg to 600 mg, 800 mg to 600 mg, or 600 mg to 400 mg when the haemoglobin (Hb)

concentration decreased to less than 10 g/dL, and was discontinued when the Hb concentration decreased to less than 8.5 g/dL. Both Peg-IFN α-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. No ferric medicine or haematopoietic growth factors, such as epoetin alpha, or granulocyte-macrophage colony stimulating factor, were administered.

### *Virologic assessment and definition of virologic response*

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 kIU/mL; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analysed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL; Roche Diagnostics). Complete early virologic response (c-EVR) was defined as the absence of detectable serum HCV RNA at treatment week 12, the late virologic response (LVR) was defined as undetectable serum HCV RNA for the first time at 13–24 weeks of treatment, and the virologic response (VR) was defined as HCV RNA negativity at week 24 and week 48. SVR was defined as the absence of detectable serum HCV RNA at week 72. Patients with less than a 2-log decrease in HCV RNA level at treatment week 12 compared with the baseline had to stop treatment according to the protocol and were regarded as nonresponders. All patients with detectable serum HCV RNA at treatment week 24 were also considered to be nonresponders and were excluded from further treatment.

### *Assessment of drug exposure*

The amounts of Peg-IFN α-2b and ribavirin actually taken by each patient during the full treatment period were evaluated by reviewing the medical records. The mean doses of Peg-IFN α-2b and ribavirin were calculated individually as averages on the basis of body weight at baseline: Peg-IFN α-2b expressed as μg/kg/week, ribavirin expressed as mg/kg/day.

### *Evaluation of impact of drug exposure on virologic relapse*

We evaluated the relationship between the drug exposure of both drugs and relapse by two different methods, univariate and multivariate analysis for relapse and independent evaluation of both drugs for relapse according to the degree of drug exposure. The former was performed with the factors of mean administration doses of both drugs, including the factors at baseline and the timing of HCV RNA negativiation. The latter was examined by classifying Peg-IFN α-2b exposure into five categories (up to 0.6 μg/kg; from 0.6 to less than 0.9 μg/kg; from 0.9 to less than 1.2 μg/kg; from 1.2 to less than 1.5 μg/kg; from 1.5 μg/kg) and ribavirin exposure into five categories (up to 6 mg/kg; from 6 to less than 8 mg/kg; from 8 to less than 10 mg/kg; from 10 to less than 12 mg/kg; from 12 mg/kg).

### *Statistical analysis*

Baseline data are expressed as means ± SD or median values. Virologic response was evaluated using per protocol (PP) analysis. To analyse the difference between baseline data including drug exposure and virologic response, univariate analysis using the Mann–Whitney *U*-test or chi-square test and multivariate analysis using logistic regression analysis were performed. The significance of trends in values was determined with the Mantel–Haenszel chi-square test. A two-tailed $P$ value <0.05 was considered significant. The analysis was conducted with SPSS version 15.0J (SPSS Inc., Chicago, IL, USA).

## RESULTS

### *Progress of patients and dose reduction of Peg-IFN α-2b and ribavirin*

The progress of patients in this study is shown in Fig. 1. Of the 984 patients, 903 completed 12 weeks of treatment and the c-EVR rate was 49% (445/903), based on PP study. To analyse for relapse, 472 patients with VR were assessed, with 178 (38%) showing Peg-IFN dose reduction without discontinuation and 246 (52%) with ribavirin dose reduction without discontinuation during the full (48 weeks) treatment period. The relapse rate was 26% (125/472) in the patients with undetectable HCV RNA level at the end of treatment. No difference was found in relapse rates between the IFN naïve patients and IFN experienced patients (IFN naïve; 25%, 72/287 *vs* IFN experienced; 29%, 53/185, $P = 0.40$). The SVR rate was 43% (347/812) in the PP study.

### *Impact of drug exposure during 0–48 weeks on relapse among patients with VR*

The mean dose of Peg-IFN α-2b actually taken during the full treatment period by each patient was 1.32 μg/kg/week (range, 0.49–2.16 μg/kg/week; median, 1.38 μg/kg/week) and that of ribavirin was 9.8 mg/kg/day (range, 3.3–16.2 mg/kg/day; median, 10.1 mg/kg/day) in patients with VR.

The result of univariate analysis for relapse among the patients with VR is shown in Table 2a. The degree of fibrosis, the timing of HCV RNA negativiation, Plt value and the mean doses of ribavirin were factors significantly associated with relapse, but those of Peg-IFN α-2b were not. The mean dose of ribavirin as well as the degree of fibrosis and the timing of HCV RNA negativiation was selected as a significant independent factor by multivariate logistic regression analysis (Table 2b).

Next, we analysed the relationship of the relapse rate and the mean ribavirin dose. The overall relapse rate among patients with VR was 26% (125/472). The

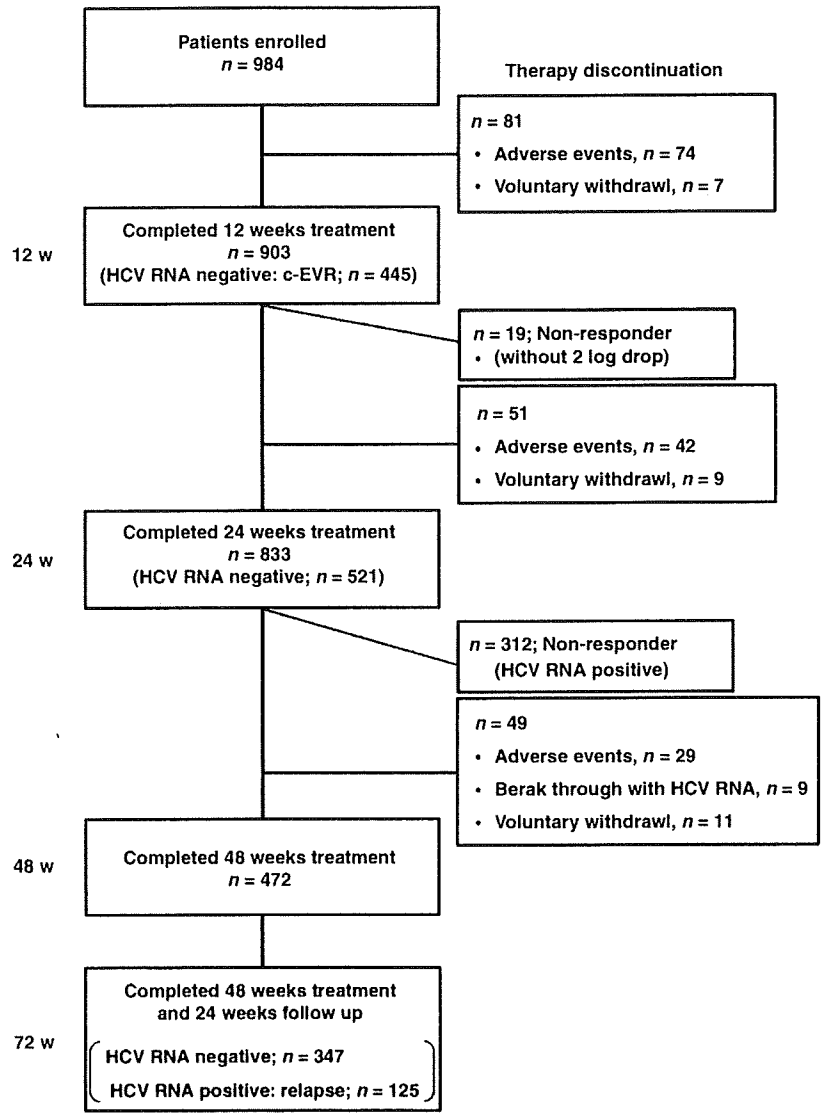


**Fig. 1** Flow of patients throughout the study.

relapse rate was 60% (9/15) in patients receiving less than 6 mg/kg/day of ribavirin, and declined to 41% (32/79) at 6–8 mg/kg/day, 27% (34/124) at 8–10 mg/kg/day, 22% (43/193) at 10–12 mg/kg/day and 11% (7/61) in patients given ≥12 mg/kg/day ($P < 0.0001$). Figure 2 shows the relationship of the relapse rate and the mean ribavirin dose for two dosage groups of Peg-IFN α-2b: the group given ≥1.4 μg/kg/week of Peg-IFN and that given <1.4 μg/kg/week (1.4 μg/kg/week was the median value). In both groups, ribavirin was dose-dependently correlated with relapse. More than 12 mg/kg/day of the mean ribavirin exposure could suppress the relapse rate to 20% (4/20) in the group given <1.4 μg/kg/week and strongly suppress it to 7% (3/41) in the group given ≥1.4 μg/kg/week of Peg-IFN.

### *Impact of drug exposure during 0–48 weeks on relapse according to the timing of HCV RNA negativiation*

#### *Relapse rates among patients with c-EVR*

The overall relapse rate among patients with c-EVR was 19% (75/391). We separately analysed the relapse rate among the patients with c-EVR according to the degree of exposure to both drugs. Table 3a shows the relapse rates among the patients with c-EVR according to the categories of Peg-IFN α-2b and ribavirin doses during the full treatment period. The relapse rate showed a decline according to the increase in the dose of ribavirin ($P = 0.0002$). The relapse rate was suppressed at an average of 15% (13–16%) in the patients who received 10–12 mg/kg/day of ribavirin, and the average was only 4% for those who received more than 12 mg/kg/day

**Table 2** Factors associated with relapse among the patients with virologic response

(a) Univariate analysis

| Factor | Nonrelapser | Relapser | *P* value |
|---|---|---|---|
| *n* | 347 | 125 | |
| Age (years) | 53.9 ± 10.7 | 56.2 ± 9.2 | 0.07 |
| Sex (male/female) | 213/134 | 66/59 | 0.09 |
| Serum HCV RNA (kIU/mL)* | 1600 | 1800 | 0.34 |
| White blood cells (/mm$^3$) | 5335 ± 1517 | 5075 ± 1428 | 0.08 |
| Neutrophils (/mm$^3$) | 2797 ± 1143 | 2625 ± 1021 | 0.17 |
| Red blood cells (×10$^4$/mm$^3$) | 450 ± 45 | 446 ± 50 | 0.25 |
| Haemoglobin (g/dL) | 14.3 ± 1.4 | 14.2 ± 1.5 | 0.45 |
| Platelets (×10$^4$/mm$^3$) | 17.6 ± 5.3 | 16.4 ± 5.1 | 0.03 |
| AST (IU/L) | 60 ± 42 | 58 ± 33 | 0.75 |
| ALT (IU/L) | 75 ± 60 | 71 ± 50 | 0.98 |
| Histology (METAVIR)† | | | |
| Fibrosis: 0–2/3–4 | 222/20 | 74/19 | 0.002 |
| Activity: 0–1/2–3 | 140/102 | 52/41 | 0.75 |
| Peg-IFN dose (μg/kg/week)‡ | 1.33 ± 0.26 | 1.27 ± 0.29 | 0.07 |
| Ribavirin dose (mg/kg/day)† | 10.1 ± 1.9 | 9.1 ± 2.1 | <0.001 |
| Virologic response§: c-EVR/LVR | 316/31 | 75/50 | <0.001 |

(b) Multivariate analysis

| Factor | Category | Odds ratio | 95% CI | *P* value |
|---|---|---|---|---|
| Platelets | By 1 × 10$^4$/mm$^3$ | – | – | NS |
| Fibrosis¶ | 0–2/3–4 | 1/3.192 | 1.515–6.725 | 0.002 |
| Ribavirin dose‡ | By 1 mg/kg/day | 0.790 | 0.696–0.896 | <0.001 |
| Virologic response§ | c-EVR/LVR | 1/6.290 | 3.385–11.690 | < 0.001 |

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HCV, hepatitis C virus; c-EVR, complete early virologic response; LVR, late virologic response; NS, not significant difference Peg-IFN, pegylated interferon.
*Data shown are median values. †137 missing. ‡Mean doses during 0–48 weeks. §The timing of HCV RNA negativiation. ¶METAVIR fibrosis score.

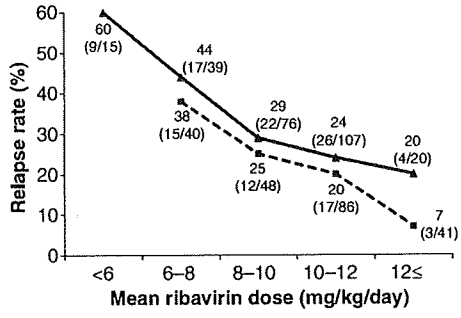


**Fig. 2** Relapse rate according to Peg-IFN α-2b and ribavirin doses during treatment of patients who completed treatment, which was stratified with the mean ribavirin doses. (–▲–) Group with the mean Peg-IFN dose <1.4 μg/kg/week; (··■··) Group with the mean Peg-IFN dose ≥1.4 μg/kg/week. The ribavirin dose was dose-dependently correlated with the virologic relapse in both groups ($P < 0.0001$). There was no significant difference between the two Peg-IFN α-2b-dose groups ($P = 0.17$).

of ribavirin. In contrast, the relapse rate was not affected by the dose of Peg-IFN α-2b when the patients were given more than 0.9 μg/kg/week of Peg-IFN α-2b. On the other hand, with respect to patients with rapid virologic response (RVR) defined as the absence of detectable serum HCV RNA at treatment week 4 ($n = 41$), none showed relapse and all attained SVR irrespective of the dose of Peg-IFN α-2b or ribavirin (prevalence of patients: the mean dose of Peg-IFN α-2b; <0.9 : 0.9–1.2 : 1.2–1.5 : 1.5 μg/kg/week ≤ =7 : 17 : 34 : 42%, the mean dose of ribavirin; <8 : 8–10 : 10–12 : 12 mg/kg/day ≤ =15 : 24 : 41 : 20%).

*Relapse rates among patients with LVR*

Among the patients with LVR, the ribavirin exposure during treatment was also the factor correlated adversely with the relapse rate ($P = 0.03$). However, the overall relapse rate was 62% (50/81), which was much higher than that of the c-EVR patients ($P < 0.0001$) and 45% (5/11) of patients with LVR relapsed even in the group given more than 12 mg/kg/day of the average ribavirin dose (Table 3b).

**Table 3** Relapse rate according to Peg-IFN and ribavirin doses during week 0–48 for patients with c-EVR and LVR who completed 48 weeks of treatment

(a) C-EVR

| Peg-IFN dose ($\mu$g/kg/week)† | Ribavirin dose (mg/kg/day)* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 12≤ | | 10–12 | | 8–10 | | <8 | | Total | |
| ≥1.5 | 0% | (0/28) | 13% | (4/31) | 14% | (3/21) | 29% | (5/17) | 12% | (12/97) |
| 1.2–1.5 | 20% | (2/10) | 16% | (16/100) | 25% | (16/65) | 23% | (7/30) | 20% | (41/205) |
| 0.9–1.2 | 0% | (0/7) | 13% | (2/15) | 15% | (2/13) | 38% | (6/16) | 20% | (10/51) |
| <0.9 | 0% | (0/5) | 15% | (2/13) | 55% | (6/11) | 44% | (4/9) | 32% | (12/38) |
| Total | 4% | (2/50) | 15% | (24/159) | 25% | (27/110) | 31% | (22/72) | 19% | (75/391) |

(b) LVR

| Peg-IFN dose ($\mu$g/kg/week)§ | Ribavirin dose (mg/kg/day)‡ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 12≤ | | 10–12 | | 8–10 | | <8 | | Total | |
| ≥1.5 | 43% | (3/7) | 50% | (1/2) | 100% | (2/2) | 100% | (4/4) | 67% | (10/15) |
| 1.2–1.5 | | (1/1) | 60% | (12/20) | 29% | (2/7) | 82% | (9/11) | 62% | (24/39) |
| <1.2 | 33% | (1/3) | 50% | (6/12) | 60% | (3/5) | 86% | (6/7) | 59% | (16/27) |
| Total | 45% | (5/11) | 56% | (19/34) | 50% | (7/14) | 86% | (19/22) | 62% | (50/81) |

Peg-IFN, pegylated interferon; c-EVR, complete early virologic response; LVR, late virologic response.
*$P$ = 0.0002 for comparison of the four ribavirin groups. †$P$ = 0.08 for comparison of the four Peg-IFN groups. ‡$P$ = 0.03 for comparison of the four ribavirin groups. §$P$ = 0.57 for comparison of the three Peg-IFN groups.

### *Impact of dose reduction after week 12 on relapse among patients with c-EVR*

Among c-EVR patients with no or little reduction of Peg-IFN α-2b (the average dose ≥1.2 μg/kg/week) during the first 12 weeks, no significant difference was found in the relapse rate between those whose average dose of Peg-IFN α-2b was reduced to 0.6–1.2 μg/kg/week during 12–48 weeks (17%, 7/41) and those without reduction of Peg-IFN α-2b (average dose ≥1.2 μg/kg/week) (18%, 53/295) ($P$ = 0.86) (Table 4a). Reducing the dose of Peg-IFN α-2b after week 12 in patients in whom HCV RNA had already become undetectable before week 12 did not appear to adversely influence virologic relapse when the average dose of Peg-IFN α-2b was more than 0.6 μg/kg/week during 12–48 weeks, irrespective of the mean dose of Peg-IFN α-2b during the first 12 weeks. On the other hand, the ribavirin dose reduction after week 12 tended to affect the relapse rate in patients given ≥10 mg/kg/day of the ribavirin dose during the first 12 weeks (Table 4b).

### *Impact of drug exposure during 0–48 weeks on relapse among VR patients with advanced fibrosis*

In the evaluation of the 39 patients with VR with progression of fibrosis or cirrhosis (METAVIR fibrosis score 3 or 4) enrolled in this study, ribavirin exposure during treatment significantly correlated with relapse (nonrelapser, 10.5 ± 2.1 mg/kg/day *vs* relapser, 8.8 ± 2.3 mg/kg/day; $P$ = 0.007). Among patients with advanced fibrosis (score 3–4), the relapse rate in patients given ≥10 mg/kg/day of the average ribavirin dose was significantly low (36%, 9/25) in comparison with that in patients given <10 mg/kg/day of ribavirin (71%, 10/14) ($P$ = 0.048).

## DISCUSSION

Previous studies have suggested that reducing the ribavirin dose within the first 12–20 weeks of treatment in patients with HCV genotype 1 was associated with a decline of SVR [7,13,14]. However, Shiffman *et al.* [8] recently reported that reducing the mean dose of ribavirin during the first 20 weeks of treatment had little impact on relapse for patients with CH-C genotype 1 and that SVR may not be adversely affected as long as the total cumulative ribavirin dose remains above 60%. As the reason for the inconsistency in the impact of reducing ribavirin on the antiviral effect, it was suggested that sample sizes of the previous studies were insufficient to assess the impact of reducing the dose of ribavirin independent of Peg-IFN. However, in Shiffman's study, while the impact of reducing the dose of Peg-IFN or ribavirin on SVR was indeed closely examined independently of each other with a large sample size, the subjects were limited to patients with advanced fibrosis or cirrhosis and prior nonresponse to Peg-IFN ± ribavirin who were enrolled in the Hepatitis Antiviral Long-term Treatment Against Cirrhosis (HALT-C) trial. Reddy *et al.* [15] analysed the drug exposure retrospectively for 569 CH-C patients with genotype 1 enrolled in clinical trials of Peg-IFN α-2a plus

**Table 4** Relapse rate according to drug doses during week 0–12 and 12–48 for patients with c-EVR who completed 48 weeks of treatment

(a) Peg-IFN

| Peg-IFN dose (mean, μg/kg/week) | | 12–48 weeks ≥1.2 | 0.9–1.2 | 0.6–0.9 | <0.6 |
|---|---|---|---|---|---|
| 0–12 weeks | ≥1.2 | 18% (53/295) | 17% (5/30) | 18% (2/11) | (1/1) |
| | 0.9–1.2 | – | 22% (4/18) | 33% (4/12) | 60% (3/5) |
| | <0.9 | (0/1) | (0/1) | 17% (2/12) | 20% (1/5) |
| Total* | | 18% (53/296) | 18% (9/49) | 23% (8/35) | 45% (5/11) |

(b) Ribavirin

| Ribavirin dose (mean, mg/kg/day) | | 12–48 weeks ≥12 | 10–12 | 8–10 | <8 |
|---|---|---|---|---|---|
| 0–12 weeks | ≥12 | 4% (2/47) | 13% (3/23) | 13% (1/8) | 33% (1/3) |
| | 10–12 | – | 15% (18/123) | 22% (12/54) | 20% (5/25) |
| | 8–10 | – | (1/1) | 26% (10/38) | 26% (10/39) |
| | <8 | – | – | – | 40% (12/30) |
| Total† | | 4% (2/47) | 15% (22/147) | 23% (23/100) | 29% (28/97) |

c-EVR, complete early virologic response; Peg-IFN, pegylated interferon.
*$P = 0.18$ for comparison of the four Peg-IFN groups. †$P < 0.0001$ for comparison of the four ribavirin groups.

ribavirin, and concluded that SVR was not affected adversely by ribavirin reduction unless the cumulative ribavirin exposure was less than 60%. This supported Shiffman's data, but in Reddy's study, the stepwise reduction in ribavirin dose was shown to be associated with a stepwise increase in relapse rate from 19% to 54%. Thus, the impact of ribavirin drug exposure on the antiviral effect (relapse) in patients with CH-C genotype 1 remains unclear. Further examination is needed to determine whether or not ribavirin can be reduced to a certain degree without adversely affecting virologic relapse or SVR in Peg-IFN and ribavirin combination therapy for CH-C genotype 1.

In order to raise the SVR rate in patients with genotype 1, two strategies are possible: one is enhancing the virologic response of HCV RNA negativity and another is reducing relapse. In Peg-IFN plus ribavirin treatment, raising the doses of either or both drugs (dose-up strategy) is the only way to enhance the virologic response of HCV RNA negativity, but this is always accompanied by a high risk and the discontinuation rate can increase with the dose-up of drug, although the virologic response among patients completing the therapy can be improved [16,17]. Therefore, in this study, we tried to manage the drug dose to reduce relapse in virologic responders with HCV RNA negativity. Large-scale clinical trials [1,2,9–12] have revealed that adding ribavirin to IFN or Peg-IFN monotherapy for patients with CH-C reduced the relapse rate from approximately 50% to under 20%. Bronowicki *et al.* [18] examined the effect of ribavirin on CH-C genotype 1 in Peg-IFN α-2a plus ribavirin treatment by randomizing patients with HCV RNA negativity by week 24 into two groups, one continuing with ribavirin and the other receiving Peg-IFN α-2a alone after week 24. As a result, the virologic responders who stopped ribavirin treatment at week 24 were found to have a significantly higher rate of breakthroughs during therapy and higher relapse rates after therapy in comparison with those who received Peg-IFN plus ribavirin for the full treatment period (relapse rate; 42% vs. 29%, $P = 0.02$). These findings indicate that ribavirin plays a very important role in reducing relapse. However, the relationship between ribavirin dose and relapse rate has not been examined in detail. Considering that ribavirin has little influence on HCV RNA negativiation [1,2,9–12], its dose impact on the antiviral effect should be carefully examined, not for the SVR rate of all patients, but for the relapse rate of patients responding to Peg-IFN plus ribavirin, as evaluating of ribavirin by SVR including HCV RNA negativiation cannot differentiate it from the strong influence of the Peg-IFN effect, which affects HCV RNA negativiation dose-dependently [19]. Here, we examined the correlation between the average dose of drugs and the virologic relapse for patients responding to the treatment.

We performed univariate and multivariate analysis for relapse among the factors of mean administration doses of both drugs, including baseline factors and the timing of HCV RNA negativiation. We found exposure to ribavirin dose, timing of HCV RNA negativiation and the degree of liver fibrosis to be the independent factors affecting the virologic relapse in patients with VR. This indicates that management

of the ribavirin dose, which is the variable factor, unlike baseline factors, plays an important role in suppressing the virologic relapse in patients with CH-C genotype 1 treated by Peg-IFN plus ribavirin treatment. This suggests that maintaining the ribavirin dose should lower the relapse rate even in patients with advanced fibrosis who are liable to relapse. In fact, among patients with advanced fibrosis (METAVIR score 3–4), the relapse rate in those given ≥10 mg/kg/day of the average ribavirin dose was significantly lower than that in patients given <10 mg/kg/day of ribavirin (36% vs. 71%). However, the sample size was too small for subsequent analysis with stratification. Further study is needed to clarify the impact of ribavirin dose on viral relapse in patients with progression of fibrosis.

The relapse rate among patients with c-EVR showed a decline according to the increase in ribavirin dose during treatment week 0–48 and was not affected by the Peg-IFN α-2b dose when the patients were given more than 0.9 μg/kg/week of Peg-IFN α-2b. Among the patients with c-EVR, none with RVR had a relapse and all attained SVR irrespective of the dose of Peg-IFN α-2b or ribavirin. Examination of the impact of dose reduction after week 12 on relapse among patients with c-EVR showed that the ribavirin dose reduction after week 12 tended to affect the relapse rate in patients given ≥10 mg/kg/day of the ribavirin dose during the first 12 weeks, while the Peg-IFN α-2b dose after week 12 could be reduced without any increase in relapse rate in patients given more than 0.6 μg/kg/week of the average dose of Peg-IFN α-2b. On the other hand, maintaining the ribavirin did not lead to reduce the relapse rate in patients with LVR. About half relapsed even when given ≥12 mg/kg/day of the average ribavirin dose. This suggested that the relapse rate could not be reduced by management of the ribavirin dose in patients with LVR. Extended therapy should be chosen in LVR patients as shown in the previous studies [20–23].

Shiffman *et al.* [24] recently reported that maintaining the Hb level with epoetin alpha did not enhance SVR if ribavirin was started at the standard dose (800–1400 mg/day, mean dose 13.3 mg/kg/day), although discontinuance and the reduction rates of ribavirin were decreased and a higher mean dose of ribavirin was administered in comparison with those treated with Peg-IFN plus ribavirin without epoetin. If these findings apply to patients with CH-C genotype 1, this would suggest that the ribavirin dose does not need to be maintained during treatment with Peg-IFN plus ribavirin, which would not agree with our findings. However, closer examination of the Shiffman *et al.* study shows that Peg-IFN plus a higher dose of ribavirin (1000–1600 mg/day, mean dose 15.2 mg/kg/day) with epoetin was found to suppress the relapse rate and enhance SVR. These data agree with ours with respect to the point that higher doses of ribavirin are associated with a lower relapse rate. What differs is the ribavirin dose needed to suppress the relapse. This is likely to be due to ethnic differences between the subjects. In Shiffman's study, approximately 40% were African-Americans in whom the virologic response is well established as being significantly lower than those of other ethnic groups [25,26], while in our study, all subjects were Japanese. In the African-Americans treated with Peg-IFN plus standard-dose ribavirin, the relapse rate (calculated from 48% of ETR and 19% of SVR) was 60%, while 18% relapse (from 38% of ETR and 31% of SVR) occurred in those given Peg-IFN plus high-dose ribavirin. The relapse rate of patients with c-EVR in our study was 19%, which was very close to that for those with Peg-IFN plus high-dose ribavirin in Shiffman's study. Ribavirin does not have a direct antiviral action against HCV [27,28], and is considered to play an important role in accelerating HCV-infected cell clearance [29] and eradicating them completely when an immune response against infected cells is induced by IFN or Peg-IFN [30,31]. Therefore, the difference between patients who are easy or difficult to treat due to ethnic differences or differences in response to Peg-IFN can result in the need for different doses of ribavirin to suppress the relapse rate in patients with CH-C genotype 1.

In conclusion, our results have demonstrated that ribavirin is dose-dependently correlated with a relapse in patients with CH-C genotype 1 responding to Peg-IFN plus ribavirin. Maintaining a high dose (≥12 mg/kg/day) of ribavirin during the full treatment period could strongly suppress the relapse in such patients, while Peg-IFN α-2b could be reduced without affecting relapse in patients with c-EVR. This possibility should be explored in a prospective study.

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## REFERENCES

1 Manns MP, McHutchison JG, Gordon SC *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358: 958–965.

2 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–982.

3 Hadziyannis SJ, Sette Jr H, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; 140: 346–355.
4 Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; 41: 17–27.
5 Zeuzem S, Hultcrantz R, Bourliere M *et al.* Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. *J Hepatol* 2004; 40: 993–999.
6 Ferenci P, Brunner H, Laferl H *et al.* A randomized, prospective trial of ribavirin 400 mg day-1 versus 800 mg day-1 in combination with peginterferon alfa-2a in hepatitis C virus genotypes 2 and 3. *Hepatology* 2008; 47: 1816–1823.
7 McHutchison JG, Manns M, Patel K *et al.* Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. *Gastroenterology* 2002; 123: 1061–1069.
8 Shiffman ML, Ghany MG, Morgan TR *et al.* Impact of reducing peginterferon alfa-2a and ribavirin dose during retreatment in patients with chronic hepatitis C. *Gastroenterology* 2007; 132: 103–112.
9 McHutchison JG, Gordon SC, Schiff ER *et al.* Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1485–1492.
10 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; 352: 1426–1432.
11 Davis GL, Esteban-Mur R, Rustgi V *et al.* Interferon alfa-2b alone or in combination with ribavirin for the treatment of relapse of chronic hepatitis C. International Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; 339: 1493–1499.
12 Lindsay KL, Trepo C, Heintges T *et al.* A randomized, double-blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. *Hepatology* 2001; 34: 395–403.
13 Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; 38: 645–652.
14 Shiffman ML, Di Bisceglie AM, Lindsay KL *et al.* Peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C who have failed prior treatment. *Gastroenterology* 2004; 126: 1015–1023.
15 Reddy KR, Shiffman ML, Morgan TR *et al.* Impact of ribavirin dose reductions in hepatitis C virus genotype 1 patients completing peginterferon alfa-2a/ribavirin treatment. *Clin Gastroenterol Hepatol* 2007; 5: 124–129.
16 Lodato F, Azzaroli F, Brillanti S *et al.* Higher doses of peginterferon alpha-2b administered twice weekly improve sustained virological response in difficult-to-treat patients with chronic hepatitis C: results of a pilot randomized study. *J Viral Hepat* 2005; 12: 536–542.
17 Lindahl K, Stahle L, Bruchfeld A, Schvarcz R. High-dose ribavirin in combination with standard dose peginterferon for treatment of patients with chronic hepatitis C. *Hepatology* 2005; 41: 275–279.
18 Bronowicki JP, Ouzan D, Asselah T *et al.* Effect of ribavirin in genotype 1 patients with hepatitis C responding to pegylated interferon alfa-2a plus ribavirin. *Gastroenterology* 2006; 131: 1040–1048.
19 Oze T, Hiramatsu N, Yakushijin T *et al.* Peginterferon alfa-2b affects early virologic response dose-dependently in patients with chronic hepatitis C genotype 1 during treatment with pegylated interferon alfa-2b plus ribavirin. *J Viral Hepat*, in press.
20 Berg T, von Wagner M, Nasser S *et al.* Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; 130: 1086–1097.
21 Sanchez-Tapias JM, Diago M, Escartin P *et al.* Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. *Gastroenterology* 2006; 131: 451–460.
22 Pearlman BL, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis c genotype 1-infected slow responders. *Hepatology* 2007; 46(6): 1688–1694.
23 Mangia A, Minerva N, Bacca D *et al.* Individualized treatment duration for hepatitis C genotype 1 patients: a randomized controlled trial. *Hepatology* 2008; 47: 43–50.
24 Shiffman ML, Salvatore J, Hubbard S *et al.* Treatment of chronic hepatitis C virus genotype 1 with peginterferon, ribavirin, and epoetin alpha. *Hepatology* 2007; 46: 371–379.
25 Layden-Almer JE, Ribeiro RM, Wiley T, Perelson AS, Layden TJ. Viral dynamics and response differences in HCV-infected African American and white patients treated with IFN and ribavirin. *Hepatology* 2003; 37: 1343–1350.
26 Jacobson IM, Brown RS Jr, McCone J *et al.* Impact of weight-based ribavirin with peginterferon alfa-2b in African Americans with hepatitis C virus genotype 1. *Hepatology* 2007; 46: 982–990.
27 Reichard O, Andersson J, Schvarcz R, Weiland O. Ribavirin treatment for chronic hepatitis C. *Lancet* 1991; 337: 1058–1061.
28 Di Bisceglie AM, Shindo M, Fong TL *et al.* A pilot study of ribavirin therapy for chronic hepatitis C. *Hepatology* 1992; 16: 649–654.
29 Hiramatsu N, Hayashi N, Haruna Y *et al.* Immunohistochemical detection of hepatitis C virus-infected hepatocytes in chronic liver disease with monoclonal antibodies to core, envelope and NS3 regions of the hepatitis C virus genome. *Hepatology* 1992; 16: 306–311.
30 Miyatake H, Kanto T, Inoue M *et al.* Impaired ability of interferon-alpha-primed dendritic cells to stimulate Th1-type CD4 T-cell response in chronic hepatitis C virus infection. *J Viral Hepat* 2007; 14: 404–412.
31 Itose I, Kanto T, Inoue M *et al.* Involvement of dendritic cell frequency and function in virological relapse in pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C patients. *J Med Virol* 2007; 79: 511–521.

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# Multiple Cytokine Profiling of the Therapeutic Responses to Ribavirin and Pegylated Interferon-α2b Using an "Induction" Approach With Natural Interferon-β in Difficult-to-Treat Chronic Hepatitis C

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Cyclic and periodic IFN treatment (CPIT) consisting of induction treatment with nIFN-β followed by maintenance treatment with IFN-α could prevent viral breakthrough and achieve rapid virological response (RVR) and early virological response (EVR) in chronic hepatitis C (CHC). The efficacy and immune response of RBV+PEG-IFN-α2b using induction approach with CPIT (novel combination treatment: NCT) in 7 CHC patients with genotype 1b and high viral load were evaluated. A biometric multiplex serum cytokine assay was utilized to characterize the immunomodulatory effect. RVR and EVR were 7/7 and 7/7, respectively. Viral titers dropped below detectable levels in five patients with sustained virological response (SVR) before the end of CPIT (early virological responder: EAVR), and two patients without SVR after the end of CPIT (late virological responder: LAVR). At baseline, in EAVR compared with the controls, IL-6 and IL-15, CXCL-8 and CXCL-10 levels were significantly higher ($P < 0.05$); IL-10 and IL-13 levels were significantly lower ($P < 0.05$); and the IL-12 level was lower. In LAVR, GM-CSF, CXCL-8 and CXCL-10, and CCL-4 levels were significantly higher ($P < 0.05$); and IL-10 and IL-12 were lower than the controls. In EAVR but not LAVR, the IL-12 increased and the CCXL-8 decreased significantly ($P < 0.05$). In conclusion, NCT-induced viral clearance leading to improvement in the innate immune response resulting in SVR in CHC with genotype 1b and high viral load.

## Introduction

WORLDWIDE MORE THAN 170 million people are chronically infected with the hepatitis C virus (HCV) (Global Surveillance and Control of Hepatitis C 1999), which is a frequent cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, and the most frequent cause of liver transplantation (Fried 2000). Chronic hepatitis C (CHC) is a serious global medical problem necessitating effective treatment. However, approximately 50% of treated patients are still not cleared of viremia when treated with pegylated (PEG)-interferon (IFN)-α plus ribavirin (RBV) (Manns and others 2001; Fried and others 2002; Berg and others 2006). Thus, more effective, more tolerable, and/or more tailored therapies are required.

Because a sustained virological response (SVR) has been shown to be more likely after favorable early viral kinetics (i.e., a more rapid and profound reduction in HCV RNA levels) (Davis and others 2003), the rapidity of the initial virus clearance augmented by induction therapy [high-dose (Tassopoulos and others 2003), more frequent IFN administrations, or combination with PEG-IFN-α, RBV, and Telaprevir (Kieffer and others 2007) for the first several months] was postulated as an approach to achieve SVR; however, the issue of how to increase the initial virological response rate has not been resolved. HCV exists as a genetically heterogeneous viral population, which are termed quasispecies. Thus, the clinical success of HCV therapies will depend on their ability to suppress all viral variants and prevent the

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emergence of resistant viruses (Herzer and others 2003). Normalization of CD4 counts in HIV-infected patients for all infected individuals might be achievable if viral suppression with combination antiretroviral therapy can be maintained for a sufficiently long period of time (Mocroft and others 2007). Thus, as with HIV, initial aggressive treatment of HCV should be considered as a means of increasing the success of therapy (Neuman and others 1998). The increased period of replication and the delay in host responses could allow for further introduction of mutations that may lead to immune escape or exhaustion of the induced response as a result of higher numbers of infected cells (Major and others 2004). If viral replication can be suppressed for a sufficient length of time, viral load should decline to a point where the continued production of quasispecies with the potential to resist drug treatment no longer occurs. In addition, increasing overall treatment duration and individual tailoring of the duration of the maintenance phase to the duration of the induction phase in patients initially cleared of HCVRNA might significantly decrease the rate of relapse.

On the other hand, viral subversion of steps both upstream and downstream of IFN induction, including IFN-α/β signaling, ISG function, and dendritic cell (DC) and T-cell responses, has now been well described (Chang and others 2008). The immune system works as a well-orchestrated interaction of different types of cells whose final goal is to eliminate invading pathogens. Chronic infection by HCV is characterized by an insufficient immune response (Kanto and others 1999). Recent research suggests that HCV interacts with and affects the function of different innate and adaptive immune cell types including DCs, monocytes, macrophages, natural killer (NK) cells, natural killer T (NKT) cells, cytotoxic T cells (CTL), and regulatory T cells (Treg). HCV downregulates NK cell activity, and readily produces mutants that are capable of evading CTLs and neutralizing antibodies to the N-terminal region of gp E2 (Taniguchi and others 2001; Crotta and others 2002; Carotenitro and others 2005; Foy and others 2005).

Moreover, HCV evades the innate immune system and turns down adaptive immune responses, and to prevent pathogen elimination through the destruction of TLR 3-induced and RIG-I-induced signaling for IFN induction in hepatocytes, inhibition of plasmacytoid DC (pDC) IFN-α production, and subversion of myeloid DC (mDC) functions in T-cell activation (Szabo and others 2007) and thus allows for persistent infection and perhaps blunts the therapeutic effects of exogenously applied IFN-α (Chang and others 2008). The rapid induction of the expression of type I IFNs (IFN-α/β) is a central event in initiating the innate antiviral response as well as enhancing cell-mediated immunity with upregulation of the production of MHC class I and class II peptides, DC maturation, and increased NK cell activity (Crotta and others 2002; Carotenitro and others 2005). The overall frequency of (IFN-α/β producing) pDCs has been found to be reduced in HCV-infected patients. Proinflammatory monocyte activation, reduced monocyte DC T-cell activation capacity, and impaired IFN production by pDCs may each contribute to ongoing liver injury as well as the inefficient elimination of HCV during antiviral therapy (Chang and others 2008). The malfunctioning of DCs in HCV-infected patients might result in insufficient priming of T-cell responses as a potential mechanism contributing to HCV-specific immune dysfunction. DCs are ubiquitous professional antigen presenting cells (APCs) that provide a critical link between innate and adaptive immunity (Wertheimer and others 2007). DCs are particularly susceptible to the inhibitory effects of HCV infection. Interestingly, the allostimulatory capacity of DCs is decreased in some animals with high viral loads, suggesting that RNA levels and the viral protein load may play a role in the modulation of DC functions (Szabo and others 2006). The pDC population is reestablished in subjects with spontaneously resolved HCV infection, suggesting that viral factors are responsible for pDC depletion. Resolution of HCV infection may restore the downregulation of innate and adaptive immunity (Urbani and others 2002; Khakoo and others 2004; Rahman and others 2004).

In the peripheral blood of patients with chronic HCV infection, a decreased frequency of mDCs, which stimulate allogenic CD4 T cells to drive the T-helper type response and produce IL-12p70, and IFN-β but not IFN-α, has been identified, and mDCs are infected with HCV with functional impairment that is associated with the development of chronic infection (Kanto and others 1999; Taniguchi and others 2001; Wertheimer and others 2004). Recent studies have shown that HCV NS3/4A protein interrupts IFN-β induction through the RIG-I pathway via the proteolytic cleavage of IFN-β promoter stimulator-1 (IPS-1; also known as MAVS, VISA, or Cardif) (Li and others 2005; Loo and others 2006; Lau and others 2008).

IFN-β has different signaling and biological activities from IFN-α, and achieves a higher rate of viral clearance than IFN-α (Platanias and others 1994; Domarski and others 1998; Da Silvia and others 2002). In hepatitis C, the efficacy of IFN-β has been investigated in several studies (Chan and others 2007). The combination of IFN-β and IFN-α enhances synergistically antiviral activity against HCV (Okuse and others 2005; Hiasa and others 2008). IFN-α and IFN-β apparently utilize different regions of the intracellular domain of the β-L subunit to generate an antiviral state similar to the mechanism proposed for the Tar protein. IFN-β signaling has two distinct features: (1) the induction of a very strong association of the α- and β-L subunits of the type I IFN receptor 1; and (2) transcriptional activation of the β-R1 gene. Previously, β-R1 was the only known ISG whose expression is selectively induced by IFN-β, but not by IFN-α or IFN-γ (Domarski and others 1998).

In a previous study (Kishida and others 2003, 2004), it was shown that cyclic and periodic IFN treatment (CPIT) consisting of induction treatment (IT) with natural (n) IFN-β followed by maintenance treatment (MT) with IFN-α could prevent virological breakthrough, and CPIT could achieve an early virological response (EVR) and an end treatment virological response (ETVR). In addition to the improvement of innate immunity because of virological clearance by CPIT during the initial course of therapy, persistent virological clearance and the restoration of downregulation of innate and adaptive immunity by RBV plus PEG-IFN-α were more likely to result in higher rapid virological response (RVR), EVR, ETVR, and SVR. On the basis of these findings, a study was conducted in seven difficult-to-treat CHC patients with genotype 1b and high viral load to assess the efficacy, tolerability, and safety of treatment with RBV plus PEG-IFN-α2b for 48 weeks using an induction approach with initial

virological clearance induced by CPIT for 24 weeks (novel combination treatment: NCT).

Cytokines are critical in all phases of the immune response to HCV infection, from the early onset of liver damage to chronic infection, viral persistence, tissue damage, and fibrosis. Chemokines play a pivotal role in the recruitment of activated/memory T cells to the HCV-infected liver, and they are important for viral clearance (Hellier and others 2003). Furthermore, the control of cytokine production is highly complex and multifactorial, and the effects of cytokines are mediated through multiple regulatory networks. Therefore, it is informative to investigate the immunopathogenesis of the disease process by analyzing multiple cytokines and chemokines. Utilizing a broad-spectrum bead-based multiplex immunoassay, an evaluation was performed with respect to the serum levels of cytokines that mediate humoral and cellular immunity and inflammation, correlated with disease activity, and characterized the immunomodulatory effects of the therapy. This study provides us with a better understanding of the role of cellular, humoral, and chemotactic immunity at a critical time in the treatment course of HCV infection, when the most significant changes in viral titer are observed.

## Materials and Methods

### Patients

Seven patients (three males and four females; mean age 53.3 ± 8.5 years, range 39–66) with CHC, positive anti-HCV antibody, genotype 1b (serotype 1), IFN sensitivity determining region (ISDR) (Watanabe and others 2001) with three wild-type, three intermediate type, and one not determined, and a high HCV viral load of 2144.3 ± 1701.2 KIU/mL (range 536 to >5000 KIU/mL) were enrolled. Patients underwent liver biopsy before IFN therapy, and the severity inflammation (Grade), and fibrosis (Stage) of liver disease (Ishak and others 1995) was evaluated and showed chronic hepatitis (Grade 1–3, Stage 1–2) (Table 1). Two patients had a history of blood transfusion. The source of infection was unknown in the remaining five patients. Serum was collected from five healthy donors, ranging in age from 28 to 58 years. Written informed consent was obtained from all participants according to the Declaration of Helsinki.

### Exclusion criteria

The following were considered as exclusion criteria: refusal by women of child-bearing age or by sexually active patients to use a safe contraceptive, pregnancy or breast-feeding, cirrhosis with signs of decompensated liver disease, coronary heart disease, the presence of overt psychiatric disorders, active alcohol or drug abuse, uncontrolled diabetes mellitus, uncontrolled hypertension, uncontrolled retinopathy, autoimmune disorders, or any other unstable medical condition not because of liver disease. All patients were negative for hepatitis B surface antigen, and frequent causes of chronic liver disease were excluded.

### Study design

*Cyclic and periodic IFN treatment (CPIT).* The patients were treated with six cycles of CPIT. One cycle of CPIT consisted of IT with nIFN-β (Feron, Toray, Chiba, Japan) at 3–6 MU/day, intravenously by drip infusion in 100 mL of saline solution, daily for 2 weeks followed by MT with nIFN-α

TABLE 1. CHARACTERISTICS OF CHRONIC HEPATITIS C WITH HIGH VIRAL LOAD, SEROTYPE-1 (GENOTYPE 1B), AND WILD OR INTERMEDIATE TYPES OF ISDR BEFORE, DURING, AND AFTER RBV PLUS PEG-IFN-α2B USING AN "INDUCTION" THERAPY WITH CYCLIC AND PERIODIC INTERFERON TREATMENT (CPIT)

| | | | | | ALT (IU/mL) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Patient No.* | *Age/ Gender* | *Body weight (kg)* | *BMI (kg/m²)* | *Liver Histology (Stage/ Grade)* | *Baseline* | *24 weeks (end of CPIT)* | *72 weeks (end of NCT)* | *96 weeks (24 weeks after the end of NCT)* | *Outcome* |
| *Early virological responders* | | | | | | | | | |
| 1 | 61/F | 46.1 | 20.4 | 1/2 | 197 | 37 | 15 | 13 | SBR |
| 2 | 47/M | 67.0 | 21.1 | 1/1 | 37 | 28 | 22 | 22 | SBR |
| 3 | 66/M | 78.0 | 24.5 | 3/2 | 57 | 29 | 29 | 17 | SBR |
| 4 | 49/M | 60.0 | 19.8 | 1/2 | 50 | 32 | 12 | 11 | SBR |
| 5 | 61/F | 48.5 | 20.9 | 1/2 | 38 | 9 | 14 | 10 | SBR |
| *Late virological responders* | | | | | | | | | |
| 6 | 39/F | 73.0 | 27.1 | 1/2 | 48 | 331 | 264 | 161 | NBR |
| 7 | 56/F | 55.0 | 20.6 | -/- | 33 | 19 | 13 | 49 | TBR |
| *Mean ± SD* | 53.3 ± 8.5 | 61.1 ± 12.1 | 22.1 ± 2.7 | 1-3/1-2 | 65.7 ± 58.5 | 69.3 ± 115.8 | 52.7 ± 93.3 | 39.0 ± 59.9 | |

Abbreviations: NCT, novel combination treatment; IFN, interferon; BMI, body mass index; ISDR, IFN sensitivity determining region; F, female; M, male; SBR, sustained biochemical response; NBR, no biochemical response; TBR, transient biochemical response.

(Sumiferon, Sumitomo, Osaka, Japan) at 6 MU/day, subcutaneously, three times weekly for 2 weeks.

*CPIT followed by treatment with RBV plus PEG-IFN-α2b (NCT).* We investigated the efficacy, tolerance to, and safety of CPIT for 24 weeks as an induction approach followed by RBV (Ribavirin; Rebetol: Schering Plough, Kenilworth, NJ, USA; 200–800 mg/day, per os, daily) plus PEG-IFN-α2b (Pegintron, Schering Plough, Kenilworth, NJ, USA; 50–120 μg/day, percutaneously inj., once weekly) for 48 weeks in a clinical trial as a potential treatment for seven difficult-to-treat CHC patients with genotype 1b (serotype I), a viral load of more than 100 KIU/mL, and a wild or intermediate types of ISDR.

### Measurement

All patients were monitored with clinical, biochemical, and virological assessments before and every 1–4 weeks during the entire treatment period, and were followed for at least a further 24 weeks.

### HCVRNA

The level of HCVRNA in serum was determined using the quantitative COBAS AMPLICOR HCV MONITOR test, ver. 2.0 (Roche Diagnostic Systems, Tokyo, Japan) and qualified using the COBAS AMPLICOR HCV test ver. 2.0 (sensitivity <50 IU/mL; Roche Diagnostic Systems). HCVRNA in serum was determined before therapy, every 4 weeks following the beginning of treatment and after the end of NCT. The detection of anti-HCV antibodies in serum was based on the use of a third-generation Ortho HCV enzyme-linked immunosorbent assay (ELISA) test system (Ortho Diagnostics Inc., Raritan, NJ, USA). Serological determination of the HCV genotype (serotyping) was conducted using a competitive EIA (Murex HCV Serotyping 1-6 $HCO_2$; Abbott Laboratories, North Chicago, IL, USA).

### IFN sensitivity determining region

As a correlation between the ISDR sequence and IFN resistance was indicated, the deduced amino acid sequence of the HCV-NS5A gene at positions 2209–2248 (ISDR) in the HCVRNA extracted from serum was compared with the NS5A 2209–2248 sequence of HCV-J, which is the prototypic sequence of HCV-1b; HCV with no mutations in the ISDR as the wild type, HCV with 1–3 mutations in the ISDR as the intermediate type, and HCV with more than 4 mutations in the ISDR as the mutant type, compared with HCV-J (Watanabe and others 2001).

### Definition of response

The HCVRNA level was used in the assessment of the efficacy of treatment. The primary efficacy end point was SVR. A RVR was defined as either undetectable HCVRNA or more than 2 log decline from the baseline in HCVRNA levels at 4 weeks of therapy. EVR was defined as more than 2 log decline from the baseline in HCVRNA levels at 12 weeks of therapy. A complete EVR (cEVR) was defined as undetectable HCVRNA levels at 12 weeks of therapy, and partial EVR (pEVR) was defined as positive HCVRNA but more than 2 log decline from the baseline in HCVRNA levels at 12 weeks of therapy. ETVR was defined as undetectable HCVRNA in serum at the end of therapy, and SVR was defined as undetectable HCVRNA (<50 IU/mL) in serum at least 24 weeks after the end of treatment. A transient virological response (TVR) was defined as the reappearance of HCVRNA in serum after the completion of a successful therapy achieving EVR and/or ETVR during IFN treatment. No virological response was defined as detectable HCVRNA in serum during treatment. A sustained biochemical response (SBR) was defined as normal alanine aminotransferase (ALT) levels 24 weeks after the end of treatment. No biochemical response was defined as elevated ALT levels in serum during and after the end of treatment.

### Assessment of cytokines and chemokines (multiplex cytokine assay)

A multiplex biometric ELISA-based immunoassay, containing dyed microspheres conjugated with a monoclonal antibody specific for each target protein, was used according to the manufacturer's instructions (Bio-Plex Human Cytokine assay; BioRad Inc., Tokyo, Japan). The cytokines measured were (1) Th1 cytokines: IFN-γ, TNF-α, IL-1α, IL-1β, IL-2, IL-12 (p70), and IL-15; (2) Th2 cytokines: IL-4, IL-6, IL-9, Il-10, and IL-13; (3) hematopoietic cytokines: GM-CSF and G-CSF; (4) CXC chemokines: CXCL-8 (IL-8) and CXCL-10 (IP-10); (5) CC chemokines: CCL-2 (MCP-1), CCL-3 (MIP-1-α), CCL-4 (MIP-1-β), CCL-5 (RANTES), and CCL-11 (Eotaxin); (6) other cytokines: VEGF and PDGF. Serum samples were diluted 1:4 and incubated with antibody-coupled beads. The complexes were washed, then incubated with biotinylated detection antibody, and, finally, with streptavidin-phycoerythrin before assessing cytokine concentration titers. Concentrated human recombinant cytokine was provided by the vendor (BioRad, Inc.). A broad range, 1.95–32,000 pg/mL, of standards was used to establish standard curves to maximize the sensitivity and dynamic range of the assay. Cytokine levels were determined using a Bio-Plex array reader (an automated flow-based microfluidics device that uses a dual-laser fluorescent detector with real-time digital signal processing for quantification; Luminex, Austin, TX). This instrument quantifies multiplex immunoassays in a 96-well format using very small serum volumes (12.5 mL). The concentrations of analytes in these assays were calculated using a standard curve with software provided by the manufacturer. Regression analysis was performed to derive an equation that was then used to predict the concentration of cytokines in serum samples (Wright and others 2005). The concentrations of cytokines and chemokines in serum were serially determined.

### Assessment of safety

Safety was assessed with laboratory tests and an evaluation of adverse events (AEs) every 1–4 weeks during and after the end of NCT. A reduction in the RBV dosage from 800 mg to 200–600 mg/day and reduction in the PEG-IFN-α2b dosage from 60–120 μg to 50–100 μg without virological breakthrough were allowed to manage AEs or laboratory abnormalities that had reached predetermined thresholds of severity. If the AEs were resolved or improved, a return to the initial dosing levels was permitted.

### Statistics

Data were expressed as the mean ± standard deviation and paired *t*-test was used to evaluate differences of the means between groups, and a *P*-value of <0.05 was considered significant.

## Results

This clinical study of NCT was carried out and follow up was completed in all patients between February 2004 and July 2008. The patient characteristics are shown in Table 1.

### Effect of NCT on HCVRNA, ALT, 2'-5'-OAS, and hyarulonate in CHC patients with genotype 1b and high viral load

*HCVRNA.* HCV viral titers decreased in all patients after 4 weeks of CPIT highlighting the efficacy of this treatment modality. None of the patients showed virological breakthrough. Serum HCVRNA [2144.3 ± 1701.2 (range 536 to >5000) KIU/mL at baseline] decreased significantly to 1.5 ± 2.4 KIU/mL ($P = 0.0157$) at the end of CPIT. The rate of RVR and EVR (pEVR and cEVR) were 7/7 (100%) and 7/7 (100%) [4/7 (57.1 %) and 3/7 (42.9%)], respectively. Viral titers dropped below detectable levels in five patients before the end of CPIT, and in two patients after the end of CPIT (after beginning of RBV plus PEG-IFN-α-2b). The rates of ETVR at the end of CPIT and NCT were 5/7 (71.4%) and 7/7 (100%), respectively. The SVR rate was 5/7 (71.4%). TVR was found in two patients who showed undetectable HCVRNA in serum after the end of CPIT. The TVR rate was 2/7 (28.6%). The two patients with TVR, who needed a dose reduction of IFN because of serum ALT elevation and hyperthyroidism during CPIT, showed late virological responses after the end of CPIT. To refine our understanding of the heterogeneity of therapeutic response, patients were classified into two statistically distinct groups based on the time of clearance of viremia, and these groups were used for further analysis. Of note, early virological responders (EAVR) with undetectable HCVRNA in serum before the end of CPIT, which included five patients (pt. No. 1–5), showed SVR. Late virological responders (LAVR) with undetectable HCVRNA in serum after the end of CPIT, which included two patients (pt. No. 6–7), showed TVR. The viral titer values in the LAVR were extremely high (>5000 and 4400 KIU/mL) (Table 3).

*ALT.* Table 1 shows the effect of NCT on serum ALT levels. Serum ALT (65.7 ± 58.5 IU/mL at baseline) decreased to 52.7 ± 93.3 IU/mL at the end of NCT and 39.0 ± 59.9 IU/mL 24 weeks after the end of NCT. The rate of SBR was 5/7 (71.4%).

*2'-5'-OAS activity.* Serum 2'-5'-OAS activity (105.6 ± 44.4 pmol/dL at baseline) increased significantly to 233.9 ± 164.3 pmol/dL ($P < 0.05$) at the end of CPIT and 207.1 ± 169.1 pmol/dL ($P < 0.05$) at the end of NCT (Table 4).

*Hyaluronate.* Serum hyaluronate (237.0 ± 104.9 ng/mL at baseline) decreased significantly to 126.0 ± 94.7 ng/mL ($P < 0.05$) at the end of CPIT, 79.9 ± 36.2 ng/mL ($P < 0.05$) at the end of NCT, and 81.4 ± 36.8 ng/mL ($P < 0.05$) 24 weeks after the end of NCT (Table 4).

### Platelets, red blood cells, hemoglobin, and white blood cells

Platelet level (19.7 ± 9.4 $\times 10^4$/μL at baseline) decreased significantly to 13.3 ± 4.6 $\times 10^4$/μL ($P = 0.0173$) at the end of CPIT but increased to 20.4 ± 5.2 $\times 10^4$/μL ($P = 0.185$) at 24 weeks after the end of NCT. Red blood cell levels (434 ± 50 $\times 10^4$/μL at baseline) decreased significantly to 346 ± 43 $\times 10^4$/μL ($P < 0.01$) at the end of NCT and recovered to 422 ± 50 $\times 10^4$/μL afterward. Hemoglobin levels (13.3 ± 2.2 g/dL at baseline) decreased to 11.0 ± 1.1 g/dL ($P = 0.053$) at the end of NCT and recovered to 12.8 ± 2.5 g/dL at 24 weeks after the end of NCT. White blood cell levels (5.5 ± 1.9 $\times 10^3$/μL at baseline) decreased significantly to 3.8 ± 1.4 $\times 10^3$/μL ($P = 0.0137$) at the end of CPIT, 3.3 ± 1.4 $\times 10^3$/μL ($P = 0.00365$) at the end of NCT, and recovered to 5.4 ± 1.9 $\times 10^3$/μL 24 weeks after the end of NCT (Table 2).

### Effect of NCT on serum cytokines and chemokines in CHC patients with genotype 1b and high viral load

Both HCV and its treatment modulate the immune response of the host. The heterogeneity of therapeutic responses may be due to differences in host responses to the virus, therapy, or a combination of the two (Figures 1-4).

### Serum cytokines and chemokines at baseline

The levels of CXCL-8, CXCL-10, CCL-4, and CCL-11 were significantly higher ($P < 0.05$) and also IFN-γ, TNF-α, IL-1α, IL-2, IL-6, IL-9, IL-15, GM-CSF, G-CSF, and CCL-2 were higher, but IL-10, IL-12, and IL-13 were significantly lower ($P < 0.05$), and VEGF was lower in all CHC patients than in the controls. The levels of IL-6, IL-15, CXCL-8, CXCL-10, and CCL-11 were significantly higher ($P < 0.05$) and IFN-γ, TNF-α, IL-1α ($P < 0.1$), IL-2, GM-CSF, G-CSF, CCL-2, and CCL-4 were higher in EAVR than in the controls. IL-10 and IL-13 were significantly lower ($P < 0.05$), and IL-12 ($P < 0.1$) and VEGF were lower in EAVR than in the controls. GM-CSF, CXCL-10, and CCL-4 were significantly higher ($P < 0.05$) and TNF-α ($P < 0.1$), IFN-γ, IL-1α, IL-1β, IL-2, IL15, IL-6, IL-9, IL-4, G-CSF, PDGF, CXCL-8 ($P < 0.1$), and CCL-11 ($P < 0.1$) were higher in LAVR than in the controls. Serum IL-10 and IL-13 were significantly lower ($P < 0.05$), and IL-12 was lower in LAVR than in the controls (Figure 1).

IFN-γ activates NK cells followed by recognition of the viral peptides MHC complexes (Frese and others 2002). IFN-γ is secreted by activated NK and NKT cells, inhibits the replication of HCV through a noncytolytic mechanism, and is associated with antiviral and immunoregulatory effects promoting other downstream protective immune responses (Biron and others 2001). IFN-γ increases and a predominantly NK-cell population rather than T cells may cause these decreases in viral titer (Major and others 2004).

IL-15 plays an important role in the innate immune system and is a stimulatory cytokine for DCs impaired in CHC (Li and others 2006). IL-15 is induced by IFN-α/β and stimulates the proliferation and accumulation of NK cells. IFN-α/β produced by DCs also modulates the activation of CD8+ T cells, which produce additional IFN-γ and represent the central players in the pathogen-specific adaptive immune response. The activation of NK cells, as well as the timing, breadth, and robustness of the subsequent