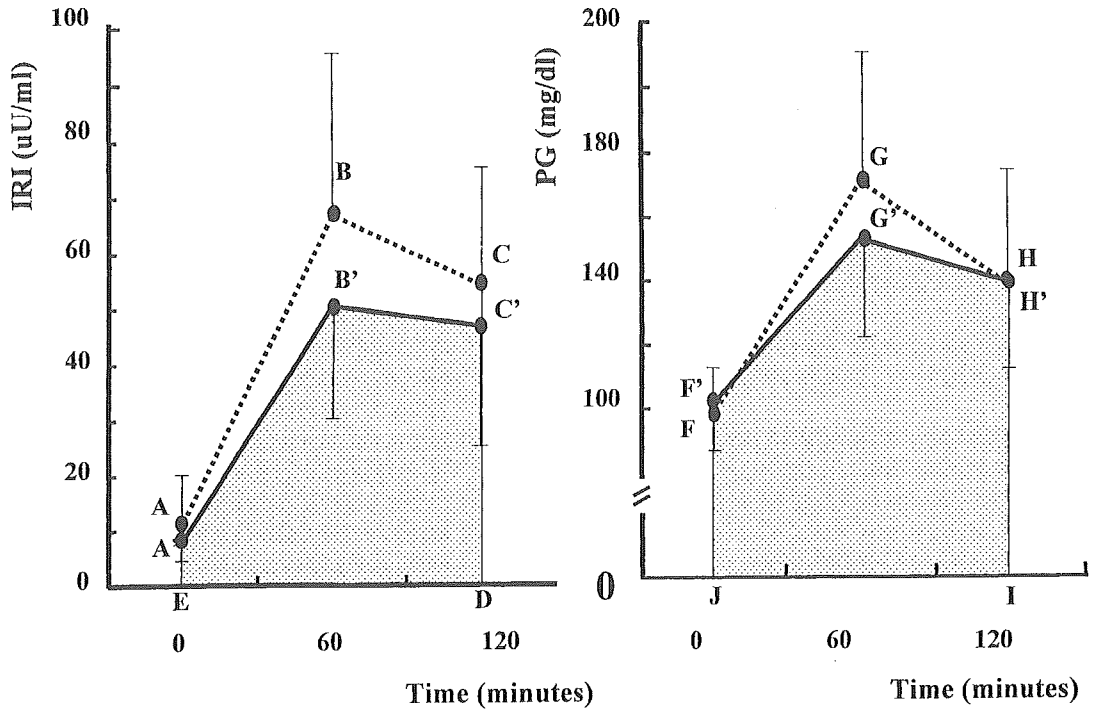

Fig. 2. PG levels and IRI levels in response to the "cookie test" at baseline and after 12 months of pravastatin treatment. The broken line indicates the baseline values and the thick line indicates the values at 12 months.
AUC-IRI at baseline = the area of ABCDE= 99.6 ± 46.6 μU•Eh/ml
AUC-IRI at 12months = the area of A'B'C'DE= 77.9 ± 39.8 μU•Eh/ml.
*$p < 0.05$ vs. baseline.
AUC-IRI × AUC-PG at baseline = the area of ABCDE × the area of FGHIJ = 29215.5 ± 15736.1 (μU•Ehr/ml) (mg•Eh/dl)
AUC-IRI × AUC-PG at 12 months = the area of A'B'C'DE × the area of F'G'H'IJ 21478.6 ±12389.8 (μU•Ehr/ml) (mg•Eh/dl).
*$p < 0.05$ vs. baseline.

tance, which may be attributed to the inhibition of proinflammatory cytokines.

Some statins, especially lipophilic statins, have been reported to have an unfavorable effect on glucose metabolism. Yada *et al.* (26) found that simvastatin, a lipophilic statin, inhibited glucose-induced cytosolic Ca^{2+} signaling and insulin secretion due to a blockade of L-type Ca^{2+} channels in rat islet β-cells; however, pravastatin, a hydrophilic statin, caused no such inhibition. Kanda *et al.* (27) reported that atorvastatin, also a lipophilc statin, significantly increased blood glucose at several time points during OGTT, however, pravastatin did not. It seems reasonable to suppose that this hydrophobic statin, pravastatin, is processed only in the liver, whereas the lipophilic statins are processed in other organs, such as the pancreas, adipose tissue, and muscle, causing a decrease in insulin secretion and exacerbation of insulin resistance.

There are potential limitations regarding the interpretation of our results. First, the small number of patients that were eligible for the analysis makes it difficult to generalize the results. Second, there is no control group that would allow us to more properly evaluate the effect of pravastatin. Further study will be required to clarify the effect of pravastatin on insulin resistance.

In conclusion, our study indicated that pravastatin improves insulin resistance in addition to reducing the serum cholesterol level and that the "cookie test", AUC-IRI, and the formula AUC-IRI × AUC-PG. were useful for evaluating glucose and lipid metabolism.

References

(1) Reaven GM: Role of insulin resistance in human disease. Diabetes, 37: 1595–1607,1988
(2) Despres JP, Lamarche B, Mauriege P, Cantin B,

Dagenais GR, Moorjani S, and Lupien PJ: Hyperinsulinemia as an independent risk factor for ischemic heart disease. N Engl J Med, 334: 952–957, 1996
(3) Pyorala K: Relationship of glucose tolerance and plasma insulin to the incidence of coronary heart disease: results from two population studies in Finland. Diabetes Care, 2: 131-141,1979
(4) Ducimetiere P, Eschwege E, Papoz L, Richard JL, Claude JR, and Rosselin G: Relationship of plasma insulin levels to the incidence of myocardial infarction and coronary heart disease mortality in a middle-aged population. Diabetologia, 19: 205–210, 1980
(5) Ohrvall M, Lithell H, Johansson J, and Vessby B: A comparison between the effects of gemfibrozil and simvastatin on insulin sensitivity in patients with non-insulin-dependent diabetes mellitus and hyperlipoproteinemia. Metabolism, 44: 212–217, 1995
(6) Farrer M, Game FL, Albers CJ, Neil HA, Winocour PH, Laker MF, Adams PC, and Alberti KG: Coronary artery disease is associated with increased lipoprotein(a) concentrations independent of the size of circulating apolipoprotein(a) isoforms. Arterioscler Thromb, 14: 1272–1283, 1994
(7) Pontrelli L, Parris W, Adeli K, and Cheung RC: Atorvastatin treatment beneficially alters the lipoprotein profile and increases low-density lipoprotein particle diameter in patients with combined dyslipidemia and impaired fasting glucose/type 2 diabetes. Metabolism, 51: 334–342, 2002
(8) Malik J, Melenovsky V, Wichterle D, Haas T, Simek J, Ceska R, and Hradec J: Both fenofibrate and atorvastatin improve vascular reactivity in combined hyperlipidaemia (fenofibrate versus atorvastatin trial—FAT). Cardiovasc Res, 52: 290–298, 2001
(9) Paolisso G, Barbagallo M, Petrella G, Ragno E, Barbieri M, Giordano M, and Varricchio M: Effects of simvastatin and atorvastatin administration on insulin resistance and respiratory quotient in aged dyslipidemic non-insulin dependent diabetic patients. Atherosclerosis, 150: 121–127, 2000
(10) Freeman DJ, Norrie J, Sattar N, Neely RD, Cobbe SM, Ford I, Isles C, Lorimer AR, Macfarlane PW, McKillop JH, Packard CJ, Shepherd J, and Gaw A: Pravastatin and the development of diabetes mellitus: evidence for a protective treatment effect in the West of Scotland Coronary Prevention Study. Circulation, 103: 357–362, 2001
(11) Coates PA, Ollerton RL, Luzio SD, Ismail I, and Owens DR: A glimpse of the 'natural history' of established type 2 (non-insulin dependent) diabetes mellitus from the spectrum of metabolic and hormonal responses to a mixed meal at the time of diagnosis. Diabetes Res Clin Pract, 26: 177–187, 1994
(12) Cozma LS, Luzio SD, Dunseath GJ, Underwood PM, and Owens DR: Beta-cell response during a meal test: a comparative study of incremental doses of repaglinide in type 2 diabetic patients. Diabetes Care, 28: 1001–1007, 2005
(13) Harano Y, Adachi T, Nabiki J, Stuji N, Taketani K, Sasaki F, Yamaguchi F, Shibati M, Miyawaki T, Ueda F, and Mori N: Development of the cookie test for the early detection and analyses of metabolic risk factors of the life style related diseases and its significance. Rinsho Byori, 52: 55–60, 2004 (article in Japanese, abstract in English)
(14) Emoto M, Nishizawa Y, Maekawa K, Hiura Y, Kanda H, Kawagishi T, Shoji T, Okuno Y, and Morii H: Homeostasis model assessment as a clinical index of insulin resistance in type 2 diabetic patients treated with sulfonylureas. Diabetes Care, 22: 818–822, 1999
(15) Takeuchi M, Kanazawa A, Suzuki M, Hasegawa M, Kimura Y, Kodama K, Ryomoto K, Hattori Y, and Harano Y: Evaluation of factors during OGTT to correlate insulin resistance in non-diabetic subjects. Endocr J, 47: 535–542, 2000
(16) Kim SH, Abbasi F, and Reaven GM: Impact of degree of obesity on surrogate estimates of insulin resistance. Diabetes Care, 27: 1998–2002, 2004
(17) Maeda N, Sawayama Y, Tatsukawa M, Shimizu C, Kashiwagi S, and Hayashi J: Chlamydia pneumoniae seropositivity and early carotid atherosclerosis in a suburban Japanese population. Atherosclerosis, 164: 313–319, 2002
(18) Sawayama Y, Shimizu C, Maeda N, Tatsukawa M, Kinukawa N, Koyanagi S, Kashiwagi S, and Hayashi J: Effects of probucol and pravastatin on common carotid atherosclerosis in patients with asymptomatic hypercholesterolemia. Fukuoka Atherosclerosis Trial (FAST). J Am Coll Cardiol, 39: 610–616, 2002
(19) Maeda N, Sawayama Y, Tatsukawa M, Okada K, Furusyo N, Shigematsu M, Kashiwagi S, and Hayashi J: Carotid artery lesions and atherosclerotic risk factors in Japanese hemodialysis patients. Atherosclerosis, 169: 183–192, 2003
(20) Okada K, Maeda N, Tatsukawa M, Shimizu C, Sawayama Y, and Hayashi J: The influence of lifestyle modification on carotid artery intima-media thickness in a suburban Japanese population. Atherosclerosis, 173: 329–337, 2004
(21) Sever PS, Dahlof B, Poulter NR, Wedel H, Beevers G, Caulfield M, Collins R, Kjeldsen SE, Kristinsson A, McInnes GT, Mehlsen J, Nieminen M, O'Brien E, Ostergren J; ASCOT investigators: Prevention of coronary and stroke events with atorvastatin in hy-

pertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial—Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial.Lancet, 361: 1149–1158, 2003
(22) Rosenson RS, Tangney CC, and Casey LC: Inhibition of proinflammatory cytokine production by pravastatin. Lancet, 353: 983–984, 1999
(23) Yudkin JS, Stehouwer CDA, Emeis JJ, and Coppack SW: C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? Arterioscler Thromb Vasc Biol,19: 972–978, 1999
(24) Ridker PM, Hennekens CH, Buring JE, and Rifai N: C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med, 342: 836–843, 2000
(25) Ridker PM, Rifai N, Pfeffer MA, Sacks FM, Moye LA, Goldman S, Flaker GC, and Braunwald E: Inflammation, pravastatin, and the risk of coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events (CARE) Investigators.Circulation, 98: 839–844, 1998
(26) Yada T, Nakata M, Shiraishi T, and Kakei M: Inhibition by simvastatin, but not pravastatin, of glucose-induced cytosolic Ca^{2+} signalling and insulin secretion due to blockade of L-type Ca^{2+} channels in rat islet beta-cells.Br J Pharmacol, 126: 1205–1213, 1999
(27) Kanda M, Satoh K, and Ichihara K: Effects of atorvastatin and pravastatin on glucose tolerance in diabetic rats mildly induced by streptozotocin. Biol Pharm Bull, 26: 1681–1684, 2003

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 67 (2005) 46–54
Antiviral Research
www.elsevier.com/locate/antiviral

Helper T cell cytokine response to ribavirin priming before combined treatment with interferon alpha and ribavirin for patients with chronic hepatitis C

Norihiro Furusyo[a,b,*], Norihiko Kubo[b], Kazuhiro Toyoda[b], Hiroaki Takeoka[b], Shigeki Nabeshima[a], Masayuki Murata[a], Makoto Nakamuta[c], Jun Hayashi[a,b]

[a] *Department of General Medicine, Kyushu University Hospital, Higashi-Ku, Fukuoka 812-8582, Japan*
[b] *Department of Environmental Medicine and Infectious Diseases, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan*
[c] *Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

Received 28 May 2004; accepted 6 April 2005

Abstract

The viral genotype and serum viral level influence the response to interferon (IFN) treatment in patients with chronic hepatitis C virus (HCV) viremia. The aim of this study was to investigate a possible relationship between early virological response and helper T (Th) cell cytokine expansion by 4 weeks of ribavirin (RIB) alone followed by IFN and RIB combined in patients with genotype 1b and a high HCV RNA level, patients reported not to respond well to IFN treatment. Eighty-one patients with genotype 1b and a high HCV RNA level, over 100 international unit per milliliter (KIU/mL) (by Amplicor HCV Monitor), were assigned to two groups: Group A ($N=40$) with a 4-week RIB administration followed by a 24-week combination treatment, and Group B ($N=41$) with a 24-week combination treatment only. Blood was obtained from each patient on the following schedule: at Baseline (4 weeks before day 0), on day 0 (initiation day of the RIB and IFN combination treatment), weeks 4 (4 weeks after the start of the combination treatment), and at the end of the combination treatment. Flow cytometry was used to investigate sequential changes of IFN-γ producing (Th1) and interleukin-4 producing (Th2) cells from whole blood samples after stimulation with PMA and ionomycin. Serum HCV RNA clearances were 32.5% at week 4, 43.2% at week 8, 85.7% at the end of the combination treatment, and 22.9% within the 24-week follow-up in Group A; and 17.1%, 27.0%, 66.7% and 19.4% in Group B, respectively. The mean Th1/Th2 ratio significantly increased from 15.9 at baseline to 17.6 at day 0 with a decrease of Th2 cells, and then significantly decreased from 17.6 at day 0 to 15.5 at week 4 in Group A, while there was no significant change in Group B between baseline and day 0. In Group A, 13 patients with HCV RNA clearance within 4 weeks had a significantly increased Th1/Th2 ratio, from 14.0 at baseline to 22.1 at day 0, and then a significantly decreased ratio, from 22.1 at day 0 to 15.0 at week 4, while the others had no significant change in the ratio. RIB administration preceding combined treatment of RIB with IFN was more effective in Th2 cell expansion than the usual combined treatment of IFN with RIB and led to a relatively early virological clearance in chronic hepatitis C patients with genotype 1b and a high HCV RNA level.

Keywords: Interferon-α (IFN-α); Ribavirin (RIB); Hepatitis C virus (HCV); Interferon-γ (IFN-γ); Interleukin-4 (Il-4)

1. Introduction

Chronic hepatitis C virus (HCV) infection is alarmingly prevalent, 2–15%, throughout the world. Of Japanese patients with HCV, 30% have a consistently abnormal alanine aminotransferase (ALT) level and develop hepatocellular carcinoma (HCC) (Hayashi et al., 1997, 2000), with the incidence of HCC rising in recent years (Taylor-Robinson et al., 1997; El-Serag and Mason, 1999). Sustained virological response (SVR) rate is 20–30% in chronic hepatitis C patients following interferon (IFN) monotherapy, but only 5% in patients with both HCV genotype 1b and a high HCV RNA level (Di Bisceglie et al., 1989; Furusyo et al., 1997, 2002; Hayashi

* Corresponding author. Tel.: +81 92 642 5909; fax: +81 92 642 5916.
E-mail address: furusyo@genmedpr.med.kyushu-u.ac.jp (N. Furusyo).

0166-3542/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2005.04.001

et al., 1998). Unfortunately, HCV genotype 1b is the most common genotype (80%) in Japan. Therefore, there are many chronic hepatitis C patients who do not respond well to IFN monotherapy in Japan.

Ribavirin (RIB) (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a synthetic guanosine analogue that possesses a broad spectrum of action against DNA and RNA viruses (Sidwell et al., 1972), including the flaviviruses (Patterson and Fernandez-Larsson, 1990). It is the only available drug that has demonstrated in many studies some beneficial effect in the treatment of chronic hepatitis C (Reichard et al., 1991; Di Bisceglie et al., 1992). However, RIB monotherapy had only a transient effect, with no significant impact on HCV replication, but reduced the ALT level in 60% of the patients and had an effect on necroinflammatory activity (Di Bisceglie et al., 1992, 1995). These findings suggest that RIB administration stimulates the activity of cytotoxic T-cells against infected hepatocytes, not directly against HCV replication. Moreover, the combination treatment with IFN and RIB leads to higher effectiveness in terms of virological response (Khakoo et al., 1998), which would enhance the activity of the immune system against HCV. Therefore, we hypothesized that RIB priming before the combination treatment would be effective for pre-setting the immune system.

An SVR rate of 40% for chronic hepatitis C patients upon combination therapy of IFN-α with RIB has been reported (Kakumu et al., 1993; Schvarcz et al., 1995), but the exact mechanism responsible for the success is unclear. IFN-α has a direct antiviral effect and induces a number of immunomodulatory activities that can enhance antiviral immune response (Peters, 1996; Hayashi et al., 1995; Furusyo et al., 1999). HCV-specific T helper (Th)-cell response has been shown to be important in the resolution of acute HCV infection, with strong T-cell reactivity found in patients who clear HCV spontaneously and a weak response in those with progressive chronic infection (Diepolder et al., 1995; Missale et al., 1996). Moreover, several studies have reported such T-cell reactivity in association with IFN treatment (Missale et al., 1997; Kawakami et al., 2000; Murata et al., 2002). These findings raise the possibility that enhancement of T-cell reactivity may be a mechanism involved in the successful antiviral effect seen with the combination IFN-α and RIB therapies.

We here report the findings of a controlled pilot study designed to assess the relationship between the virological efficacy of and immunological response to IFN-α and RIB combined after 4 weeks of RIB pre-treatment of Japanese chronic hepatitis C patients.

2. Patients and methods

2.1. Patients

This controlled pilot study was designed to assess the relationship between the virological efficacy of and immunological response to IFN-α and RIB combined after 4 weeks of RIB pre-treatment of Japanese patients with chronic HCV viremia. All 81 patients with chronic hepatitis C (49 men and 32 women, mean age 56.7 years, age range 27–66 years) were randomly allocated by sealed envelope to the following treatment groups: Group A, RIB pre-treatment followed by a combination therapy of IFN-α and RIB, and Group B, combination therapy of IFN-α and RIB without RIB pre-treatment. Data on patient characteristics at entry are given in Table 1. All patients were positive for antibody to HCV and HCV RNA. No patient positive for hepatitis B virus surface antigen or antibody to human immunodeficiency virus or having other possible causes of hepatocellular injury such as autoimmunity or drug-induced liver disease were included. No patient had received antiviral or corticosteriod therapy within the 12 months prior to inclusion. All patients were infected with HCV of genotype 1b and had a high HCV RNA level, over 100 KIU/mL, determined by Amplicor HCV Monitor. The mean ALT level at entry was 88.8 IU/L (range, 41–333 IU/L). Needle biopsy of the liver was done for each patient within 2 months of the start of therapy, and two pathologists examined the biopsy specimens independently without prior knowledge of the patients. All patients were diagnosed with chronic active hepatitis with piecemeal necrosis or fibrosis formation of portal–portal bridging. Cirrhosis and non-cirrhosis were histologically diagnosed by biopsy. No significant differences were observed between Group A and B patients at entry. Serum ALT and HCV RNA level changes were measured during the observation. All patients gave their informed consent, and the study was approved by the ethics committee of Kyushu University Hospital. All the patients were followed for 6 months after cessation of IFN treatment.

Table 1
Patient characteristics at entry

Characteristics	Group A ($N=40$)	Group B ($N=41$)
Male:female (N)	24:16	25:16
Age (years)	57.5 ± 8.5	55.9 ± 12.6
Weight > 60 kg N (%)	21 (48.8)	22 (52.4)
Previous IFN N (%)	22 (51.2)	16 (38.1)
HCV RNA level (kilocopies/mL)	733.7 ± 228.1	719.0 ± 241.0
ALT (IU/L)	89.2 ± 39.2	89.1 ± 40.1
Hb (g/dL)	14.4 ± 1.2	13.9 ± 1.4
Non-cirrhosis:cirrhosis N (%)	40:0	40:1

IFN, interferon; HCV, hepatitis C virus; ALT, alanine aminotransferase; Hb, hemoglobin. No significant differences in each characteristic were observed between Groups A and B patients. All patients were chronically infected with HCV genotype 1b.

2.2. Treatment protocol

Both groups of patients were given IFN-α 2b (Intron-A, Schering-Plough, Kenilworth, NJ) intramuscularly at a dose of 6 million units (MU) daily for 2 weeks, then 6 MU thrice weekly for 22 weeks (total dose 480 MU). Patients received RIB (Rebetol, Schering-Plough) 600–800 mg orally twice daily according to weight: 600 mg for patients less than 60 kg and 800 mg for those 60 kg or over. Fig. 1 shows the intervention and study timeline of the IFN plus RIB com-

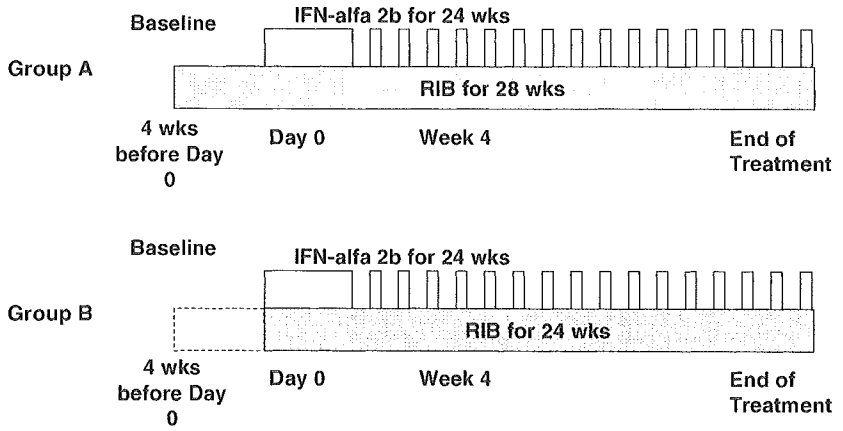

Fig. 1. Intervention protocol and study timeline of the IFN plus RIB combination treatment. IFN, interferon-α; RIB, ribavirin. Blood samples were taken at baseline (4 weeks before day 0), on day 0 (initiation day of RIB and IFN combination treatment), at week 4 (4 weeks after the start of the combination treatment), and at the end of the combination treatment.

bination treatment. The Group A patients were treated with RIB for 4 weeks, after which they received the combination therapy of IFN plus RIB for 24 weeks. The Group B patients received the combination therapy of IFN plus RIB for 24 weeks. Whole blood was obtained from each patient, to investigate sequential changes of IFN-γ producing (Th1) and interleukin-4 producing (Th2) cells on the following schedule: at baseline (4 weeks before day 0), on day 0 (IFN plus RIB combination therapy, initiation day), week 4 (4 weeks after the start of combination therapy), and at the end of the combination therapy.

The above duration and dose of IFN and RIB were approved by the Japanese Minister of Health, Labour and Welfare. The 48 weeks of IFN and RIB and the RIB dosage of 1000–1200 mg recommended by the international guideline were not permitted under the rules of the Japanese national health insurance system during the period of this study.

2.3. Definition of virological response during treatment

In this study, an early HCV RNA clearance response (ER) was defined as a undetectable HCV RNA test at 4 weeks after the initiation of the combination therapy of IFN plus RIB and Non-ER as a positive HCV RNA test at this time point. Sustained virological response (SVR) was defined as undetectable HCV RNA and a normal ALT level (under 36 IU/L) at 6 months after the cessation of treatment.

2.4. Serum assay methods

Serum samples were also drawn at monthly intervals while on treatment and at 4, 12, and 24 weeks after completion of treatment, stored at −20 °C, and frozen and thawed only once before doing the HCV RNA analysis.

2.5. HCV RNA determination by PCR

RNA was extracted from 50 μL of serum by Sep Gene RV (Sanko Junyaku, Tokyo, Japan). Complementary DNA was synthesized by use of random primers and reverse transcriptase (Super Script II; Life Technologies, Gaithersburg, MD). HCV RNA was detected by 2-stage PCR with primers from the 5′NC of the HCV genome (Choo et al., 1989): 5′-CTGTGAGGAACTACTGTCTT-3′ (sense) and 5′-AACACTACTCGGCTAGCAGT-3′ (antisense) in the first stage and 5′-TTCACGCAGAAAGCGTCTGT-3′ (sense) and 5′-GTTGATCCAAGAAAGGACCC-3′ (antisense) in the second stage.

2.6. HCV RNA genotyping

The HCV RNA genotype of each patient with HCV viremia was determined by 2-stage PCR using universal and type-specific primers from the putative core gene of the HCV genome by a modification of the method of Okamoto et al. (1992) and our previous report (Hayashi et al., 2000). The genotype nomenclature was based on the system proposed by Simmonds et al. (1994).

2.7. Quantity of serum HCV RNA

Serum HCV RNA levels were determined by the second-generation Cobas Amplicor HCV Monitor assay (COBAS v2.0, Roche Diagnostics Systems, Meylan, France) (Amplicor monitor). The range of the linear relationship provided was 0.5×10^3 KIU/mL to 850 KIU/mL for Amplicor monitor (Doglio et al., 1999). Samples over 850 KIU/mL by Amplicor monitor were re-measured after 10 and 100 times dilution to determine the accurate HCV RNA level.

2.8. IFN-γ and interleukin-4 producing peripheral CD4+ T cells by flow cytometric analysis

Identification of the capacity for cytokine production by CD4+ T cells for all the studied patients was done by three-color flow cytometry. The following analysis, as previously described elsewhere (Kawakami et al., 2000; Murata et al.,

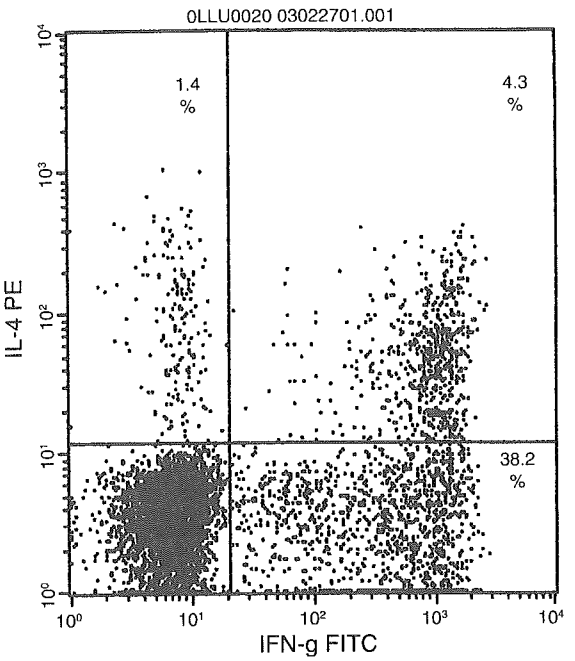

Fig. 2. A representative three-color flow cytometric pattern of the peripheral CD4+ T cells of one patient. Intracellular cytokines in CD4+ T cells after stimulation with mitogen were stained with anti-IFN-γ and IL-4 mAbs. The percentage of cytokine-positive cells to CD4+ T cells is shown in each quadrant.

2002), was immediately done after sampling whole blood from the patients. Based on these primary experiments, the system using IFN-γ and interleukin (IL)-4 (IL-4) proved to be the most stable and representative markers of Th1 and Th2 cell cytokines, respectively. Aliquots of diluted whole blood (1 mL) were cultured in a 24-well culture plate (Becton Dickinson, NJ) with 50 ng/mL PMA and 1 mg/mL of ionomycin in the presence of a protein-secretion inhibitor, GolgiPlug™, containing brefeldin A, and then incubated at 37 °C in 5% CO_2 for 4 h. Fixation, permeabilization, and intracellular cytokine staining of cultured cells were done with CytoStain™ Kits according to the Manufacturer's instructions (whole blood method). Cells were stained with both anti-IFN-γ and anti-IL-4 monoclonal antibodies (mAbs), and then incubated at 4 °C for 60 min. Stained cells were analyzed by flow cytometry, CYTORON ABSOLUTE with ImmunoCount 2 software (Ortho Diagnostic Systems, Raritan, NJ). Live-gating of lymphocytes was done, and up to 30,000 events were acquired for each analysis.

Fig. 2 shows a representative three-color flow cytometric pattern of the peripheral CD4+ T cells of one of the patients studied. Intracellular cytokines in CD4+ T cells after stimulation with mitogen were stained with anti-IFN-γ and IL-4 mAbs. The percentage of cytokine-positive cells to CD4+ T cells is shown in each quadrant. The percentages of IFN-γ and IL-4 producing cells in CD4+ lymphocytes were 38.2% and 1.4%, respectively. We used this method to investigate sequential changes of IFN-γ producing (Th1) and IL-4 producing (Th2) cells from whole blood samples of each patient.

2.9. Statistical analysis

Continuous data were expressed as mean values ± standard deviation (S.D.) of the mean. Statistical differences in the continuous data were determined by paired *t*-test, unpaired *t*-test, or Kruskal–Wallis test, and categorical data were compared by chi-square test and Fisher's exact test. Statistical analysis of the IFN-γ and IL-4 producing cell percentages and the ratio classified by response to treatment was done by analysis of variance with repeated measures. For each level of IFN-γ and IL-4 producing cell, percentage and ratio, a model that involves the fixed effects of the response-group to treatment (early HCV viremia clearance response or no response), time and a random effect of patients was assumed as follows:

$$Y_{ijk} = M + A_i + B_j + G_{ik} + E_{ijk}$$

where Y_{ijk} is the jth value of each level of IFN-γ and IL-4 producing cell percentages and the ratio for the kth patient in the ith response-group; M the mean of all observations; A_i the fixed effect of the response-group; B_j the fixed effect of time; G_{ik} a random effect of the kth patient in the ith response-group; E_{ijk} a random effect corresponding to error.

Stepwise logistic regression analysis was done using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA) for the IBM (Yorktown Heights, NY) 3090 computer system. The BMPD program LR was used to evaluate the complicated relationship between the clinical features and the rates of ER and SVR to IFN among patients.

A P-value less than 0.05 was regarded as being statistically significant.

3. Results

3.1. HCV RNA level change during the 4-week RIB administration

In Group A, the mean serum HCV RNA level significantly decreased, from 733.7 ± 228.1 KIU/mL at baseline to 510.3 ± 224.0 KIU/mL at day 0, despite no patient having cleared HCV RNA, but the mean ALT did not significantly change from baseline (89.2 ± 39.2 IU/L) to day 0 (82.0 ± 47.7 IU/L) The mean Hb level did not significantly change in Group A (14.4 ± 1.2 g/dL to 13.2 ± 1.6 g/dL). In Group B, no significant difference in HCV RNA, ALT, or Hb level was observed during the period, and no patient had HCV RNA clearance (HCV RNA, 719.0 ± 241.0 KIU/mL to 705.2 ± 259.5 KIU/mL: ALT, 89.1 ± 40.1 IU/L to 95.8 ± 46.2 IU/L: Hb, 13.9 ± 1.4 g/dL to 13.6 ± 1.6 g/dL).

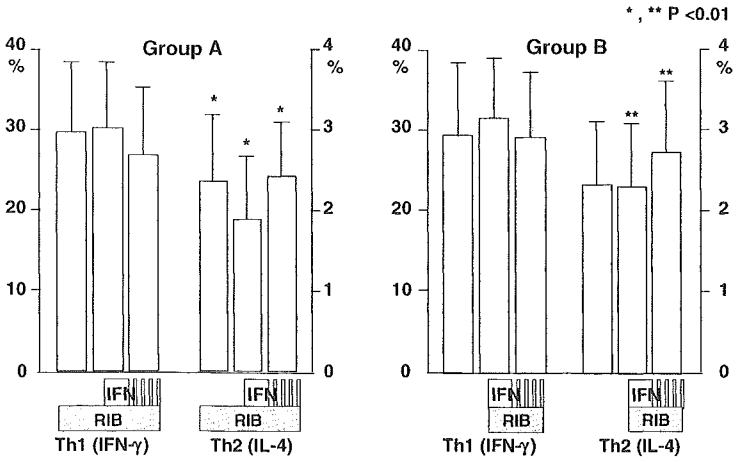

Fig. 3. Changes in the percentages of IFN-γ (Th1) and IL-4 (Th2) producing CD4+ T cells of 40 Group A and 41 B patients during the 4-week RIB administration and the combination treatment with IFN-α (at baseline, day 0, and week 4). IFN, interferon; Th, helper T; IL, interleukin; RIB, ribavirin.

3.2. IFN-γ and IL-4 producing peripheral CD4+ T cell percentage changes

Fig. 3 shows the changes in the percentages of IFN-γ (Th1) and IL-4 (Th2) producing CD4+ T cells of Group A and B patients during the 4-week RIB alone administration and the combination therapy of IFN plus RIB. In Group A patients during the 4-week RIB administration, the mean percentage of Th1 cells did not significantly change from baseline, but the mean percentage of Th2 cells significantly decreased, from 2.3% at baseline to 1.9% at day 0. In Group B patients, neither Th1 nor Th2 cells changed during the study period. On the other hand, in both Group A and B patients the mean percentage of Th1 cells did not significantly change between day 0 and week 4 of the combination therapy, but the mean percentage of Th2 cells significantly increased in Group A and B patients, from 1.9% and 2.4% at day 0 to 2.3% and 2.7% at week 4, respectively.

3.3. Th1/Th2 ratio change

Fig. 4 shows the changes in the ratio of IFN-γ (Th1) to IL-4 (Th2) producing CD4+ T cells of Group A and B patients at baseline, day 0 (after 4 weeks of RIB alone administration) and at week 4 of the combination therapy of IFN plus RIB. In Group A patients, the mean Th1/Th2 ratio significantly increased, from 15.4 at baseline to 18.6 at day 0, and significantly decreased between day 0 till week 4, from 18.6 to 14.6. In Group B patients, the Th1/Th2 ratio did not change during the study period (14.9 at baseline, 15.0 at day 0, and 13.3 at week 4).

Fig. 5 shows the relationship between early HCV RNA clearance and changes in the ratio of IFN-γ (Th1) to IL-4 (Th2) producing CD4+ T cells at baseline, day 0, and week 4. Interestingly, 13 of the Group A patients with an ER had a significantly increased Th1/Th2 ratio, from 14.0 at baseline to 22.1 at day 0, and then a significantly decreased ratio, from

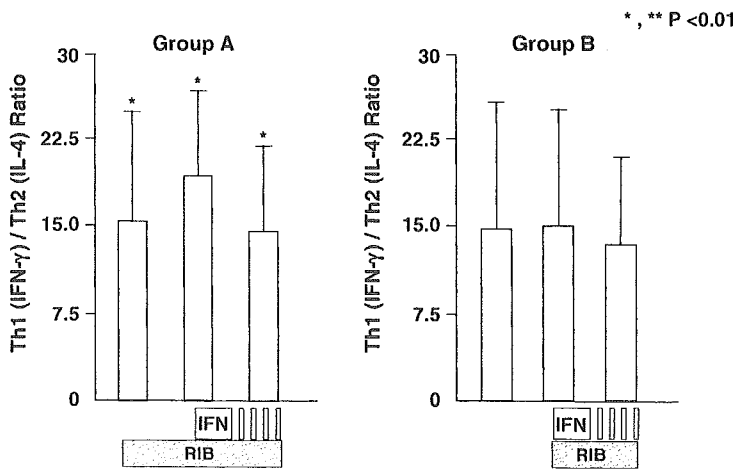

Fig. 4. Changes in the ratio of IFN-γ (Th1) to IL-4 (Th2) producing CD4+ T cells of 40 Group A and 41 B patients during the 4-week RIB administration and the combination treatment with IFN-α (at baseline, day 0, and week 4). IFN, interferon; Th, helper T; IL, interleukin; RIB, ribavirin.

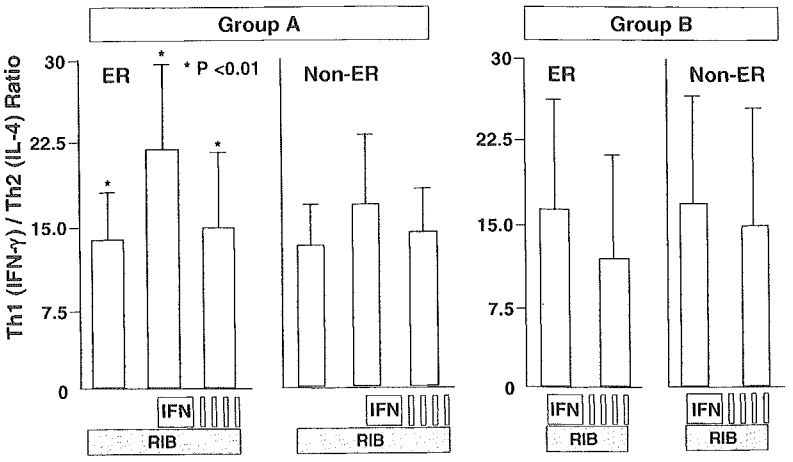

Fig. 5. Relationship between early HCV viremia clearance and changes in the ratio of IFN-γ (Th1) to IL-4 (Th2) producing CD4+ T cells during the 4-week RIB administration and the combination treatment with IFN-α (at baseline, day 0, and week 4). IFN, interferon; Th, helper T; IL, interleukin; RIB, ribavirin.

22.1 at day 0 to 15.0 at week 4. The other patients, without ER, had no significant change in the Th1/Th2 ratio during the observation period.

In order to determine the factors contributing to the virological response, a stepwise logistic regression analysis was done. However, no significant predictive factors of SVR or EVR were found.

3.4. Clinical course

No group A patient discontinued treatment during the 4-week RIB alone administration. Within 4 weeks of the start of the combination therapy, no Group A or B patients discontinued treatment, however, several patients discontinued after 4-weeks of combination therapy: 3 from Group A and 4 from Group B within 8 weeks of the start of the combination therapy, then 2 more Group A patients and one more in Group B patient between week 8 and the end of the treatment. In total, 12.5% of the Group A patients (5 of 40) and 12.2% of the Group B patients (5 of 41) discontinued treatment. No significant difference in the rate of discontinuation was found between Groups A and B. The reasons for discontinuation were mainly general fatigue and hematological disorders.

Fig. 6 shows the HCV RNA clearance rates of Group A and B patients during the combination therapy of IFN plus RIB and the 24 weeks of follow-up. Virological response (negative HCV RNA by PCR) was relatively high in Group A during the combination therapy and the 24 weeks of follow-up, although no statistical difference in virological response between Groups A and B was found.

Fig. 7 shows the changes in the mean ALT level of Group A and B patients after the start of therapy. The mean ALT level of Group A was lower than Group B, even though there was no significant difference in virological response between the two groups.

With regard to hematological disorders during treatment, no significant difference in hemoglobin (g/dL) change was

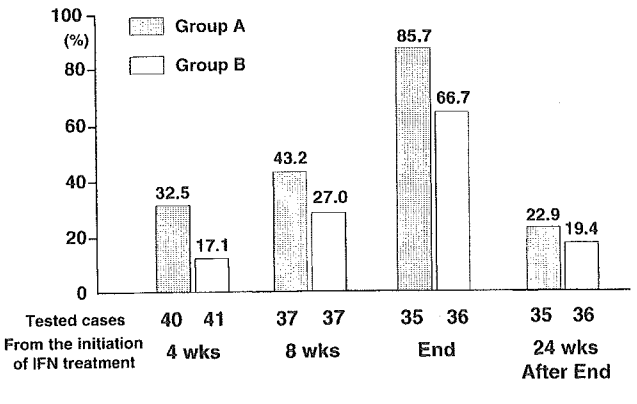

Fig. 6. Clearance rate of HCV viremia in Group A and B patients during the combination RIB and IFN treatment and at 24 weeks after the end of treatment. HCV, hepatitis C virus; RIB, ribavirin; IFN, interferon. Of the patients in each group, five did not complete treatment.

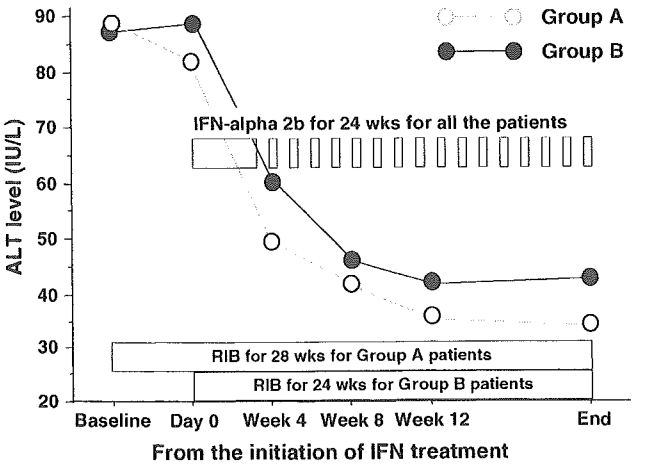

Fig. 7. Changes in the mean ALT level of Group A and B patients during the treatment. ALT, alanine aminotransferase.

found between Groups A and B. Group A patients were 14.4 ± 1.2 at baseline, 13.2 ± 1.6 on day 0, 12.3 ± 2.0 at week 4, 11.5 ± 2.2 at week 8, 11.9 ± 2.0 at week 12, and 12.0 ± 2.0 at the end. Group B patients were 13.9 ± 1.4 at baseline, 14.1 ± 1.4 on day 0, 12.8 ± 2.0 at week 4, 12.1 ± 2.3 at week 8, 12.4 ± 2.0 at week 12, and 12.6 ± 2.1 at the end. No significant difference in the changes of leukocyte or platelet counts was observed (data not shown).

4. Discussion

In the present study, a shift to a higher Th1 cytokine profile was found during the 4-week RIB alone administration, followed by a shift to a higher Th2 cytokine profile during the combination therapy of IFN plus RIB, which was significantly correlated with ER in these chronic hepatitis C patients. We previously reported that ER to IFN monotherapy was found in all patients with SVR, suggesting that monitoring serum HCV RNA during IFN administration is useful in evaluating the antiviral effect (Furusyo et al., 1999, 2002; Yamaji et al., 1998). Patients with both HCV genotype 1b and high HCV RNA levels do not respond well to IFN treatment. Therefore, the present study focused on ER among chronic hepatitis C patients with both genotype 1b and a high viral load.

The study would have been strengthened if some of the classic predictive factors of good viral response, for example, age, stage, and viral load, could have been explored using Th1 and Th2 monitoring. In order to find the factors contributing to virological response, stepwise logistic regression analysis was done. Because our patients had a high viral level and were all chronically infected with genotype 1b, which is known to be highly IFN-resistant, and also the groups were small, no significant predictive factors of SVR or EVR were found.

A pharmacokinetic study showed that the range of mean half-lives following multiple dosing of 600 mg RIB orally twice daily was 274–298 h, and that the steady state assessment from the trough concentrations in 3, 4, and 5 weeks of multiple dose administration indicated no significance between RIB alone treatment and a combination treatment with IFN (Khakoo et al., 1998). Therefore, a 4-week RIB pretreatment was chosen so that the plasma RIB concentration would be at steady state in the present study.

The two Th cell subsets, Th1 and Th2, are generally characterized by distinct and mutually exclusive patterns of cytokine production with different functions. Th1 cells produce IFN-γ and IL-2, as well as other cytokines, and promote cellular immune reaction, while Th2 cells produce IL-4, IL-6, IL-10, and other cytokines, and enhance humoral immune response. The Th1 and Th2 subsets have been proposed to play a pivotal role in the development of chronic viral infections and autoimmune diseases (Mosmann and Sad, 1996). With respect to chronic HCV infection, the data are still controversial because different methodologies have been used for evaluating the cytokine levels (Masaki et al., 2002). Recently, identification of Th subsets at the single cell level has become practical with the development of an intracellular cytokine assay using flow cytometry, as we already reported elsewhere (Kawakami et al., 2000; Murata et al., 2002). The stability of IFN-γ and the IL-4 cytokine system were shown in our primary studies. Therefore, this methodology was used in the present study to investigate the possible relationship between ER and expansion of Th cells by 4 weeks of RIB alone followed by a combination therapy of IFN plus RIB.

Immune response by a shift from the Th1 to the Th2 subset has been described in patients with chronic HCV infection during an IFN and RIB combination treatment (Lee et al., 2002). However, to our knowledge, no study has been done on the effect of a 4-week RIB administration followed by a combination therapy of IFN plus RIB. RIB in vitro was reported to promote or preserve Th1 cytokine-immune response, but to inhibit Th2 cytokines (Ning et al., 1998; Tam et al., 1999). We documented that a shift to a Th1 cytokine profile occurred during the 4-week RIB administration, followed by a shift to a Th2 cytokine profile during the IFN and RIB combination treatment, which was significantly correlated with early virological clearance. Other researchers, using the same methodology as ours, reported that a lower Th1/Th2 ratio before IFN monotherapy was a significant factor for long-term virological response in Japanese patients with chronic hepatitis C (Masaki et al., 2002). Moreover, an increase in Th1 profile and a decrease in Th2 after IFN-α therapy were observed in SVR patients, whereas the opposite result was obtained in non-SVR patients (Piazzolla et al., 2001). These findings may reflect our data that Th1/Th2 changes occurred and were related to viral clearance after preceding RIB pre-treatment followed by the combined treatment.

Crotty and colleagues proposed that RIB may act as an RNA mutagen, an effect that mutates the virus and reduces its infectivity, thus inducing the production of defective HCV particles (Crotty et al., 2000). Indeed, the mean HCV RNA level significantly decreased in our patients after a 4-week RIB alone administration, although no significant impact on HCV replication was reported (Di Bisceglie et al., 1992, 1995). This discrepancy can be explained by differences in the study population. Our patients had both high a HCV RNA level and genotype 1b.

The efficacy of a pegylated formulation of IFN-α (Peg-IFN) plus RIB combination treatment was reported to be superior to IFN-α plus RIB combination treatment and Peg-IFN monotherapy (Manns et al., 2001). A 48-week combination treatment of Peg-IFN and RIB has replaced the 24-week IFN monotherapy. Although no statistically significant difference in virological clearance was found between the two treatment schedules, the 4-week RIB administration followed by a 24-week IFN-α and RIB combination treatment (Group A), showed a higher frequency of virological clearance during treatment than the standard 24-week combination treatment (Group B) which is the most common in Japan and which has been considered the most effective. Peg-IFN plus RIB com-

bination treatment has not yet been approved for clinical use in patients with chronic HCV viremia by the Japanese Ministry of Health, Labour and Welfare at the time of the present study. In December 2004, the Peg-IFN plus RIB combination treatment received the official approval. Moreover, RIB administration for over 6 months has also not yet been approved for use with these patients, although IFN treatment for over 6 months was allowed. So far, the most effective and available treatment is the 6-month IFN-α plus RIB combination. The 4-week RIB pre-treatment did not increase the rate of discontinuation or the incidence of hematological disorders. Modifying this RIB pre-treatment regimen to take advantage of the potent antiviral effect seems necessary to obtain the highest possible rate of sustained response to IFN treatment. We, therefore, feel that RIB priming combined with peg-IFN-α plus RIB treatment may be beneficial for obtaining the most potent antiviral effects to improve the sustained response rate.

In conclusion, RIB administration followed by a combined therapy with IFN resulted in greater Th2 cell expansion than the usual combined treatment of IFN with RIB and led to relatively early virological clearance in chronic hepatitis C patients with genotype 1b and a high HCV RNA level.

Acknowledgements

This study was supported by a grant from the 21st century COE program of the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan. We greatly thank Hironori Ebihara for valuable advice.

References

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244, 359–362.

Crotty, S., Maag, D., Arnold, J.J., Zhong, W., Lau, J.Y., Hong, Z., Andino, R., Cameron, C.E., 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat. Med. 6, 1375–1379.

Di Bisceglie, A.M., Martin, P., Kassianides, C., Lisker-Melman, M., Murray, L., Waggoner, J., Goodman, Z., Banks, S.M., Hoofnagle, J.H., 1989. Recombinant interferon alfa therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. N. Engl. J. Med. 321, 1506–1510.

Di Bisceglie, A.M., Shindo, M., Fong, T.L., Fried, M.W., Swain, M.G., Bergasa, N.V., Axiotis, C.A., Waggoner, J.G., Park, Y., Hoofnagle, J.H., 1992. A pilot study of ribavirin therapy for chronic hepatitis C. Hepatology 16, 649–654.

Di Bisceglie, A.M., Conjeevaram, H.S., Fried, M.W., Sallie, R., Park, Y., Yurdaydin, C., Swain, M., Kleiner, D.E., Mahaney, K., Hoofnagle, J.H., 1995. Ribavirin as therapy for chronic hepatitis C. A randomized, double-blind, placebo controlled trail. Ann. Intern. Med. 123, 897–903.

Diepolder, H.M., Zachoval, R., Hoffmann, R.M., Wierenga, E.A., Santantonio, T., Jung, M.C., Eichenlaub, D., Pape, G.R., 1995. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 346, 1006–1007.

Doglio, A., Laffont, C., Caroli-Bosc, F.X., Rochet, P., Lefebvre, J., 1999. Second generation of the automated Cobas Amplicor HCV assay improves sensitivity of hepatitis C virus RNA detection and yields results that are more clinically relevant. J. Clin. Microbiol. 37, 1567–1569.

El-Serag, H.B., Mason, A.C., 1999. Rising incidence of hepatocellular carcinoma in the United States. N. Engl. J. Med. 340, 745–750.

Furusyo, N., Hayashi, J., Ueno, K., Sawayama, Y., Kawakami, Y., Kishihara, Y., Kashiwagi, S., 1997. Human lymphoblastoid interferon treatment for patients with hepatitis C virus-related cirrhosis. Clin. Ther. 19, 1352–1367.

Furusyo, N., Hayashi, J., Ohmiya, M., Sawayama, Y., Kawakami, Y., Ariyama, I., Kinukawa, N., Kashiwagi, S., 1999. Differences between interferon-alpha and -beta treatment for patients with chronic hepatitis C virus infection. Dig. Dis. Sci. 44, 608–617.

Furusyo, N., Hayashi, J., Kashiwagi, K., Nakashima, H., Nabeshima, S., Sawayama, Y., Kinukawa, N., Kashiwagi, S., 2002. Hepatitis C virus (HCV) RNA level determined by second-generation branched-DNA probe assay as predictor of response to interferon treatment in patients with chronic HCV viremia. Dig. Dis. Sci. 47, 535–542.

Hayashi, J., Kishihara, Y., Yamaji, K., Yoshimura, E., Ohmiya, M., Tani, Y., Ikematsu, H., Kashiwagi, S., 1995. Serum levels of soluble interleukin-2 receptors and effects of interferon-alpha for patients with chronic hepatitis C virus. Dig. Dis. Sci. 40, 1837–1841.

Hayashi, J., Kishihara, Y., Yamaji, K., Furusyo, N., Yamamoto, T., Pae, Y., Etoh, Y., Ikematsu, H., Kashiwagi, S., 1997. Hepatitis C viral quasispecies and liver damage in patients with chronic hepatitis C virus infection. Hepatology 25, 697–701.

Hayashi, J., Kishihara, Y., Ueno, K., Yamaji, K., Kawakami, Y., Furusyo, N., Sawayama, Y., Kashiwagi, S., 1998. Age-related response to interferon alfa treatment in women vs. men with chronic hepatitis C virus infection. Arch. Intern. Med. 158, 177–181.

Hayashi, J., Furusyo, N., Ariyama, I., Sawayama, Y., Etoh, Y., Kashiwagi, S., 2000. A relationship between the evolution of hepatitis C virus variants, liver damage, and hepatocellular carcinoma in patients with hepatitis C viremia. J. Infect. Dis. 181, 1523–1527.

Kakumu, S., Yoshioka, K., Wakita, T., Ishikawa, T., Takayanagi, M., Higashi, Y., 1993. A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. Gastroenterology 105, 507–512.

Kawakami, Y., Nabeshima, S., Furusyo, N., Sawayama, Y., Hayashi, J., Kashiwagi, S., 2000. Increased frequency of interferon-gamma-producing peripheral blood CD4+ T cells in chronic hepatitis C virus infection. Am. J. Gastroenterol. 95, 227–232.

Khakoo, S., Glue, P., Grellier, L., Wells, B., Bell, A., Dash, C., Murray-Lyon, I., Lypnyj, D., Flannery, B., Walters, K., Dusheiko, G.M., 1998. Ribavirin and interferon alfa-2b in chronic hepatitis C: assessment of possible pharmacokinetic and pharmacodynamic interactions. Br. J. Clin. Pharmacol. 46, 563–570.

Lee, S., Macquillan, G.C., Keane, N.M., Flexman, J., Jeffrey, G.P., French, M.A., Brochier, J., Price, P., 2002. Immunological markers predicting outcome in patients with hepatitis C treated with interferon-alfa and ribavirin. Immunol. Cell. Biol. 80, 391–397.

Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M., Albrecht, J.K., 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358, 958–965.

Masaki, N., Fukushima, S., Hayashi, S., 2002. Lower Th-1/Th-2 ratio before interferon therapy may favor long-term virological responses in patients with chronic hepatitis C. Dig. Dis. Sci. 47, 2163–2169.

Missale, G., Bertoni, R., Lamonaca, V., Valli, A., Massari, M., Mori, C., Rumi, M.G., Houghton, M., Fiaccadori, F., Ferrari, C., 1996. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J. Clin. Invest. 98, 706–714.

Missale, G., Cariani, E., Lamonaca, V., Ravaggi, A., Rossini, A., Bertoni, R., Houghton, M., Matsuura, Y., Miyamura, T., Fiaccadori, F., Ferrari, C., 1997. Effects of interferon treatment on the antiviral T cell response in hepatitis C virus genotype 1b- and genotype 2c-infected patients. Hepatology 26, 792–797.

Mosmann, T.R., Sad, S., 1996. The expanding universe of T cell subsets: Th1, Th2 and more. Immunology. Today 17, 138–146.

Murata, M., Nabeshima, S., Maeda, N., Nakashima, H., Kashiwagi, S., Hayashi, J., 2002. Increased frequency of IFN-gamma-producing peripheral CD8+ T cells with memory-phenotype in patients with chronic hepatitis C. J. Med. Virol. 67, 162–170.

Ning, Q., Brown, D., Parodo, J., Cattral, M., Gorczynski, R., Cole, E., Fung, L., Ding, J.W., Liu, M.F., Rotstein, O., Phillips, M.J., Levy, G., 1998. Ribavirin inhibits viral-induced macrophage production of TNF, IL-1, the procoagulant fgl2 prothrombinase and preserves Th1 cytokine production but inhibits Th2 cytokine response. J. Immunol. 160, 3487–3493.

Okamoto, H., Sugiyama, Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Sato, K., Tsuda, F., Miyakawa, Y., Mayumi, Y., 1992. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J. Gen. Virol. 73, 673–679.

Patterson, J.L., Fernandez-Larsson, R., 1990. Molecular mechanisms of action of ribavirin. Rev. Infect. Dis. 12, 1139–1146.

Peters, M., 1996. Actions of cytokines on the immune response and viral interactions: an overview. Hepatology 23, 909–916.

Piazzolla, G., Tortorella, C., Fiore, G., Fanelli, M., Pisconti, A., Antonaci, S., 2001. Interleukin-12 p40/p70 ratio and in vivo responsiveness to IFN-alpha treatment in chronic hepatitis C. J. Interferon Cytokine. Res. 21, 453–461.

Reichard, O., Andersson, J., Schvarcz, R., Weiland, O., 1991. Ribavirin treatment for chronic hepatitis C. Lancet 337, 1058–1061.

Schvarcz, R., Yun, Z.B., Sonnerborg, A., Weiland, O., 1995. Combined treatment with interferon alpha-2b and ribavirin for chronic hepatitis C in patients with a previous non-response or non-sustained response to interferon alone. J. Med. Virol. 46, 43–47.

Sidwell, R.W., Huffman, J.H., Khare, G.P., Allen, L.B., Witkowski, J.T., Robins, R.K., 1972. Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science 177, 705–706.

Simmonds, P., Alberti, A., Alter, H.J., Bonino, F., Bradley, D.W., Brechot, C., Brouwer, J.T., Chan, S.W., Chayama, K., Chen, D.S., Choo, Q.L., Colombo, M., Cuypers HTM, Date, T., Dusheiko, G.M., Esteban, J.I., Fay, O., Hadzyannis, S.J., Han, J., Hatzakis, A., Holmes, E.C., Hotta, H., Houghton, M., Irwine, B., Kohara, M., Kolberg, J.A., Kuo, G., Lau JYN, Lelie, P.N., Maertens, G., McOmish, F., Snedecar, G.W., 1994. A proposed system for the nomenclature of hepatitis C virus genotypes. Hepatology 19, 1321–1324.

Tam, R.C., Pai, B., Bard, J., Lim, C., Averett, D.R., Phan, U.T., Milovanovic, T., 1999. Ribavirin polarizes human T cell responses toward a Type 1 cytokine profile. J. Hepatol. 30, 373–382.

Taylor-Robinson, S.D., Foster, G.R., Arora, S., Hargreaves, S., Thomas, H.C., 1997. Increase in primary liver cancer in the UK, 1979–1994. Lancet 350, 1142–1143.

Yamaji, K., Hayashi, J., Kawakami, Y., Furusyo, N., Sawayama, Y., Kishihara, Y., Etoh, Y., Kashiwagi, S., 1998. Hepatitis C viral RNA status at two weeks of therapy predicts the eventual response. J. Clin. Gastroenterol. 26, 193–199.

European Journal of Epidemiology (2005) 20: 251–261
DOI 10.1007/s10654-004-6516-5

HEPATITUS EPIDEMIOLOGY

Strenuous physical labor is important as a cause of elevated alanine aminotransferase levels in Japanese patients with chronic hepatitis C viremia

Norihiko Kubo[1], Norihiro Furusyo[1,2], Hisashi Nakashima[2], Kenichiro Kashiwagi[2] & Jun Hayashi[1,2]

[1]*Department of Environmental Medicine and Infectious Diseases, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan;* [2]*Department of General Medicine, Kyushu University Hospital, Fukuoka, Japan*

Accepted in revised form 22 November 2004

Abstract. To clarify the influence of lifestyle habits on the elevated alanine aminotransferase (ALT) levels deterioration of Japanese patients with chronic hepatitis C virus (HCV) viremia, we investigated the effects of smoking, drinking, and physical labor on the disease course of the residents living in a rural area of Kyushu, Japan. The data of patients with chronic HCV viremia and control subjects without HCV infection were analyzed retrospectively from 1986 to 1992 and prospectively from 1993 to 2000. In 2000, a questionnaire was given to 268 HCV-infected patients and 275 control subjects to survey for the lifestyle habits. The data of serial ALT level testing during the observation period was used as a measure of liver damage: 183 HCV patients (68.3%) and 10 control subjects (3.6%) had abnormal ALT levels greater than 35 IU/l for more than half of their observation period. The percentage of HCV patients with elevated ALT levels significantly increased with the daily consumption of alcohol ($p < 0.0001$), the length of time spent in strenuous physical labor per day ($p = 0.0056$), and the number of cigarettes smoked per day ($p = 0.0003$). A stepwise logistic regression analysis showed male sex ($p = 0.003$), platelet counts ($p < 0.001$), strenuous physical labor ($p = 0.002$), and drinking history ($p = 0.007$) to be significantly associated with the elevated ALT levels of HCV patients. When strenuous physical labor was done for over 2 h, the probability of elevated ALT levels was increased compared with patients engaging in strenuous physical labor under 2 h (estimated odds ratio = 1.82 [under 2 h], 20.60 [over 2 h]). Interestingly, strenuous physical labor was extracted before alcohol consumption as a significant factor in the elevated ALT levels. Among the control subjects, only the amount of alcohol consumed per day ($p = 0.0001$) was significantly associated with the elevated levels. These data suggests that strenuous physical labor over a long period of time might be related to elevated ALT levels in patients with chronic HCV viremia as well as drinking.

Key words: Alcohol consumption, Cigarette smoking, Hepatitis C virus, Lifestyle habits, Physical labor

Introduction

Chronic hepatitis C virus (HCV) infection has become the most frequent cause of chronic liver disease, since HCV discovery in 1989. Population based surveys in Japan have reported the prevalence of HCV to be 0.7–3.7% [1–3]. Chronic HCV viremia often follows a progressive course over many years and can ultimately result in cirrhosis and hepatocellular carcinoma (HCC) [4, 5]. During the past decade, more than 80% of Japanese patients with HCC had chronic HCV viremia [6, 7]. The risk of developing HCC has been reported to be higher in HCV-infected patients who have biochemically and histologically active chronic hepatitis, suggesting that necroinflammation and its associated regenerative processes play a pivotal role in hepatic carcinogenesis [8]. Interferon (IFN) has been shown to eliminate HCV viremia and to reduce serum alanine aminotransferase (ALT) [9–11]. However, IFN is not effective for all patients, and is sometimes not possible because of its high cost and side effects. Therefore, it is important to know the possibility whether lifestyle may be related to liver damage in patients with chronic HCV viremia, because physicians can give the patients accurate guidance.

Data from various epidemiological studies suggest that other risk factors such as male sex, HCV genotype 1 infection, and alcohol consumption contribute to HCV-related liver diseases and the development of HCC [12–14]. However, the relation between physical activity and ALT levels is as yet unclear [15–17]. If these lifestyle habits independently influence the elevated ALT levels of patients with chronic HCV viremia, it is important to understand the mechanisms of their influence in order to develop secondary

prevention regimens other than IFN treatment. Few serial or prospective, clinical or epidemiological studies of the interaction between lifestyle habits and elevated ALT levels among patients with chronic HCV viremia who have never received IFN treatment have been done. To evaluate this issue, we investigated the relationship between elevated ALT levels and the lifestyle habits of 268 patients with chronic HCV viremia and 275 control subjects without HCV infection, living in a rural area of Japan.

Methods

Study population

The area studied was Hoshino village in Fukuoka Prefecture, Japan, on the north part of Kyushu Island, one of the main islands of Japan. Hoshino village is surrounded by mountains and is relatively isolated from other communities. According to the census bureau, the population of the village was 4250 in 1993 and 3881 in 2000. The main sources of income are farming, forestry, and the tourist industry. The farming population of the village in 2000 was about 650 persons, about 100 persons were forestry workers, and the tourist industry employed about 400 persons. The farmers mainly cultivate rice, Japanese tea, and trees. They plant the saplings, scatter the disinfections, foster, and wholesale to the market. Most inhabitants are middle class, Buddhism is the predominant religion, and the lifestyle does not seem to differ from that in other parts of Japan.

The public office of Hoshino village provides free yearly health examinations, announced by distributing written notices to residents 30 years of age and older. Since we had started to test 2046 residents of the village who reported for a free examination in 1993, 334 (16.3%) of whom were confirmed to have chronic HCV viremia, as already reported in our previous report [18]. Because of excluding 3 moved out or 10 deceased residents, we were able to prospectively follow 321 (96.1%) of the 334 HCV-infected patients twice in every year, village's health examination and the 6 month-later liver examination of ours, from 1993 to 2000 [19]. The 10 excluded study participants, three died from HCC (30%), two from subarachnoid hemorrhage (20%), and one each from hepatic failure, lung cancer, gastric cancer, and myocardial infarction (10%, respectively). After excluding 10 hepatitis B surface antigen (HBs Ag) positive HCV patients and 46 HCV-infected patients who had had IFN treatment, a total of 268 HCV-infected patients remained for the present study. The 104 HCV patients (38.8%) all worked in farming or forestry. In the same manner as with the HCV patients, we were able to prospectively follow 1273 (74.4%) of the 1712 non-HCV subjects from 1993 to 2000. We selected 275 (21.6%) non-HCV subjects into the present study matched with age and sex of the HCV patients as the control subjects, after excluding 25 HBs Ag positive subjects.

The 268 HCV patients studied included 152 men (56.7%) and 116 women (43.3%) (age range, 30–84 years; mean ± SD age, 55.1 ± 9.9 years at entry). All patients antibody to HCV (anti-HCV) positive were tested for HCV RNA at least twice, at an interval of 6 months or longer, before being classified as having chronic HCV viremia. ALT and platelet counts were tested in all patients and control subjects twice a year between 1986 and 2000. The results of the biochemical liver tests were analyzed retrospectively from 1986 to 1992, and prospectively from 1993 to 2000. The mean (± SD) tracking period of the study patients was 11.2 ± 2.9 years.

The results of biochemical liver tests were considered abnormal if the ALT level was over 35 IU/l. Liver damage was defined as an abnormal ALT levels for more than half of the patient's observation period. Patients with ALT always normal throughout the observation period or who had ALT levels greater than 35 IU/l for less than half of their observation period were defined as having always normal or mildly abnormal liver function. We categorized the HCV patients into 2 groups; 183 patients with liver damage (114 men and 69 women) and 85 with always normal or mildly abnormal liver function (38 men and 47 women), according to the results of biochemical liver tests during the observation period. All of the 268 HCV patients had refused IFN treatment because of its high cost or concerns about the possible side effects.

The lifestyle habits of 275 residents without HCV infection, as control subjects, from Hoshino village were also evaluated (153 men and 122 women) (age range, 32–81 years; mean ± SD age, 58.4 ± 10.2 years at entry). In the same manner as the HCV patients, we categorized the control subjects into 2 groups; 10 subjects with liver damage (10 men) and 265 with always normal or mildly abnormal liver function (143 men and 122 women), according to the ALT level pattern during the observation period. We could know the causes of death among 12 residents of 43 excluded non-HCV residents from 1993 to 2000. The 12 excluded study participants without HCV, six died from malignant tumor (3: gastric cancer, 2: lung cancer, 1: colon cancer), three from brain stroke, two from myocardial infarction, and one from traffic accident. The causes of death among the other 31 non-HCV residents were unknown.

Lifestyle questionnaire

Public health nurses who well know the life of the inhabitants of Hoshino village gave a questionnaire-based interview about lifestyle habits to each patient in 2000. The questionnaire included height, weight, occupation, health condition, amount and type of

physical labor, alcohol consumption, and cigarette smoking. The amount of physical labor for the most recent 5 years was queried, the history of alcohol consumption and cigarette smoking were also queried. The average time of standing and walking per day was asked. Work primarily requiring muscular activity or walking for lengthy periods of time was defined as strenuous physical labor. All patients were classified as follows: no strenuous physical labor, strenuous physical labor under 2 h per day, and strenuous physical labor over 2 h per day. The number of grams of alcohol consumed per day was calculated using information from the United States Department of Agriculture Handbook 456 [20]. The following ethanol content figures were used for calculation: beer, 22.3 g/bottle; sake, 23.8 g/cup; shochu (a distilled Japanese liquor) 35.7 g/cup; and wine, 72.0 g/bottle. From these estimates, we computed the total grams of ethanol consumed in an average day. The amount of alcohol consumption per day was divided by the period of alcohol consumption to create an alcohol consumption index. All patients were classified as follows: non drinkers, light drinkers consuming less than 60 g of ethanol per day, and heavy drinkers consuming 60 g or more of ethanol per day. The patients were asked how many cigarettes they smoked each day and how many years they had smoked. All patients were classified as follows: non smokers, cigarette smoking under 20 pieces per day, and cigarette smoking over 20 pieces per day. The number of cigarettes smoked per day was divided by the period of cigarette smoking to create a cigarette smoking index (Brinkmann Index).

Assay methods

All serum samples collected from 1993 to 2000 were separated, stored at −20 °C until testing, and screened for HCV and hepatitis B virus markers. Anti-HCV was tested by a second-generation enzyme-linked immunosorbant assay (ELISA) (HCV EIA II, Abbott Laboratories, North Chicago, IL). Antibody to hepatitis B core antigen (anti-HBc) and HBs Ag were tested for by reserved passive hemagglutination assay (MyCell, Institute of Immunology, Tokyo, Japan).

HCV RNA by PCR

To identify currently infected anti-HCV-positive patients and residents, serum HCV RNA was detected as follows: RNA was extracted from 50 μl of serum by Sepa Gene RV (Sanko Junyaku, Tokyo, Japan), and complementary DNA was synthesized using random primers and reverse transcriptase (Super Script II; Gibco BRL, Gaithersburg, MD). The HCV RNA was detected by two-stage PCR using primers from the 5′-noncoding region of the HCV genome, as previously described [21].

HCV RNA genotype

The HCV RNA genotype was determined by two-stage PCR using universal and type-specific primers from the putative C gene of the HCV genome with modifications of the methods of Hayashi et al. [21] and Okamoto et al. [22]. The genotype nomenclature was based on the system proposed by Simmonds et al. [23].

HCV RNA level

The HCV RNA level was determined by a second-generation branched DNA probe assay (Quantiplex HCV RNA 2.0 assay; Chiron Corporation, Emeryville, CA). The range of linear relationship provided by the branched DNA probe assay was 0.2 to 120×10^6 genome equivalent (Meq)/ml, and the lower cutoff was 0.2 Meq/ml. This assay accurately quantifies the HCV RNA level for the major HCV genotypes [21].

Serum HA and IV-C measurement

Serum levels of hyaluronic acid (HA) and IV-collagen (IV-C) as hepatic fibrogenesis markers were measured in all HCV patients in 2000 with a commercial sandwich enzyme binding assay (Hyaluronic Acid Chugai, Chugai Diagnostics Science Co., Ltd., Osaka, Japan) and a commercial one-step sandwich radioimmunoassay (Pannassy IV-C, Daiichi Pure Chemical Co., Tokyo, Japan) [24]. The normal range of serum HA was 30 ± 16 (mean ± SD) ng/ml, the upper limit being 50 ng/ml. The normal range of serum IV-C was 99.3 ± 22.8 (mean ± SD) ng/ml, the upper limit being 140 ng/ml.

Diagnosis of HCC development

During the observation period, all patients had yearly abdominal ultrasonographic examination. At each visit, the alpha-fetoprotein (AFP) level was tested. A diagnosis of HCC was made on the basis of the appearance of focal lesions on ultrasonographic examination of the liver or an AFP level higher than 400 ng/ml. Confirmation was by dynamic computed tomography, hepatic angiography, or tumor biopsy in hospitals.

Statistical analysis

Continuous data were expressed as mean values ± SD of the mean. The unpaired t test and Mann–Whitney U test were used to compare the means of samples between two groups. The chi-square test or Fisher's exact test was used for comparison of categorical variables between two groups. The Cochran–Armitage's trend test was used to evaluate the correlation between the percentage of residents with

Table 1. Lifestyle differences by sex among 268 patients with chronic hepatitis C virus viremia in Hoshino village, Fukuoka, Japan

Characteristics	Men (n = 152)	Women (n = 116)	p Value
Mean age at entry (years)	54.8 ± 9.3	55.4 ± 10.5	NS
Amount of alcohol consumption per day (grams)	32.8 ± 2.8	10.2 ± 2.2	<0.0001
Period of alcohol consumption (years)	26.3 ± 1.7	9.1 ± 1.5	<0.0001
Alcohol Consumption Index	1293.4 ± 116.8	382.6 ± 84.4	<0.0001
Number of cigarettes smoked per day (pieces)	11.5 ± 0.8	3.4 ± 0.7	<0.0001
Period of cigarette smoking (years)	22.2 ± 1.6	8.2 ± 1.5	<0.0001
Brinkmann Index	374.8 ± 28.7	124.3 ± 25.4	<0.0001
Strenuous physical labor n (%)	52 (34.2)	22 (19.0)	0.0086

Continuous data were expressed as mean values ± SD of the means.
Alcohol Consumption Index is the amount of alcohol consumed per day multiplied by the period of alcohol consumption.
Brinkmann Index (Index of Cigarette Smoking) is the number of cigarettes smoked per day multiplied by the period of cigarette smoking.
Strenuous physical labor means work primarily requiring muscular activity or walking for lengthy periods of time.
NS, not significant.

liver damage and the daily consumption of alcohol. The Kruskal–Wallis test was used for independent samples. Stepwise logistic regression analysis was done using a commercially available software package (BMDP Statistical Software Inc., Los Angeles, CA) for the IBM (Yorktown Heights, NY) 3090 computer system. The BMPD program LR was used to evaluate the complicated relationship between liver damage and lifestyle habits. A p value less than 0.05 was considered to be statistically significant.

Results

Lifestyle habits of all the surveyed patients and control subjects

Of all 268 HCV patients, 131 (48.9%) were drinkers, 74 (27.6%) were patients doing physical labor, and 127 (47.4%) were smokers. Of 152 male HCV patients, 100 (65.8%) were drinkers, 52 (34.2%) were patients doing physical labor, and 100 (65.8%) were smokers. Of 116 female HCV patients, 31 (26.7%) were drinkers, 22 (19.0%) were patients doing physical labor, and 27 (23.3%) were smokers. These habits were found significantly more often in males than in females ($p < 0.0001, p = 0.0086, p < 0.0001$, respectively).

Of all 275 control subjects without HCV infection, 138 (50.2%) were drinkers, 133 (48.4%) were subjects doing physical labor, and 123 (44.7%) were smokers. Of 153 male subjects, 124 (81.0%) were drinkers, 55 (35.9%) were subjects doing physical labor, and 119 (77.8%) were smokers. Of 122 female control subjects, 14 (11.5%) were drinkers, 87 (71.3%) were subjects doing physical labor, and 4 (3.3%) were smokers. These habits were found significantly more often in males than in females (all $p < 0.0001$), as well as among HCV patients.

Table 1 shows differences in lifestyle among the HCV patients by sex. Age at entry showed no significant between group differences. Mean alcohol consumption per day, the mean period of alcohol consumption, and the mean alcohol consumption index were all significantly higher for men than for women ($p < 0.0001$, respectively). The mean number of cigarettes smoked per day ($p < 0.0001$), the mean period of cigarette smoking ($p < 0.0001$), mean Brinkmann index ($p < 0.0001$), and the percentage doing strenuous physical labor ($p < 0.01$) were all significantly higher for men than for women among the HCV patients.

Relationship between liver damage and the characteristics of patients and control subjects

Table 2 shows the clinical and lifestyle characteristics of all 268 HCV patients. The percentage of men with liver damage (62.3%) was significantly higher than that of those with always normal or mild abnormal liver function (44.7%) ($p = 0.0101$). The percentage engaging in alcohol consumption and the mean amount of alcohol consumed per day, period of alcohol consumption, and alcohol consumption index were significantly higher in HCV patients with liver damage than in those with always normal or mild abnormal liver function (all $p < 0.05$). The percentage engaging in cigarette smoking and the mean number of cigarettes smoked per day, period of cigarette smoking, and Brinkmann index were significantly higher in HCV patients with liver damage than in those with always normal or mild abnormal liver function (all $p < 0.001$). The percentage engaging in strenuous physical labor was significantly higher for HCV patients with liver damage than for those with always normal or mild abnormal liver function ($p < 0.05$). Age at entry, history of blood transfusion, body mass index (BMI), anti-HBc positivity,

Table 2. Clinical and lifestyle characteristics of 268 patients with chronic hepatitis C virus viremia in Hoshino village, Fukuoka, Japan

Characteristics	Liver damage (n = 183)	Always normal or mild abnormal liver function (n = 85)	p Value
Mean age at entry (years)	55.0 ± 9.6	55.2 ± 10.4	NS
Men n (%)	114 (62.3)	38 (44.7)	0.0101
History of blood transfusion n (%)	20 (11.6)	7 (10.6)	NS
Body Mass Index (kg/m^2)	21.7 ± 2.7	21.7 ± 2.6	NS
anti-HBc positive n (%)	80 (43.7)	37 (43.5)	NS
HCV-RNA level (Meq/ml)[a]	9.9 ± 12.9	11.1 ± 15.0	NS
HCV genotype 1b n (%)	162 (88.5)	74 (88.1)	NS
Alcohol consumption n (%)	106 (57.9)	25 (29.4)	<0.0001
Amount of alcohol consumption per day (grams)	25.1 ± 2.3	17.6 ± 3.6	0.0306
Period of alcohol consumption (years)	20.5 ± 1.5	14.4 ± 2.3	0.0243
Alcohol consumption Index	977.1 ± 94.6	690.9 ± 151.3	0.0268
Cigarette smoking n (%)	101 (55.2)	26 (30.6)	0.0003
Number of cigarettes smoked per day (pieces)	9.4 ± 0.7	5.0 ± 0.9	0.0002
Period of cigarette smoking (years)	18.7 ± 1.5	10.6 ± 2.0	0.0006
Brinkmann Index	307.9 ± 25.6	176.9 ± 35.2	0.0006
Strenuous physical labor n (%)	59 (32.2)	15 (17.6)	0.0193

Continuous data were expressed as mean values ± SD of the means.
Alcohol Consumption Index is the amount of alcohol consumed per day multiplied by the period of alcohol consumption.
Brinkmann Index (Index of Cigarette Smoking) is the number of cigarettes smoked per day multiplied by the period of cigarette smoking.
Liver damage means patients with ALT levels >35 IU/L for more than half of each patient's observation period.
Always normal or mild abnormal liver function means patients with always normal ALT levels throughout the observation period or who had ALT levels >35 IU/L for less than half of their observation period.
NS, not significant.
[a]HCV RNA level was determind by branched DNA probe assay.

HCV-RNA level, and HCV genotype showed no significant between group differences.

Among 275 control subjects, the percentage of men with liver damage (100%) was significantly higher than that of those with always normal or mild abnormal liver function (54.0%) ($p = 0.0107$). The percentage engaging in alcohol consumption (100% vs. 48.3%, $p = 0.0039$) and cigarette smoking (100% vs. 42.6%, $p = 0.0011$) were significantly higher in control subjects with liver damage than in those with always normal or mild abnormal liver function. The period of alcohol consumption (37.6 ± 4.0 years vs. 15.3 ± 1.2 years, $p = 0.0003$), mean number of cigarettes smoked per day (19.3 ± 4.4 pieces vs. 7.4 ± 0.6 pieces, $p = 0.0005$), period of cigarette smoking (30.6 ± 5.2 years vs. 13.0 ± 1.1 years, $p = 0.0005$), and Brinkmann index (588.0 ± 143.8 vs. 227.0 ± 20.7, $p = 0.0004$) were significantly higher in control subjects with liver damage than in those with always normal or mild abnormal liver function. Mean alcohol consumption index was significantly higher in control subjects with liver damage than in those with normal liver function (2269.5 ± 374.4 vs. 590.1 ± 859.6, $p < 0.0001$). The percentage engaging in strenuous physical labor was not significantly higher in control subjects with liver damage than in those with always normal or mild abnormal liver function (60.0% vs. 47.9%, $p = 0.6679$). Age at entry (56.1 ± 11.5 years vs. 59.5 ± 10.2 years) and BMI (23.0 ± 3.8 kg/m^2 vs. 21.9 ± 2.9 kg/m^2) showed no significant between group differences among the control subjects.

Evaluation of the relationship between liver function and the development of HCC

The relationships of serum HA and IV-C levels, as hepatic fibrogenesis markers, and platelet count to biochemical liver status were as follows among the HCV patients. The mean serum HA and IV-C levels of HCV patients with normal liver function ($n = 85$) were 107.4 ± 21.5 ng/ml and 149.5 ± 17.8 ng/ml, respectively, and those of HCV patients with liver damage ($n = 183$) were 174.6 ± 19.1 ng/ml and 184.6 ± 15.0 ng/ml, respectively, both significantly higher in HCV patients with liver damage than in those with normal liver function ($p < 0.0001$, respectively). The mean platelet count of the HCV patients with liver damage (16.8 ± 0.5 × 10^6/μl) was significantly lower than that of those with normal liver function (20.7 ± 1.0 × 10^6/μl) ($p = 0.0002$). In the development of HCC, 24 of 183 (13.1%) patients with liver damage were significantly more found than none of 85 patients without liver damage ($p = 0.0011$). In the decompensated liver cirrhosis, one of 183 (0.5%) patient with liver damage was

more found than none of 85 patients without liver damage, although no significant difference was found. No control subjects developed HCC during the observation period.

Relationship between lifestyle habits and liver damage, classified by sex

Table 3 shows the relationship between lifestyle habits and liver damage, classified by sex among the HCV patients. The percentages of all HCV patients with liver damage in the no alcohol consumption, light alcohol consumption, and heavy alcohol consumption groups were 56.2%, 76.9%, and 90.0%, respectively, significantly increasing with the daily consumption of alcohol ($p < 0.0001$). For men, the rates of liver damage were 63.5%, 77.9%, and 87.5%, respectively, significantly increasing with the daily consumption of alcohol ($p = 0.0105$). For women, the rates of liver damage were 51.8%, 73.9%, and 100.0%, respectively, significantly increasing with the daily consumption of alcohol ($p = 0.0020$). Regardless of sex, the more alcohol consumed, the more liver function deteriorated.

The percentages of HCV patients with liver damage in the no physical labor, strenuous physical labor under 2 h, and strenuous physical labor over 2 h groups were 63.9%, 73.0%, and 86.5%, respectively, significantly increasing with the time of strenuous physical labor per day ($p = 0.0056$). For women, the rates of liver damage were 53.2%, 83.3%, and 90.0%, respectively, significantly increasing with the time of strenuous physical labor per day ($p = 0.0054$). No significant differences were found for men.

The percentage of HCV patients with liver damage in the non smoking, smoking under 20 pieces, and smoking over 20 pieces groups were 58.2%, 77.8%, and 81.3%, respectively, significantly increasing with the number of cigarettes smoked per day ($p = 0.0003$). For men, the rates of liver damage were 65.4%, 78.7%, and 81.1%, respectively, with a non-significant increase with the number of cigarettes smoked per day. There were no significant differences by the number of cigarettes smoked. For women, the rates of liver damage were 53.9%, 75.0%, and 81.8%, respectively, significantly increasing with the number of cigarettes smoked per day ($p = 0.0289$).

Distribution of overlapping lifestyle habits

The distribution of HCV patients engaging in the three surveyed factors, alcohol consumption, cigarette smoking, and physical labor, was diagramed to show overlaps. Figures 1 and 2 show the number of patients with chronic HCV viremia, 152 male and 116 female respectively, reporting engaging in the three

Table 3. Alcohol consumption, strenuous physical labor, and cigarette smoking classified by sex and liver damage in 268 patients with chronic hepatitis C virus viremia in Hoshino village, Fukuoka, Japan

	Men		Women		All	
	Total *n*	Liver damage *n* (%)	Total *n*	Liver damage *n* (%)	Total *n*	Liver damage *n* (%)
Alcohol consumption/day						
Non drinkers	52	33 (63.5)[a]	85	44 (51.8)[b]	137	77 (56.2)[c]
Light drinkers	68	53 (77.9)[a]	23	17 (73.9)[b]	91	70 (76.9)[c]
Heavy drinkers	32	28 (87.5)[a]	8	8 (100.0)[b]	40	36 (90.0)[c]
Strenuous physical labor/day						
No	100	74 (74.0)	94	50 (53.2)[d]	194	124 (63.9)[e]
Under 2 h	25	17 (68.0)	12	10 (83.3)[d]	37	27 (73.0)[e]
Over 2 h	27	23 (85.2)	10	9 (90.0)[d]	37	32 (86.5)[e]
Cigarette smoking/day						
No	52	34 (65.4)	89	48 (53.9)[f]	141	82 (58.2)[g]
Under 20 Pieces	47	37 (78.7)	16	12 (75.0)[f]	63	49 (77.8)[g]
Over 20 Pieces	53	43 (81.1)	11	9 (81.8)[f]	64	52 (81.3)[g]
Total	152	114 (75.0)	116	69 (59.5)	268	183 (68.3)

Liver damage means patients with ALT levels >35 IU/l for more than half of each patient's observation period.
Light drinkers means consumption less than 60 g of ethanol per day.
Heavy drinkers means consumption 60 g or more of ethanol per day.
[a] $p = 0.0105$.
[b] $p = 0.0020$.
[c] $p < 0.0001$.
[d] $p = 0.0054$.
[e] $p = 0.0056$.
[f] $p = 0.0289$.
[g] $p = 0.0003$ by the Cochran–Armitage's trend test.

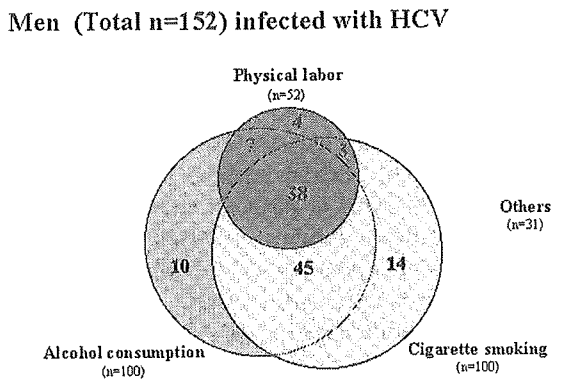

Figure 1. The distribution of moderate and extreme lifestyle habits of 152 male patients with chronic hepatitis C virus viremia in Hoshino village, Fukuoka, Japan, 2000. We divided the male patients into three groups by lifestyle habits; physical labor, alcohol consumption, and cigarette smoking. The circles indicate the number of patients engaging in each habit and areas of convergence. "Others" are patients who reported no physical labor, no alcohol consumption, and no cigarette smoking.

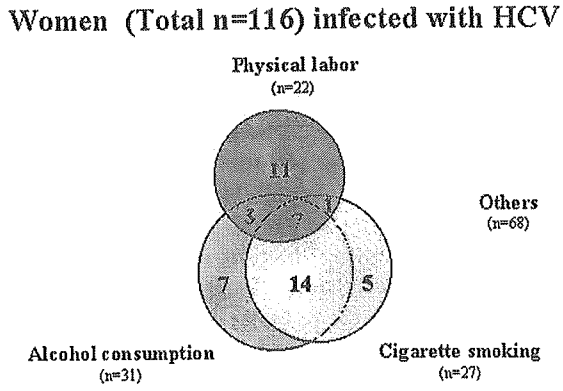

Figure 2. The distribution of moderate and extreme lifestyle habits of 116 female patients with chronic hepatitis C virus viremia in Hoshino village, Fukuoka, Japan, 2000. We divided the female patients into three groups by lifestyle habits; physical labor, alcohol consumption, and cigarette smoking. The circles indicate the number of patients engaging in each habit and areas of convergence. "Others" are patients who reported no physical labor, no alcohol consumption, and no cigarette smoking.

factors. The percentages of male and female HCV drinkers with other overlapping habits were 90.0% (90 of 100) and 77.4% (24 of 31), respectively, with no significance. The percentages of male and female HCV patients engaging in physical labor with other overlapping habits were 92.3% (48 of 52) and 50.0% (11 of 22), respectively, a significant difference ($p = 0.0001$). The percentages of male and female HCV smokers with other overlapping habits were 86.0% (86 of 100) and 81.5% (22 of 27), respectively, with no significance. The percentages of male and female HCV patients engaging in all three habits were 25.0% (38 of 152) and 6.0% (7 of 116), respectively, a significant difference ($p < 0.0001$). The percentages of male and female HCV patients not engaging in any of the three habits were 20.4% (31 of 152) and 58.6% (68 of 116), respectively, a significant difference ($p < 0.0001$).

Stepwise logistic regression analysis

Table 4 shows the results of stepwise logistic regression analysis done to determine the relationship between liver damage and lifestyle habits among the HCV patients. The factors incorporated were age at entry, male sex, history of blood transfusion, BMI, platelet counts, HA level, IV-C level, HCV-RNA level, HCV genotype, history of alcohol consumption, amount of alcohol consumed per day, period of alcohol consumption, alcohol consumption index, history of cigarette smoking, number of cigarettes smoked per day, period of cigarette smoking, Brinkmann index, and strenuous physical labor. Male sex ($p = 0.003$), platelet counts ($p < 0.001$), strenuous physical labor ($p = 0.002$), and period of alcohol consumption ($p = 0.007$) were significantly associated with the liver damage of 268 patients with chronic HCV viremia. Men had an increased probability of liver damage. When strenuous physical labor was done for over 2 h, the probability of liver damage was increased compared with patients engaging in strenuous physical labor under 2 h (estimated odds ratio = 1.82 [under 2 h], 20.60 [over 2 h]). The

Table 4. Factors contributing to the liver damage of 268 patients with chronic hepatitis C virus viremia in Hoshino village, Fukuoka, Japan: result of a stepwise logistic regression analysis

Factors	Category	Coefficient	Coefficient/ Standard error	Odds ratio	95% Confidence odds ratio interval	*p* Value
Sex	Men[a]	1.79	2.79	5.98	1.69–21.2	0.003
Platelet counts (μl)		0.22	4.16	1.25	1.12–1.39	<0.001
Strenuous physical labor	under 2 h[b]	0.60	0.94	1.82	0.52–6.30	0.002
	over 2 h[b]	3.02	3.24	20.60	3.25–130.0	
Period of alcohol consumption (years)		0.04	2.53	1.04	1.01–1.08	0.007

[a]Compared with women.
[b]Compared with non strenuous physical labor.

probability of liver damage increased with the period of alcohol consumption.

Among control subjects without HCV infection, only amount of alcohol consumed per day ($p = 0.0001$) was significantly associated with the liver damage. As the amount of alcohol consumed per day increased, there was a tendency for the probability of liver damage to increase (odds ratio = 1.06).

We did a similar analysis, classified by sex. Among the HCV male patients, alcohol consumption index ($p = 0.002$) and age at entry ($p = 0.022$) were significantly associated with the liver damage. As the alcohol consumption index increased, there was a tendency for the probability of the liver damage to increase (odds ratio = 8.46). As age at entry increased, there was a tendency for the probability of the liver damage to increase (odds ratio = 1.08). Among the HCV female patients, no significant factor was found in this analysis.

Discussion

Among patients with chronic HCV viremia, elevated ALT levels was associated with male sex, drinking, and strenuous physical labor, but not associated with viral properties such as genotype and viral load. This study showed an interaction between lifestyle habits and elevated ALT levels among patients with chronic HCV viremia, based on a community, not a hospital sample. The HCV-infected patients of the present study also had never received IFN or other antiviral treatment. Because liver damage and HCC development rates are reduced by IFN treatment [9–11], we excluded such treated patients from the present study and analyzed to evaluate the relationship between lifestyle habits and liver damage in the natural course of chronic HCV infection.

The Japanese Ministry of Health, Labor and Welfare reported in 1999 a large population study including about 6000 Japanese persons aged 20 to over 70 years that showed that 52.7% of Japanese males drank, 49.2% of males smoked, 8.1% of females drank, and 10.3% of females smoked [25]. Both the drinking and smoking rates of the residents of the studied area were relatively high. To our knowledge, no epidemiological studies of daily physical labor are to be found in the literature. The present study was the first report showing a relationship between daily physical labor and the elevated ALT levels of patients with chronic HCV viremia.

Several reports have indicated that the factors involved in deteriorating liver function do not include virus markers, but include male sex, alcohol consumption, cigarette smoking, and physical exercise among patients with chronic HCV viremia [13, 14, 26]. Because physical labor causes elevation of ALT levels liver damage by decreased hepatic blood flow, rest is generally thought to be desirable [27]. Hypoxia of the hepatic cells, permeability accentuation of the hepatic cell membrane, and hepatic cell necrosis are caused by decreases in hepatic blood flow, resulting in changes in serum hepatic enzyme production [28]. The hypoxia involved in physical labor brings about increases in catecholamine secretion, permeability accentuation of the hepatic cell membrane, and high levels of serum hepatic enzymes [29]. Although ALT is a marker for inflammation of the liver it is also an enzyme that can be found in skeletal muscle [30]. In fact, exercise testing by ergometer and treadmill induced increases in serum aminotransferase levels [17]. Changes in aminotransferase levels have also been found in healthy sporters after strenuous physical exercise [31, 32]. Thus, the observed raises in ALT levels after strenuous physical labour may also be due to muscle injury instead of liver deterioration. Analysis of physical labor and elevated ALT levels in our female HCV patients showed that elevated ALT levels increased with the length of time spent doing strenuous physical labor. On comparison of the data, no relationship was found between liver damage and physical labor among control subjects without HCV infection, although the study number was very small. We concluded that strenuous physical labor over a long period of time might be related to elevated ALT levels in patients with chronic HCV viremia.

Light physical labor or exercise would not seem to be detrimental to liver function. It is reported that physical exercise improved the health of European HCV patients [33]. In patients with liver cirrhosis, light physical exercise may be of benefit. With the minor increases in muscular activity seem in light physical exercise, the levels of serum ammonia do not rise and hepatic encephalopathy does not appear. All physical labor is not detriment to liver function, and physical exercise is often healthy. However, as strenuous physical labor goes beyond 2 h in our HCV patients, the hepatic blood flow rate lowers, and the adverse effects are seemed [27].

It is important to analyze the effect of education. It is thought that many unschooled workers are blue-collar workers, and the academic background may relate consciousness of disease. Most unschooled workers can be doing strenuous labor, while it is also known that people with less education are likely to be less healthy and to die younger. Investigating these relationships may clarify new factors of elevation of ALT levels.

Alcohol consumption has been widely reported to be an important lifestyle risk factor that causes liver damage [12, 34, 35]. We previously reported that the prevalence of liver abnormalities in hepatitis B virus carriers increased with alcohol consumption [36]. The causes of the liver damage include various metabolites involved in alcohol metabolism, especially acetaldehyde, hypoxia of the hepatic cell, microcir-