David S. Ludwig, MD, PhD
david.ludwig@childrens.harvard.edu
Department of Medicine
Children's Hospital
Boston, Mass

1. Ford ES, Giles WH, Mokdad AH. Increasing prevalence of the metabolic syndrome among U.S. adults. *Diabetes Care*. 2004;27:2444-2449.
2. St-Onge MP, Janssen I, Heymsfield SB. Metabolic syndrome in normal-weight Americans: new definition of the metabolically obese, normal-weight individual. *Diabetes Care*. 2004;27:2222-2228.
3. Salmeron J, Manson JE, Stampfer MJ, Colditz GA, Wing AL, Willett WC. Dietary fiber, glycemic load, and risk of non-insulin-dependent diabetes mellitus in women. *JAMA*. 1997;277:472-477.
4. Liu S, Willett WC, Stampfer MJ, et al. A prospective study of dietary glycemic load, carbohydrate intake, and risk of coronary heart disease in US women. *Am J Clin Nutr*. 2000;71:1455-1461.
5. Esposito K, Marfella R, Ciotola M, et al. Effect of a Mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome. *JAMA*. 2004;292:1440-1446.

Carvedilol, Metoprolol, and Insulin Resistance

To the Editor: The study by Dr Bakris and colleagues[1] comparing the metabolic effects of carvedilol vs metoprolol in patients with type 2 diabetes mellitus and hypertension found that insulin sensitivity improved with carvedilol (–9.1%; $P=.004$) but not metoprolol (–2.0%; $P=.48$), with a between-group difference of –7.2% (95% confidence interval [CI], –13.8% to –0.2%). The reference the article cites supporting the use of the Homeostasis Model Assessment–Insulin Resistance (HOMA-IR) as the tool to measure insulin resistance was the study by Haffner et al.[2] This was a prospective study assessing predictors of development of diabetes mellitus in 3.5 years of follow-up. Jayagopal et al[3] studied the variation of HOMA indices and showed that to consider a change of HOMA as clinically significant, the new value must represent at least a 90% increase or a 47% decrease. The mean improvement in HOMA-IR of 9% in the present study may be statistically significant but not clinically significant.

Edgard Gonzalez-Feldman, MD
egonzal2@hfhs.org
Endocrinology Division
Henry Ford Hospital
Detroit, Mich

1. Bakris GL, Fonseca V, Katholi RE, et al. Metabolic effects of carvedilol vs metoprolol in patients with type 2 diabetes mellitus and hypertension: a randomized controlled trial. *JAMA*. 2004;292:2227-2236.
2. Haffner SM, Kennedy E, Gonzalez C, Stern MP, Miettinen H. A prospective analysis of the HOMA model: the Mexico City Diabetes Study. *Diabetes Care*. 1996; 19:1138-1141.
3. Jayagopal V, Kilpatrick ES, Jennings PE, Hepburn DA, Atkin SL. Biological variation of homeostasis model assessment-derived insulin resistance in type 2 diabetes. *Diabetes Care*. 2002;25:2022-2025.

In Reply: Dr Gonzalez-Feldman raises concerns about interpretation of changes in HOMA-IR. The study by Haffner et al[1] was cited to document the validity of HOMA-IR as a measure of insulin resistance in large numbers of patients. HOMA-IR correlates well with more complex testing such as the euglycemic clamp method ($r^2=-0.88$; $P<.001$) and the minimal model ($r=0.7$; $P<.001$).[2,3] A review of this test in the context of large studies documents its validity.[4]

Although a change in 2 measurements in the same patient may be due to biological variation, as suggested by Jayagopal et al,[5] this is not applicable to large groups, as in the Glycemic Effects in Diabetes Mellitus: Carvedilol-Metoprolol Comparison in Hypertensives (GEMINI) study.[6] This is exemplified by data from the UK Prospective Diabetes Study,[7] in which metformin increased insulin sensitivity by 11% in the group that received it; although the group sensitivity remained at that higher level for 6 years, individuals within the group had varying results. Similarly, in the GEMINI study,[6] the difference in insulin sensitivity over time was maintained and closely paralleled differences in glycosylated hemoglobin (HbA_{1c}) between the groups. Because of the congruency of data between changes in HOMA-IR and HbA_{1c} in this and other studies, large magnitudes of change in HOMA-IR are not needed to support a clinically important benefit. Thus, we believe that the difference in insulin sensitivity between carvedilol and metoprolol was clinically as well as statistically significant.

David S. Bell, MD
dshbell@uab.edu
University of Alabama School of Medicine
Birmingham
George L. Bakris, MD
Rush University Medical Center
Chicago, Ill

1. Haffner SM, Kennedy E, Gonzalez C, Stern MP, Miettinen H. A prospective analysis of the HOMA model: the Mexico City Diabetes Study. *Diabetes Care*. 1996; 19:1138-1141.
2. Garcia-Estevez DA, Araujo-Vilar D, Fiestras-Janeiro G, Saavedra-Gonzalez A, Cabezas-Cerrato J. Comparison of several insulin sensitivity indices derived from basal plasma insulin and glucose levels with minimal model indices. *Horm Metab Res*. 2003;35:13-17.
3. Bonora E, Targher G, Alberiche M, et al. Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. *Diabetes Care*. 2000;23:57-63.
4. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. *Diabetes Care*. 2004;27:1487-1495.
5. Jayagopal V, Kilpatrick ES, Jennings PE, Hepburn DA, Atkin SL. Biological variation of homeostasis model assessment-derived insulin resistance in type 2 diabetes. *Diabetes Care*. 2002;25:2022-2025.
6. Bakris GL, Fonseca V, Katholi RE, et al. Metabolic effects of carvedilol vs metoprolol in patients with type 2 diabetes mellitus and hypertension: a randomized controlled trial. *JAMA*. 2004;292:2227-2236.
7. UK Prospective Diabetes Study Group. UK prospective diabetes study 16: overview of 6 years' therapy of type II diabetes: a progressive disease. *Diabetes*. 1995; 44:1249-1258.

RESEARCH LETTER

Ribavirin and Use of Clotting Factors in Patients With Hemophilia and Chronic Hepatitis C

To the Editor: Ribavirin has been used in combination with interferon (IFN) alfa to treat chronic hepatitis C. This combination therapy has been reported to be more effective than IFN monotherapy for eradicating hepatitis C virus (HCV),[1-3] including that occurring in patients with concomitant hemophilia.[4,5] We are unaware of previous reports of reduction in the use of clotting factors in HCV-

©2005 American Medical Association. All rights reserved.

Table. Patient Characteristics and Treatment Responses*

Patient No.	Age, y	Hemophilia†			HCV‡				
		Type	Severity	Duration, y	HCV-RNA Level, kIU/mL	HCV Genotype	Duration of HCV Infection, y	Ribavirin Load, mg/d	Eradication of HCV
1	28	A	Moderate	28	44	3a	27	800	Yes
2	61	A	Severe	61	640	3a	29	800	Yes
3	50	A	Severe	50	850	1b	34	600/400§	No
4	42	B	Mild	42	510	2a + 1b	30	800	Yes
5	44	A	Severe	44	600	3a	26	800	Yes
6	52	A	Mild	52	750	2b	22	600	Yes
7	37	A	Mild	37	59	1a	29	800/600§	No
8	44	B	Moderate	44	310	1a	33	800	Yes

*Human immunodeficiency virus (HIV) infection status, as determined by HIV antibody detection using a particle agglutination assay kit (Fuji Rebio, Tokyo, Japan), was negative for all patients except patient 7.
†Severity of hemophilia was classified as mild when clotting factor was above 5%, moderate when clotting factor was 1 to 5%, and severe when clotting factor was below 1%.
‡Serum hepatitis C virus (HCV) RNA levels were measured by Amplicor HCV assay version 2.0 (Roche Diagnostic Systems, Tokyo) at start of treatment. Hepatitis C virus genotype was determined by direct sequence of the 5'UTR region. Eradication of HCV was considered positive when the absence of serum HCV RNA was maintained for 24 weeks after treatment was completed.
§Patient 3 received 600 mg/d for 12 weeks and 400 mg/d for 12 weeks. Patient 7 received 800 mg/d for 8 weeks and 600 mg/d for 16 weeks.

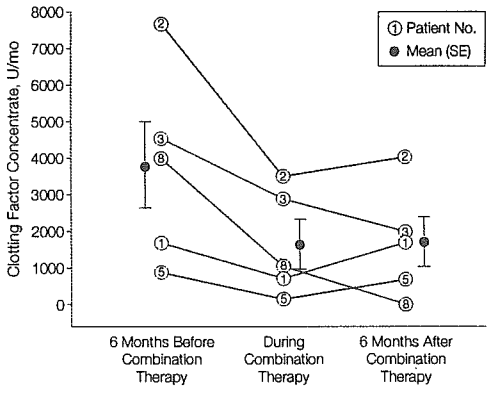
positive patients with hemophilia treated with ribavirin and IFN.

Methods. Eight consecutive patients with hemophilia were treated as outpatients for HCV infection with IFN and ribavirin between June 2002 and November 2003 at Nagoya University Hospital (TABLE). All patients were men with a mean (SD) age of 44.8 (10.0) years. Six patients had hemophilia A, and 2 had hemophilia B. Hemophilia was severe in 3 patients, moderate in 2, and mild in 3. Four patients (patients 1, 2, 3, and 5) had been previously treated with IFN alfa-2b alone (Intron A; Schering Plough K. K., Osaka, Japan) (6 MU/d for 2 weeks, followed by 3 times/wk for 22 weeks), but HCV had not been eradicated. During this study, all patients were treated with the same 24-week regimen of IFN. Oral ribavirin (Rebetol; Schering-Plough, Kenilworth, NJ) was administered at a dose of 600 mg/d for patients who weighed 60 kg or less and 800 mg/d for those who weighed more than 60 kg during 24 weeks. The dose of ribavirin was reduced by 200 mg/d if the patient's hemoglobin concentration decreased to below 10 g/dL due to hemolytic anemia induced by ribavirin; the hemoglobin level did not fall this low from any other causes.

The use of clotting factors was assessed by patient logs. Because the patients with mild hemophilia rarely used clotting factors, we compared the average use of clotting factors from the 5 patients with moderate and severe hemophilia before, during, and after treatment. Pairwise comparisons were made by 2-sided t test, with statistical significance defined as $P<.05$. All statistical analyses were done using JMP version 5.0.1 (SAS Institute Inc, Cary, NC). Ability to perform activities of daily living was assessed by interview. The protocol was approved by the Nagoya University institutional review board and written informed consent was obtained from each patient before treatment.

Results. In the 5 patients requiring clotting factor, the mean (SD) use of clotting factors per month for the 6 months prior to combination therapy was 3783 [2646] U/mo; during therapy it was 1605 [1488] U/mo; and for the 6 months after therapy it was 1667 [1528] U/mo (FIGURE). The mean use during treatment was significantly lower than before treatment ($P=.03$). The mean use after treatment was lower than before treatment, approaching statistical significance ($P=.06$). Seven of 8 patients noted no limitation of life activities before or after the start of IFN and ribavirin. One patient (patient 1) noted slight limitation of daily activities because of fatigue associated with combination therapy. The use of clotting factors in this patient was reduced during treatment but returned to the pretreatment level afterward. The average

Figure. Use of Clotting Factor Concentrates 6 Months Before, During, and 6 Months After Combination Therapy With Ribavirin and Interferon for the 5 Patients Requiring Clotting Factor During the Study



Differences in each period were analyzed by paired t test. $P=.03$ for comparison of pretreatment and active treatment; $P=.06$ for comparison of pretreatment and posttreatment. Large data markers indicate mean clotting factor use; error bars, SEs.

©2005 American Medical Association. All rights reserved.

numbers of bleeding episodes treated per month in the 6 months before combination therapy, during the therapy period, and in the 6 months after therapy, respectively, were 1.7, 0.7, and 1.6 (patient 1); 6.0, 3.0, and 4.1 (patient 2); 4.0, 2.6, and 2.2 (patient 3); 1.0, 0.2, and 0.7 (patient 5); and 3.6, 0.8, and 0.0 (patient 8). Most bleeding episodes were hemarthroses; a few involved mucosal or intramuscular bleeding.

Comment. In our hospital, 47 patients with hemophilia who had been treated for chronic hepatitis C with IFN alone had no observed reduction in the use of clotting factor, including 4 of the patients in this study. This strongly suggests that the reduced use of clotting factors was associated with the addition of ribavirin.

The reason for the reduction in the use of clotting factors by these patients with hemophilia is not clear. One suggestion comes from a case report that described an increase in warfarin dose requirement in a patient with a heart valve prosthesis after the start of this combination therapy,[6] and a change in coagulation status after starting ribavirin could be associated with our observations.

In 2 patients in this study, the decreased use of clotting factors continued after combination therapy ended. Being able to increase physical activities during treatment might strengthen muscles recovering from atrophy caused by joint bleeding. This increased strength could reduce stress on joints, further decreasing the risk of spontaneous hemarthrosis.

Based on this case series, ribavirin in combination with IFN, and possibly alone, may reduce the use of clotting factors in patients with hemophilia and chronic hepatitis C. Further studies, including replication of these findings and clinical trials, would help to clarify the potential role of ribavirin in these patients.

Takashi Honda, MD, PhD
Hidenori Toyoda, MD, PhD
Kazuhiko Hayashi, MD, PhD
Yoshiaki Katano, MD, PhD
Motoyoshi Yano, MD, PhD
Isao Nakano, MD, PhD
Kentaro Yoshioka, MD, PhD
Hidemi Goto, MD, PhD
Department of Gastroenterology
Nagoya University School of Medicine
Nagoya, Japan

Koji Yamamoto, MD, PhD
Junki Takamatsu, MD, PhD
jtkmtsu@med.nagoya-u.ac.jp
Department of Transfusion Medicine
Nagoya University School of Medicine

Financial Disclosures: None reported.

1. McHutchison JG, Gordon SC, Schiff ER, et al; Hepatitis Interventional Therapy Group. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. *N Engl J Med*. 1998;339:1485-1492.
2. Poynard T, Marcellin P, Lee SS, et al; International Hepatitis Interventional Therapy Group (IHIT). 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. *Lancet*. 1998;352:1426-1432.
3. 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.
4. Santagostino E, Rumi MG, Rivi M, Colombo M, Mannucci PM. Sustained suppression of hepatitis C virus by interferon and ribavirin in hemophilic patients not responding to interferon monotherapy. *Blood*. 2002;99:1089-1091.
5. Fried MW, Peter J, Hoots K, et al. Hepatitis C in adults and adolescents with hemophilia: a randomized, controlled trial of interferon alfa-2b and ribavirin. *Hepatology*. 2002;36:967-972.
6. Schulman S. Inhibition of warfarin activity by ribavirin. *Ann Pharmacother*. 2002;36:72-74.

©2005 American Medical Association. All rights reserved.

Journal of Thrombosis and Haemostasis, 4: 469–487

LETTERS TO THE EDITOR

Anti-HCV agent, ribavirin, elevates the activity of clotting factor VII in patients with hemophilia: a possible mechanism of decreased events of bleeding in patients with hemophilia by ribavirin

K. YAMAMOTO,* T. HONDA,† T. MATSUSHITA,‡ T. KOJIMA§ and J. TAKAMATSU*

**Department of Transfusion Medicine, Nagoya University Hospital, Nagoya; †Department of Gastroenterology, Nagoya Graduate School of Medicine, Nagoya; ‡Department of Hematology, Nagoya Graduate School of Medicine, Nagoya; and §Department of Medical Technology, Nagoya University School of Medicine, Nagoya, Japan*

To cite this article: Yamamoto K, Honda T, Matsushita T, Kojima T, Takamatsu J. Anti-HCV agent, ribavirin, elevates the activity of clotting factor VII in patients with hemophilia: a possible mechanism of decreased events of bleeding in patients with hemophilia by ribavirin. *J Thromb Haemost* 2006; 4: 469–70.

The combination therapy with ribavirin and interferon-α (IFN-α) has been reported to be more effective than IFN-α monotherapy for eradicating hepatitis C virus (HCV) [1,2], including patients with concomitant hemophilia [3]. We observed significant decreases in doses of clotting factors used for hemostatic therapy in hemophiliacs during ribavirin administration (e.g. 3780 units per month before ribavirin treatment and 1600 units per month during ribavirin on the average) [4]. In our hospital, 47 hemophilic patients who had been treated for chronic hepatitis C with IFN-α alone demonstrated no significant reduction in the use of clotting factor. This observation strongly suggests that the addition of ribavirin leads to the reduction of clotting factors used for bleeding in hemophiliacs. One suggestion comes from a case report that described an increase in warfarin dose requirement in a patient with heart valve prosthesis after starting this anti-HCV combination therapy [5].

These observations led us to investigate the ribavirin-induced change in vitamin K-dependent coagulation factors. To this purpose, we have measured the clotting activity of factor (F)VII, X, and prothrombin in hemophilic patients who were receiving the anti-HCV combination therapy. The protocol of therapy and analysis was approved by the Nagoya University institutional review board and written informed consent was obtained from each patient before treatment. Nine hemophilic patients, including seven hemophilia A and two hemophilia B (mean age ± SD: 42.5 ± 10.4 years old), whose characteristics were previously described [4], were entered in this study. The liver biopsy performed before starting the combination therapy did not show cirrhosis but chronic hepatitis in all patients analyzed. During this study, all patients were treated with the same 24-week regimen of IFN-α 2b (Intron A®, Schering Plough, K.K., Osaka, Japan) and oral ribavirin (600–800 mg day^{-1} of Rebetol; Schering-Plough, Kenilworth, NJ, USA). All statistical analyses were performed with STATA ver.7 software (STATA Corp., College Station, TX, USA) and the P-value < 0.05 was considered statistically significant.

The procoagulant activity of FVII in plasma has been elevated in all of nine ribavirin-treated hemophilic patients in comparison with that before ribavirin administration (Fig. 1A). The average and standard deviation for the elevation of FVII activity was 15.7% ± 8.8% ($P < 0.04$ in before vs. during ribavirin treatment; max. 28%; min. 5%). This elevation of FVII activity was independent of improvement of liver function (i.e. albumin, total billirubin, cholinesterase) in the patients (not shown). Only two patients, one has HIV infection and the other has hepatitis B virus concomitant with HCV, did not show a substantial elevation of FVII activity (i.e. 5% and 8%, respectively). We then measured activated FVII (FVIIa) levels in patients' plasma before and during ribavirin treatment using STACLOT® VIIa-rTF (Diagnostica Stago, Asnieres, France) [6] and observed substantial increases in FVIIa (e.g. 25.3 ± 14.8 mU mL^{-1}), which were almost compatible with elevation of FVII clotting activity. The plasma levels of FX and prothrombin were unchanged by ribavirin treatment in all of nine hemophilic patients (not shown). The elevation of FVII clotting activity by ribavirin is consistent with the previous observation of warfarin resistance in a ribavirin-treated patient [5].

To investigate the mechanism of ribavirin-induced elevation of FVII activity, we analyzed the gene expression of FVII in cultured normal human hepatocytes (Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) or human hepatoma cell line, HepG2 cells (ATCC, Manassas, VA, USA),

Correspondence: Koji Yamamoto, Department of Transfusion Medicine, Nagoya University Hospital, 65 Tsurumai, Showa, Nagoya 466-8550, Japan.
Tel.: +81 52 744 2576; fax: +81 52 744 2610; e-mail: kojiy@med.nagoya-u.ac.jp

Received 17 July 2005, accepted 18 October 2005

© 2006 International Society on Thrombosis and Haemostasis

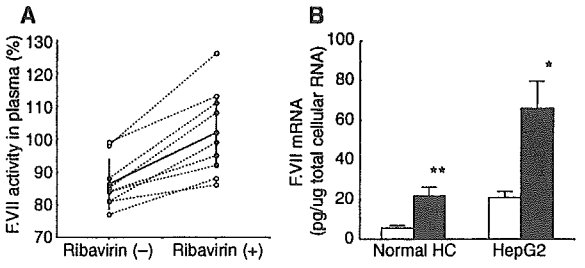


Fig. 1. Clotting activity of FVII in plasma of hemophilic patients and the mRNA expression of FVII in cultured human hepatocytes with or without ribavirin. (A) Each clotting activity of FVII in plasma of nine hemophilic patients before and at 4 weeks after starting ribavirin therapy was shown as open circle and dashed line, respectively. The average and SD of all patients was expressed as closed circle and error bar (without ribavirin: 86.3% ± 7.6%; with ribavirin: 102.0% ± 10.3%; $P < 0.04$). (B) Normal human hepatocytes or HepG2 cells had been cultured with (■) or without (□) ribavirin for 48 h. The mRNA expression of FVII was quantitated by real-time RT-PCR assay. Each value is expressed as the mean and SD from three sets of experiments. All of real-time RT-PCR assays were performed in duplicate. $*P < 0.02$; $**P < 0.01$.

which were cultured in medium with ribavirin at clinically therapeutic concentration (150 μg mL^{-1}) in the presence of IFN-α 2b (0.75 μg mL^{-1}; kindly provided by Schering Plough, K.K.). The expression level of mRNA for FVII, FX, and prothrombin, was determined by real-time quantitative RT-PCR with the ABI Prisms 7700 Sequence Detection (Perkin-Elmer Biosystems, Foster City, CA, USA) and SYBR Green PCR Kit (Perkin-Elmer Biosystems), according to the manufacturer's recommendations. The sequences of primer pairs used to quantify mRNA of the above genes were described in the NCBI Sequence Viewer. Variations in sample loading were assessed by measuring β-actin mRNA. Comparison of quantitative RT-PCR results between two groups was performed with the two-sample *t*-test. Welch's method was applied when variance between two groups was unequal (statistical significance: $P < 0.05$).

Significant induction of FVII mRNA was demonstrated in cultured normal hepatocytes (fourfold; $P < 0.01$) or HepG2 cells (threefold; $P < 0.02$) at 48 h after ribavirin treatment (Fig. 1B). No significant induction of mRNAs for FX and prothrombin was detected in ribavirin-treated cultured hepatocytes or HepG2 cells (not shown). In hepatocytes, ribavirin may stimulate to synthesize FVII by binding specifically to the promoter region of FVII gene (under current investigation).

It is possible that not only the induction of FVII but also changes in other coagulation factors during ribavirin therapy may be responsible for the decreased events of bleeding in hemophiliacs. However, the elevation of FVII activity in plasma could contribute most to the increased hemostatic potential in hemophilic patients because the cell-based tissue factor-activated FVII would play a central role in initiating coagulation and in activating platelets followed by large scale thrombin generation [7]. Clinically, recombinant activated FVII has been widely used as an antidote to control and prevent excessive hemorrhage in hemophilic patients with inhibitors [8]. Meanwhile, it was reported that even 10–20% of increase in plasma FVII/FVIIa would be an independent risk factor for coronary heart disease in healthy individuals [9,10], suggesting that a substantial elevation of endogenous FVII levels could result in an increased thrombotic potential. In general, the occurrence of spontaneous bleeding events in hemophiliacs is dependent on the critical hemostatic balance. In these conditions, 15–20% elevation of intrinsic FVII activity in plasma (Fig. 1A), because of the continuous induction of endogenous FVII by ribavirin (Fig. 1B), would contribute to the prevention from spontaneous bleeding in hemophiliacs. As a half-life of FVII in plasma is the shortest in all of coagulation factors, the continuous induction of FVII can maintain or increase the hemostatic value *in vivo*. If the prophylaxis to bleeding in hemophilic patients by ribavirin treatment were executable, it would result in much improvement of quality of their life and in large reduction of medical expenses in the country.

Acknowledgements

We thank Satoshi Suzuki, Tomomi Narita and Tomoko Nashida for their excellent technical assistance.

References

1 McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. 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**: 1485–92.

2 Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 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**: 958–65.

3 Santagostino E, Rumi MG, Rivi M, Colombo M, Mannucci PM. Sustained suppression of hepatitis C virus by interferon and ribavirin in hemophilic patients not responding to interferon monotherapy. *Blood* 2002; **99**: 1089–91.

4 Honda T, Toyoda H, Hayashi K, Katano Y, Yano M, Nakano I, Yoshioka K, Goto H, Yamamoto K, Takamatsu J. Ribavirin and the use of clotting factors in patients with hemophilia and chronic hepatitis C. *JAMA* 2005; **293**: 1190–2.

5 Schulman S. Inhibition of warfarin activity by ribavirin. *Ann Pharmacother* 2002; **36**: 72–4.

6 Morrissey JH, Macik BG, Neuenschwander PF, Comp PC. Quantitation of activated factor VII in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. *Blood* 1993; **81**: 734–44.

7 Roberts HR, Monroe DM, Oliver JA, Chang JY, Hoffman M. Newer concepts of blood coagulation. *Haemophilia* 1998; **4**: 331–4.

8 Roberts HR, Monroe DM, White GC. The use of recombinant factor VIIa in the treatment of bleeding disorders. *Blood* 2004; **104**: 3858–64.

9 Junker R, Heinrich J, Schulte H, van de Loo J, Assmann G. Coagulation factor VII and the risk of coronary heart disease in healthy men. *Arterioscler Thromb Vasc Biol* 1997; **17**: 1539–44.

10 Lowe GD, Rumley A, McMahon AD, Ford I, O'Reilly DS, Packard CJ for the West of Scotland Coronary Prevention Study Group. Interleukin-6, fibrin D-dimer, and coagulation factors VII and XIIa in prediction of coronary heart disease. *Arterioscler Thromb Vasc Biol* 2004; **24**: 1529–34.

© 2006 International Society on Thrombosis and Haemostasis

Eur J Clin Microbiol Infect Dis (2005) 24: 645–646
DOI 10.1007/s10096-005-0020-z

BRIEF REPORT

H. Toyoda · T. Honda · Y. Katano
H. Goto · J. Takamatsu

Clearance of GB virus C during highly active antiretroviral therapy and course of HIV disease progression in HIV-infected patients with hemophilia

Published online: 1 October 2005
© Springer-Verlag 2005

Highly active antiretroviral therapy (HAART) has been shown to improve the prognosis for HIV-infected patients through dramatic improvement of immune function. In the long-term follow-up of individuals receiving HAART, the status of other concomitant viruses, such as hepatitis C virus (HCV) or GB virus C (GBV-C), may also be altered [1]. It was recently reported that GBV-C viremia has a favorable effect on the prognosis of HIV infection [2–5], but a more recent study reported that loss of GBV-C viremia during follow-up of HIV-infected patients suggests a poor prognosis [6]. The prevalence of GBV-C clearance during HAART and the effect of GBV-C clearance on the clinical course of HIV infection have not yet been clarified. In this report, GBV-C persistence and clearance in HIV-infected patients receiving HAART and the effect of GBV-C clearance on HIV disease are discussed.

In a previous study, we investigated the presence of GBV-C RNA in sera obtained prior to 1988 from 41 HIV-infected patients with hemophilia [2]. GBV-C RNA was detected in 11 of these 41 patients. Two of the 11 patients died from AIDS before HAART was introduced, and the remaining nine patients began receiving HAART between 1996 and 1997. We monitored these nine patients until September 2004, and the findings of this long-term follow-up are presented here.

H. Toyoda (✉) · T. Honda · Y. Katano · H. Goto
Department of Gastroenterology,
Nagoya University School of Medicine,
Nagoya, Japan
e-mail: tkumada@he.mirai.ne.jp
Tel.: +81-584-813341
Fax: +81-584-755715

H. Toyoda
Department of Gastroenterology,
Ogaki Municipal Hospital,
Ogaki, Japan

J. Takamatsu
Department of Transfusion Medicine,
Nagoya University School of Medicine,
Nagoya, Japan

All nine patients tested positive for persistence of GBV-C RNA and negative for E2 antibody against GBV-C at the start of HAART. Annual serum samples from all patients were tested for the presence of GBV-C RNA using nested reverse transcription polymerase chain reaction [7]. HIV RNA levels, determined using an Amplicor HIV Monitor Assay (Roche Molecular Systems, Branchburg, NJ, USA) and CD4+ T-cell counts were also monitored annually. In patients in whom GBV-C RNA disappeared during follow-up, GBV-C E2 antibody was measured annually thereafter using an enzyme-linked immunosorbent assay [8]. Written informed consent was obtained from all nine patients before HAART was started. The study was approved by the University Ethics Committee and carried out in compliance with the Helsinki Declaration.

HIV RNA concentrations, CD4+ T-cell counts and GBV-C viremia status for all nine patients are summarized in Table 1. All of the patients were followed up for more than 7.5 years. In all patients, HIV RNA levels decreased after the start of HAART and fell below the detection limit within 2 years. CD4+ T-cell counts increased accordingly and were within the normal range, also within 2 years. All patients tolerated the therapy well and were compliant.

As of the end of September 2004, no patient had discontinued therapy. In three of the nine patients (patients 2, 8, and 9), GBV-C RNA was no longer detected in sera obtained after the start of HAART, whereas it was detected throughout the therapy in the remaining six patients. Clearance of GBV-C was accompanied by the appearance of GBV-C E2 antibody in two patients (patients 2 and 9). In contrast, in patient 8, E2 antibody was not detected in any of the annual serum samples obtained after the clearance of GBV-C, and serum was still negative at the end of September 2004. During observation, no patient showed HAART failure or advancement to AIDS, and no patient died. HIV RNA levels remained below the detection limit and CD4+ T-cell counts were normal in all patients regardless of GBV-C RNA and E2 antibody status.

It has been reported that 40–70% of immunocompetent persons show spontaneous clearance of GBV-C with the development of E2 antibody [9, 10]. Therefore, it should be

Table 1 Clinical features of HIV-infected patients with GB virus C who received highly active antiretroviral therapy (HAART)

Patient	Age (year)	Status at start of HAART		Clearance of GBV-C[a]	Appearance of E2 antibody	Status at end of September 2004	
		HIV RNA concentration (copies/ml)	CD4+ T-cell counts (cells/mm^3)			HIV RNA concentration (copies/ml)	CD4+ T-cell counts (cells/mm^3)
1	24	160,000	15	No	NT	UDL	553
2	15	5,800	110	Yes (2.5 years)	Yes	UDL	748
3	36	14,000	275	No	NT	UDL	474
4	13	810	228	No	NT	UDL	810
5	42	26,000	362	No	NT	UDL	445
6	19	58,000	36	No	NT	UDL	594
7	28	28,000	379	No	NT	UDL	540
8	21	1,100	476	Yes (0.5 year)	No[b]	UDL	852
9	39	77,000	260	Yes (2.0 years)	Yes	UDL	676

GBV-C GB virus C, *NT* not tested, *UDL* under the detection limit
[a]Number of years of GBV-C clearance after start of HAART is given in parentheses
[b]E2 antibody was not detected in annual serum samples between 1998 and 2004, and the enzyme immunosorbent assay was still negative at the end of September 2004

clarified whether GBV-C clearance during HAART also has an adverse effect on the clinical course of HIV infection. We observed clearance of GBV-C in three of nine HIV-infected patients. Of the three patients with GBV-C clearance, two had developed an E2 antibody response to GBV-C, whereas the remaining patient had not. A previous study reported a poorer prognosis for HIV-infected patients in whom GBV-C had been cleared and who failed to develop E2 antibodies [6]. We found no differences in immune status or HIV RNA concentration between patients with and without clearance of GBV-C RNA, regardless of the development of E2 antibody.

In conclusion, GBV-C clearance was observed during HAART in one-third of our cohort of HIV-infected patients, but this had no adverse effect on HIV disease progression. Larger studies with longer observation periods may clarify the effect of GBV-C clearance and presence of E2 antibody on HIV disease.

References

1. Takamatsu J, Toyoda H, Fukuda Y, Nakano I, Yokozaki S, Hayashi K, Saito H (2001) Effects of HAART on hepatitis C, hepatitis G, and TT virus in multiply coinfected HIV-positive patients with haemophilia. Haemophilia 7:575–581
2. Toyoda H, Fukuda Y, Hayakawa T, Takamatsu J, Saito H (1998) Effect of GB virus C/hepatitis G virus coinfection on the course of HIV infection in hemophilia patients in Japan. J Acquir Immune Defic Syndr Hum Retrovirol 17:209–213
3. Yeo AE, Matsumoto A, Hisada M, Shih JW, Alter HJ, Goedert JJ (2000) Effect of hepatitis G virus infection on progression of HIV infection in patients with hemophilia. Multicenter Hemophilia Cohort Study. Ann Intern Med 132:959–963
4. Xiang J, Wunschmann S, Diekema DJ, Klinzman D, Patrick KD, George SL, Stapleton JT (2001) Effect of coinfection with GB virus C on survival among patients with HIV infection. N Engl J Med 345:707–714
5. Tillmann HL, Heiken H, Knapik-Botor A, Heringlake S, Ockenga J, Wilber JC, Goergen B, Detmer J, McMorrow M, Stoll M, Schmidt RE, Manns MP (2001) Infection with GB virus C and reduced mortality among HIV-infected patients. N Engl J Med 345:715–724
6. Williams CF, Klinzman D, Yamashita TE, Xiang J, Polgreen PM, Rinaldo C, Liu C, Phair J, Margolick JB, Zdunek D, Hess G, Stapleton JT (2004) Persistent GB virus C infection and survival in HIV-infected men. N Engl J Med 350:981–990
7. Toyoda H, Fukuda Y, Hayakawa T, Takamatsu J, Saito H, Okamoto H (1998) GB virus C/hepatitis G virus isolates in Japanese haemophiliacs and their origins. Thromb Haemost 80:242–245
8. Tacke M, Kiyosawa K, Stark K, Schlueter V, Ofenloch-Haehnle B, Hess G, Engel AM (1997) Detection of antibodies to a putative hepatitis G virus envelope protein. Lancet 349:318–320
9. Thomas DL, Vlahov D, Alter HJ, Hunt JC, Marshall R, Astemborski J, Nelson KE (1998) Association of antibody to GB virus C (hepatitis G virus) with viral clearance and protection from reinfection. J Infect Dis 177:539–542
10. Hanley JP, Jarvis LM, Hayes PC, Lee AJ, Simmonds P, Ludlam CA (1998) Patterns of hepatitis G viraemia and liver disease in haemophiliacs previously exposed to non-virus inactivated coagulation factor concentrates. Thromb Haemost 79:291–295

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Hepatology Research 27 (2003) 18–22
Hepatology Research
www.elsevier.com/locate/ihepcom

Detection, using a novel method, of a high prevalence of cryoglobulinemia in persistent hepatitis C virus infection

Chiaki Okuse [a], Hiroshi Yotsuyanagi [a,*], Toshio Okazaki [b], Kiyomi Yasuda [c], Takahiro Fujioka [d], Masai Tomoe [a], Kiyoe Hashizume [a], Takeshi Hayashi [a], Michihiro Suzuki [a], Shogo Iwabuchi [a], Tatsuo Nagai [b], Shiro Iino [a]

[a] *Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University School of Medicine, 2-6-11, Sugao, Miyamae-ku, Kawasaki 216-8512, Japan*
[b] *Department of Clinical Hematology, Kitasato University School of Allied Health Sciences, 1-15-1, Kitasato, Sagamihara, Kanagawa 288-8555, Japan*
[c] *Research Center of Liver Diseases, Kiyokawa Hospital, 2-31-12, Asagaya-minami, Suginami, Tokyo 166-0004, Japan*
[d] *Department of Internal Medicine, Mishuku Hospital, 5-33-12, Kamimeguto, Meguro, Tokyo 153-0051, Japan*

Received 18 June 2002; received in revised form 19 March 2003; accepted 26 March 2003

Abstract

To elucidate precisely the prevalence and significance of cryoglobulinemia in hepatitis C, we examined the prevalence of serum cryoglobulin (CG) among 232 consecutive hepatitis C virus carriers (23 asymptomatic carriers, 164 with chronic hepatitis, 45 with cirrhosis), 30 consecutive hepatitis B virus carriers and 100 age- and sex-matched healthy volunteers. We used a gel-diffusion procedure that detects CG with greater sensitivity and specificity than the conventional precipitation method. Among the 232 patients, 166 were tested for the presence or absence of CG by the precipitation method also, which showed 60 (36.1%) patients to be positive for CG. On the other hand, 164 of the 232 patients (70.7%) were positive for CG using the diffusion method. 5 (16.7%) of the 30 HBV carriers and 2 (2%) of the healthy volunteers also were positive for CG using the gel-diffusion procedure. CG was detected more frequently among the patients with chronic hepatitis or cirrhosis than the asymptomatic carriers. In spite of the high prevalence of CG positivity, only one patient had symptoms related to cryoglobulinemia. Positivity for CG was not related to viral serogroup, viral load or the presence of antinuclear antibody, but it was related closely to CH50: 58 of 63 (92.1%) patients with lower levels of CH50 were positive for serum CG. In conclusion, cryoglobulinemia is a very common feature of chronic hepatitis C.
© 2003 Elsevier B.V. All rights reserved.

Keywords: Cryoglobulinemia; Hepatitis C; Gel-diffusion procedure; Precipitation method

1. Introduction

Cryoglobulinemia frequently complicates HCV infection. Although the mechanisms which generate cryoglobulin (CG) in HCV infection remain unclear, monoclonal or polyclonal activation of B cells by viral proteins has been proposed to be an important step [1]. It also has been proved that monoclonal B cell activation, expressed as a monoclonal rheumatoid factor (RF), is driven by one common immunological mechanism among those who share a particular cross-reacting idiotype [2]. In contrast, the mechanisms of polyclonal B cell activation remain obscure.

The prevalence of cryoglobulinemia in chronic hepatitis C differs widely among various reports [3–12], and this may be attributed to the varying sensitivity of standard methods. At present, CG is detected by cold precipitation of serum samples that are collected at 37 °C. The samples are stored at 4 °C for about a week to detect cryoprecipitation. Although this method is simple, it requires significant time and a small amount of CG may be overlooked. The lack of a standard method may obscure the significance of CG in chronic hepatitis C.

We have established a gel-diffusion procedure that detects CG more sensitively and precisely than the

* Corresponding author. Tel.: +81-44-977-8111; fax: +81-44-977-2696.
E-mail address: hyotsu@marianna-u.ac.jp (H. Yotsuyanagi).

1386-6346/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S1386-6346(03)00189-X

conventional precipitation method [13]. Using this method, we investigated the prevalence and significance of CG among patients with chronic hepatitis C and found that positivity for CG among HCV carriers is very high.

2. Materials and methods

2.1. Patients

We analyzed prospectively 232 consecutive patients with chronic liver disease type C who visited our hospital from December 1998 to March 1999. Diagnosis of a chronic HCV infection was made by continuous serum HCV-RNA for more than 6 months. The patients included 23 asymptomatic carriers (AsC), 164 with chronic hepatitis (CH) and 45 with cirrhosis (LC). Diagnosis of an asymptomatic carrier was made by continuous normal ALT levels for more than 1 year in serum samples tested every 3–4 months. Chronic hepatitis was diagnosed by monthly blood examinations as continuous elevation of ALT for more than 6 months. Chronic hepatitis and cirrhosis were differentiated using the calculation formula of Ikeda et al. [14]. 30 consecutive patients with chronic hepatitis B virus infection and 100 healthy volunteers, whose age and sex were matched with the patients, were enrolled in the study as controls. Informed consent was taken from each patient and volunteer, based on the rules of the ethical committees in our hospitals.

2.2. Viral and serological markers

Levels of HCV-RNA were determined using an Amplicor-M kit (Roche Diagnostics, Tokyo, Japan). The serogroup of HCV was determined using a commercial ELISA kit (Tonen Chemistry, Tokyo, Japan).

To elucidate further the significance of cryoglobulinemia, levels of IgG, IgM, IgA and rheumatoid factor were determined. Because cold activation of serum complement also is found frequently in HCV infection [15,16], CH50 also was measured in each patient.

2.3. Detection of cryoglobulin

Cryoglobulin was detected using the precipitation and gel-diffusion methods. Serum samples were collected and separated at 37 °C. Samples were kept at 4 °C for 1 week for the detection of CG using the conventional cryoprecipitation method. Any cryoprecipitate detected was confirmed as CG if the precipitate dissolved after warming to 37 °C.

The procedure for the gel-diffusion method has been described previously [13]. In short, 10 μl of the serum sample collected at the time of conventional analysis was applied to a glycin-containing gel and left at 4 °C for 48 h. Visualization of a ring around the well indicated CG positivity.

2.4. Statistical analysis

The data were analyzed using the Chi-square test, Mann–Whitney U-test or Fisher's exact test where appropriate. P-values less than 0.05 were regarded as significant.

3. Results

3.1. Positivity of cryoglobulin (Table 1)

The conventional precipitation method showed that 63 of the 166 HCV carriers (36.1%) were positive for CG. On the other hand, 164 of 232 patients (70.7%) were positive for cryoglobulin using the diffusion method. All but one patient who tested positive for cryoglobulin using the precipitation method also was positive by the diffusion method. 5 of the 30 patients (16.7%) with HBV infection and 2% (2/100) of the healthy volunteers were positive for CG by the diffusion method.

Table 1 compares the positivity for CG as detected by the two procedures. We examined 166 samples in total. 59 of 60 samples (98.3%) that were positive for CG by the precipitation method also were positive by the gel-diffusion method. 22 of 23 samples (95.7%) that were negative for CG by the gel-diffusion method also were negative by the precipitation method. These data show that the gel-diffusion method can detect CG with greater sensitivity and specificity than the precipitation method ($P < 0.0001$). Therefore, we carried out the following CG analysis using data from the gel-diffusion method.

3.2. Relationship between progression of disease and CG positivity (Table 2)

Table 2 shows the positivity for CG (using the gel-diffusion method) among each subset of chronic hepatitis C. Serum CG was detected in 30.4% of AsC, 81.9% of CH and 97.8% of LC patients. Positivity for CG increased with the progression of disease ($P < 0.0001$).

3.3. Relationship between biochemical, serological and viral markers and CG positivity (Table 3)

The correlation between positivity or negativity for CG and biochemical, serological and viral markers among patients with chronic hepatitis C is shown in Table 3. As shown in Table 3, levels of γ-GTP and hyalulonic acid were higher in CG-positive patients than CG-negative patients (γ-GTP: 51.7±45.5 IU/l vs. 37.3±

Table 1
Positivity of cryoglobulin by the precipitation method and the gel-diffusion method

	Positive by gel-diffusion method	Negative by gel-diffusion method	Total
Positive by gel-diffusion method	59 (98.3%)	1 (1.7%)	60 (36.1%)
Negative by gel-diffusion method	84 (79.2%)	22 (20.8%)	106 (63.9%)
Total	143 (86.1%)	23 (13.9%)	166

$P < 0.001$ by Chi-square test.

Table 2
Relation between progression of disease and CG positivity

Disease subset	Positive by gel-diffusion method	Negative by gel-diffusion method	Total
Asymptomatic carrier	7 (30.4%)	16 (69.6%)	23
Chronic hepatitis	133 (81.1%)	31 (18.9%)	164
Liver cirrhosis	44 (97.8%)	1 (2.2%)	45
Total	184 (70.7%)	48 (29.3%)	232

31.5 IU/l, $P = 0.04$; hyalulonic acid: 148.4±379.0 g/l vs. 51.5±60.8 g/l, $P = 0.004$), although levels of ALT did not differ (54.0±40.3 IU/l vs. 49.5±41.7 IU/l, $P = 0.69$). As shown in Table 3, levels of HCV-RNA did not differ between the CG-positive patients and CG-negative patients ($10^{5.48}$ copies/ml vs. $10^{5.43}$ copies/ml, $P = 0.47$). Positivity for CG also did not differ between patients with serogroup 1 and those with serogroup 2 HCV (86.9% vs. 91.9%, $P = 0.41$; Table 3).

Table 3
Relation between viral markers and CG positivity

	Cryoglobulin-Positive	Cryoglobulin-Negative	P
	($n = 143$)	($n = 23$)	
Biochemical and serological tests			
ALT (IU/l)	54.0±40.3	49.5±41.7	0.69
γ-GTP (IU/l)	51.7±45.5	37.3±31.5	0.04
Hyalulonic acid (g/l)	148.4±379.0	51.5±60.8	0.004
	($n = 143$)	($n = 17$)	
HCV-RNA			
HCV-RNA (log - KIU/ml)	2.48±0.75	2.43±0.91	0.47
	($n = 140$)	($n = 19$)	Total
HCV serogroup			
Serogroup 1	106 (86.9%)	16 (13.1%)	122
Serogroup 2	34 (91.9%)	3 (8.1%)	37
Total	140	19	159

3.4. Relationship between cryoglobulinemia-related signs and CG positivity (Table 4)

The correlation between signs related to essential cryoglobulinemia (purpura, proteinuria, hematuria) and positivity or negativity for cryoglobulin is shown in Table 4. The signs could be checked in 134 CG-positive and 17 CG-negative patients. All the patients who showed such signs were positive for cryoglobulin. Hematuria was found in 11 (7.3%), and proteinuria in 6 (4.0%), of 151 patients. Skin manifestation was found only in one patient (0.7%). Positivity for the three signs did not differ between CG-positive and CG-negative patients (purpura, proteinuria and hematuria: $P = 0.99$, 0.99 and 0.61, respectively).

3.5. Relationship between immunological markers and CG positivity (Table 5)

The correlation between serum immunological markers and positivity or negativity for cryoglobulin is shown in Table 5. All patients whose sera were reactive for rheumatoid factor were positive for cryoglobulin, although the positivity for rheumatoid factor did not differ between the CG-positive and CG-negative groups

Table 4
Relation between cryoglobulinemia-related signs and CG positivity

CG-related sign	Cryoglobulin-negative	Total	P
Purpura	0/17	1/151 (0.7%)	0.99*
Proteinuria	0/17	6/151 (4.0%)	0.99*
Hematuria	0/17	11/151 (7.3%)	0.61*

* Fisher's exact test.

Table 5
Relation between immunological markers and CG positivity

Immunological marker	Cryoglobulin-positive	Cryoglobulin-negative	Total	P
Antinuclear antibody	22/177 (12.5%)	2/32 (6.3%)	24/209 (11.5%)	0.31*
CH50	58/126 (46.0%)	5/23 (21.7%)	63/149 (42.3%)	0.03*
Rheumatoid factor	16/142 (11.3%)	0/26	16/168 (9.5%)	0.13*

* Chi-square test.

($P = 0.13$). Moreover, 58 of 126 (46.0%) CG-positive patients had decreased complement activity, a higher prevalence than the CG-negative patients (5 out of 23, 21.7%) ($P = 0.03$). 58 of 63 (92.1%) patients with lower levels of CH50 were positive for serum CG. Anti-nuclear antibody positivity did not differ between the CG-positive and CG-negative groups (12.5% vs. 6.3%, $P = 0.31$).

4. Discussion

There have been several reports examining the extra-hepatic manifestations that may accompany HCV infection [4,5,9–11]. Cryoglobulinemia was the most frequent clinical manifestation in each study. The prevalence of cryoglobulinemia among HCV carriers is reported to be between 21 and 59%. This high prevalence, irrespective of ethnic origin, suggests that cryoglobulinemia is a common phenomenon in individuals with HCV infection. Our study shows that CG may be found in a very high proportion of HCV carriers using a gel-diffusion method.

As shown in Table 1, the gel-diffusion method is more sensitive than the conventional precipitation method for the detection of CG. Although the gel-diffusion method missed one serum sample that was found to be CG-positive using the precipitation method, it may be said that the gel-diffusion method is superior to the precipitation method.

Table 2 shows that CG positivity increases with the progression of disease, which is compatible with previous reports [7]. Furthermore, CG is detected even during the asymptomatic stage in about one-third of patients. Recent studies have shown that the number of CD5+ B-cells and the expression of CD81 on B-cells are increased in chronic HCV carriers compared with healthy controls. Furthermore, they are closely associated with the production of rheumatoid factor and mixed cryoglobulins [17,18]. Because HCV can infect peripheral blood mononuclear cells [19–26] and CD81 is a putative co-receptor of HCV [27,28], HCV-associated CG production may start even at an early stage of chronic infection. A longer duration of infection may lead to a higher probability of CG, as observed in our patients with cirrhosis.

As shown in Table 3, levels of HCV-RNA and viral serogroup did not affect CG positivity. The major HCV serogroups found in Japan are serogroups 1 and 2. Furthermore, the number of studied patients is not sufficiently large. Although our data show that CG is common irrespective of viral type and amount, further studies are necessary to examine our hypothesis.

As shown in Table 4, skin manifestations, proteinuria and hematuria were not observed more frequently among the CG-positive groups than the CG-negative groups. The lack of a difference may be attributable to the low frequency of the three symptoms. Most of chronic hepatitis C patients with cryoglobulinemia are asymptomatic, as shown in previous reports [7–9].

Table 5 shows that CG positivity is related closely to low complement activity. Although not statistically different, rheumatoid factor was detected exclusively in CG-positive patients. Because CG consists of immunoglobulin that has rheumatoid factor activity, it is logical that CG and RF are correlated strongly. The low frequency of positivity for the two markers probably is attributable to the sensitivities of the two tests.

It was reported that cold activation of complement was observed frequently among HCV carriers [15,16]. It has been proposed that IgG–IgM rheumatoid factor (RF) complex induced by HCV may be the main causal factor for cold-dependent activation of complement in patients with rheumatic disease [29], which suggests that cold activation is associated closely with cryoglobulinemia. Our study shows that positivity for CG using the gel-diffusion method is higher than that of hypocomplementemia. One study of blood donors has shown that cold activation of complement is a phenomenon observed frequently among HCV carriers.

We have shown already that the gel-diffusion method may be used for analysis of CG. Melting the gel cut from the area with the CG ring may lead to a simple method for calculating the amount of CG. Diffusion with anti-immunoglobulin antibodies enables us to determine the components of CG. The gel-diffusion method is a useful tool for large-scale study.

In conclusion, cryoglobulinemia is a common extra-hepatic manifestation of HCV infection, especially in patients with liver injury. The gel-diffusion method is useful to study cryoglobulinemia and may substitute for the precipitation method.

References

[1] Agnello V. The etiology and pathophysiology of mixed cryoglobulinemia secondary to hepatitis C virus infection. Springer Semin Immunopathol 1997;19:111–29.

[2] Fornasieri A, Bernasconi P, Ribero ML, et al. Hepatitis C virus (HCV) in lymphocyte subsets and in B lymphocytes expressing rheumatoid factor cross-reacting idiotype in type II mixed cryoglobulinaemia. Clin Exp Immunol 2000;122:400–3.

[3] Cacoub P, Fabiani FL, Musset L, et al. Mixed cryoglobulinemia and hepatitis C virus. Am J Med 1994;96:124–32.

[4] Pawlotsky JM, Roudot-Thoraval F, Simmonds P, et al. Extrahepatic immunologic manifestations in chronic hepatitis C and hepatitis C virus serotypes. Ann Intern Med 1995;122:169–73.

[5] Clifford BD, Donahue D, Smith L, et al. High prevalence of serological markers of autoimmunity in patients with chronic hepatitis C. Hepatology 1995;21:613–9.

[6] Tanaka K, Aiyama T, Imai J, Morishita Y, Fukatsu T, Kakumu S. Serum cryoglobulin and chronic hepatitis C virus disease among Japanese patients. Am J Gastroenterol 1995;90:1847–52.

[7] Hartmann H, Schott P, Polzien F, et al. Cryoglobulinemia in chronic hepatitis C virus infection: prevalence, clinical manifestations, response to interferon treatment and analysis of cryoprecipitates. Z Gastroenterol 1995;33:643–50.

[8] Wong VS, Egner W, Elsey T, Brown D, Alexander GJ. Incidence, character and clinical relevance of mixed cryoglobulinaemia in patients with chronic hepatitis C virus infection. Clin Exp Immunol 1996;104:25–31.

[9] Lee YH, Ji JD, Yeon JE, Byun KS, Lee CH, Song GG. Cryoglobulinaemia and rheumatic manifestations in patients with hepatitis C virus infection. Ann Rheum Dis 1998;57:728–31.

[10] Cacoub P, Poynard T, Ghillani P, et al. Extrahepatic manifestations of chronic hepatitis C. MULTIVIRC Group. Multidepartment Virus C. Arthritis Rheum 1999;42:2204–12.

[11] Cacoub P, Renou C, Rosenthal E, et al. Extrahepatic manifestations associated with hepatitis C virus infection. A prospective multicenter study of 321 patients. The GERMIVIC Groupe d'Etude et de Recherche en Medecine Interne et Maladies Infectieuses sur le Virus de l'Hepatite C Medicine (Baltimore) 2000;79:47–56.

[12] Cicardi M, Cesana B, Del Ninno E, et al. Prevalence and risk factors for the presence of serum cryoglobulins in patients with chronic hepatitis C. J Viral Hepat 2000;7:138–43.

[13] Okazaki T, Nagai T, Kanno T. Gel-diffusion procedure for the detection of cryoglobulins in serum. Clin Chem 1998;44:1558–9.

[14] Ikeda K, Saitoh S, Kobayashi M, et al. Distinction between chronic hepatitis and liver cirrhosis in patients with hepatitis C virus infection: practical discriminant function using common laboratory data. Hepatol Res 2000;18:252–66.

[15] Ueda K, Nakajima H, Nakagawa T, Shimizu A. The association of complement activation at a low temperature with hepatitis C virus infection in comparison with cryoglobulin. Clin Exp Immunol 1995;101:284–7.

[16] Nomura H, Ogo T, Rikimaru N, et al. Hepatitis C virus-related liver damage is related to cold activation of complement. J Clin Gastroenterol 1997;25:529–34.

[17] Zuckerman E, Slobodin G, Kessel A, Sabo E, Yeshurun D, Halas K, Toubi E. Peripheral B-cell CD5 expansion and CD81 overexpression and their association with disease severity and autoimmune markers in chronic hepatitis C virus infection. Clin Exp Immunol 2002;128:353–8.

[18] Curry MP, Golden-Mason L, Nolan N, Parfrey NA, Hegarty JE, O'Farrelly C. Expansion of peripheral blood CD5+ B cells is associated with mild disease in chronic hepatitis C virus infection. J Hepatol 2000;32:121–5.

[19] Wang JT, Sheu JC, Lin JT, Wang TH, Chen DS. Detection of replication form of HCV RNA in peripheral blood mononuclear cells. J Infect Dis 1992;166:1167–9.

[20] Müller HM, Pfaff E, Goeser T, Kallinowski B, Solbach C, Theilmann L. Peripheral blood leukocytes serve as a possible extrahepatic site for hepatitis C virus replication. J Gen Virol 1993;74:669–76.

[21] Zignego AL, Carli MD, Monti M, Careccia G, Villa GL, Giannini C, D'Elios MM, Prete GD, Gentilini P. HCV infection of mononuclear cells from peripheral blood and liver infiltrates in chronically infected patients. J Med Virol 1995;47:58–64.

[22] Cribier B, Schmitt C, Bingen A, Kirn A, Keller F. In vitro infection of peripheral blood mononuclear cells by HCV. J Gen Virol 1995;76:2485–91.

[23] Mihm S, Hartmann H, Ramadori G. A reevaluation of the association of hepatitis C virus replicative intermediates with peripheral blood cells including granulocytes by a tagged reverse transcriptase/polymerase chain reaction technique. J Hepatol 1996;24:491–7.

[24] Lerat H, Berby F, Trabaud MA, Vidalin O, Major M, Trepo C, Inchauspe G. Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J Clin Invest 1996;97:845–51.

[25] Laskus T, Radkowski M, Wang LF, Vargas H, Rakela J. Search for hepatitis C virus extrahepaytic replication sites in patients with acquired immunodeficiency syndrome: specific detection of negative-strand viral RNA in various tissues. Hepatology 1998;28:1398–401.

[26] Torres B, Martin JL, Caballero A, Villalobos M, Olea N. HCV in serum, peripheral blood mononuclear cells and lymphocyte subpopulations in C-hepatitis patients. Hepatol Res 2000;18:141–51.

[27] Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. Science 1998;282:938–41.

[28] Wack A, Soldaini E, Tseng C, Nuti S, Klimpel G, Abrignani S. Binding of the hepatitis C virus envelope protein E2 to CD81 provides a co-stimulatory signal for human T cells. Eur J Immunol 2001;31:166–75.

[29] Wei G, Yano S, Kuroiwa T, Hiromura K, Maezawa A. Hepatitis C virus (HCV)-induced IgG–IgM rheumatoid factor (RF) complex may be the main causal factor for cold-dependent activation of complement in patients with rheumatic disease. Clin Exp Immunol 1997;107:83–8.

Intervirology

Intervirology 2003;46:367–372
DOI: 10.1159/000074994

Short-Term Lamivudine for the Treatment of Chronic Hepatitis B

Hiroshi Yotsuyanagi[a] Kiyomi Yasuda[b] Shiro Iino[a,b]

[a]Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University, Kawasaki, and [b]Seizankai Kiyokawa Hospital, Tokyo, Japan

Key Words
Chronic hepatitis · Hepatitis B e antigen · Hepatitis B virus · Lamivudine

Abstract
Short-term lamivudine and its withdrawal were evaluated as regards an immunomodulatory therapy of chronic hepatitis B. Lamivudine was given for 3 or 6 months to 23 patients with chronic hepatitis B who were infected with hepatitis B virus (HBV) genotype C, including 15 with hepatitis B e antigen (HBeAg) and 8 without it. It decreased serum levels of alanine aminotransferase (ALT) and HBV DNA in HBeAg-positive patients. Flare-ups of ALT and HBV DNA after treatment were observed in most patients, and 4 of the 12 (33%) patients with 6-month lamivudine treatment remained in remission 6 months after withdrawal of the therapy. In HBeAg-negative patients, however, flare-ups of ALT and HBV DNA were mild. Normalization of ALT and a decrease in serum HBV DNA were accomplished in 6 of the 9 (75%) patients. Breakthroughs or serious side effects were not observed in any patients. Short-term lamivudine is safe and may offer a therapeutic option to patients with chronic hepatitis B.

Copyright © 2003 S. Karger AG, Basel

Introduction

Lamivudine is a nucleotide analogue that inhibits reverse transcription of the pregenome of hepatitis B virus (HBV). Soon after the start of lamivudine treatment, serum levels of HBV DNA and alanine aminotransferase (ALT) decrease in the patients who receive it. Lamivudine, however, cannot clear HBV infection in hosts.

The reduction of viral loads by means of nucleotide analogues can upregulate the immune response to HBV. In fact, the withdrawal of lamivudine frequently causes posttreatment flares (PTF) of hepatitis [1–4]. In some patients with PTF, serum HBV DNA decreases subsequently, which is caused presumably by an increased immunoreactivity of the hosts [5]. Withdrawal of lamivudine intended for the induction of immune responses, therefore, may suppress HBV replication after PTF and be used for treatment of chronic hepatitis B.

In this study, the usefulness of short-term lamivudine and its withdrawal was evaluated as regards its efficacy as an immunomodulatory therapy in patients with chronic hepatitis B.

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2003 S. Karger AG, Basel
0300–5526/03/0466–0367$19.50/0
Accessible online at
www.karger.com/vir

Hiroshi Yotsuyanagi, MD
Department of Internal Medicine, Division of Gastroenterology and Hepatology
St. Marianna University School of Medicine
2-16-1, Sugao, Miyamae-ku, Kawasaki, 216-8512 (Japan)
Tel. +81 44 977 8111, Fax +81 44 977 2698, E-Mail hyotsuy@marianna-u.ac.jp

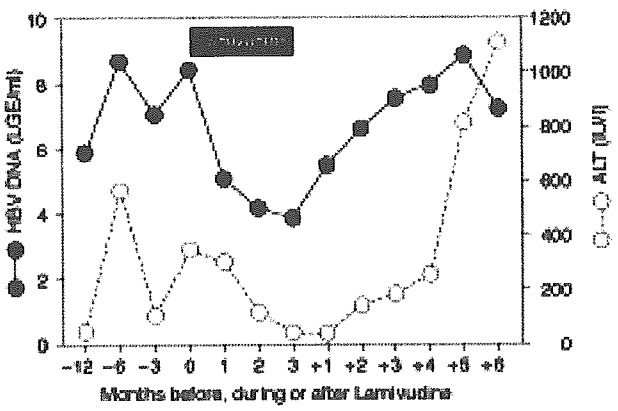


Fig. 1. Changes in HBV DNA and ALT levels in the representative HBeAg-positive patients who received lamivudine for 3 months. Mild decrease of HBV DNA and ALT occurred during the treatment period. Mild flare-ups of ALT and HBV DNA occurred after the withdrawal of lamivudine.

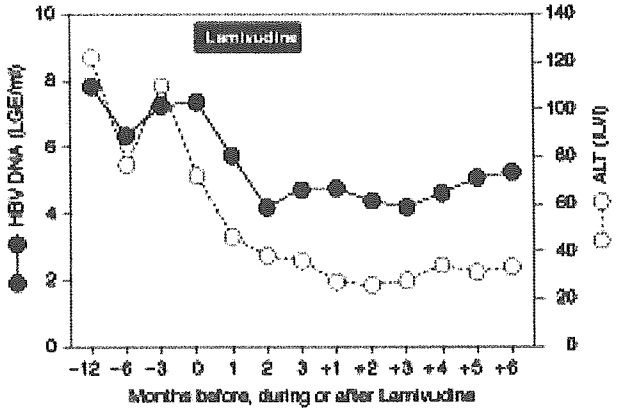


Fig. 2. Changes in HBV DNA and ALT in the representative anti-HBe-positive patients who received lamivudine for 3 months. ALT levels decreased during the treatment period and remained low even after the withdrawal of lamivudine.

Patients and Methods

Twenty-four patients were enrolled in the study. Since hepatic decompensation can occur in association with PTF in patients with cirrhosis [5–7], only patients were entered into the study who had (1) bilirubin ≤2 mg/dl, (2) hyaluronic acid ≤110 μg/l, and (3) platelet counts ≥12 × 10^4/mm^3.

Sixteen patients were positive for hepatitis B e antigen (HBeAg) and negative for the corresponding antibody (anti-HBe), while 8 patients were negative for HBeAg and positive for anti-HBe. Lamivudine 100 mg/day was given to 6 patients (4 with HBeAg and 2 with anti-HBe) for 3 months and to 18 patients (12 with HBeAg and 6 with anti-HBe) for 6 months. All the patients were monitored monthly for 6 months before the start of lamivudine till 6 months after its withdrawal.

Levels of HBV DNA were measured by transcription-mediated hybridization assay (Chugai Diagnostics Science, Tokyo, Japan) and expressed as log genome equivalents (LGE)/ml. The range of detection for this assay was from 3.7 LGE/ml (5.0 × 10^3 copies/ml) to 8.7 LGE/ml (5.0 × 10^8 copies/ml) [8]. Genotypes of HBV were determined by enzyme-linked immunosorbent assay involving epitopes on the pre-S2 region products [9, 10] with commercial kits (HBV Genotype EIA; Institute of Immunology, Tokyo, Japan) in accordance with manufacturer's instructions.

Informed consent was obtained from each patient enrolled in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

Collected data were analyzed using the χ^2 test, Fisher's exact test, Student's t test or the Mann-Whitney test where they were applicable. A p value <0.05 was considered statistically significant.

Results

All the patients were infected with HBV genotype C except 1 who harbored HBV genotype B in serum. In order to avoid the influence of genotypes, our analysis was confined to 23 patients who were infected with HBV genotype C. They were positive for HBeAg and treated for 3 months.

Figure 1 shows the changes in HBV DNA and ALT in the representative patient of 3 with HBeAg who were given lamivudine for 3 months. Levels of ALT decreased in all of them during lamivudine treatment. After the withdrawal of lamivudine, flare-ups of ALT were observed in 2 patients. HBV DNA returned to high levels in all of them 6 months after the withdrawal of therapy, while ALT returned to pretreatment levels except in 1 patient who did not have flare-ups.

Figure 2 shows the changes in HBV DNA and ALT in the representative patient of 2 negative for HBeAg who were given lamivudine for 3 months. After the withdrawal of lamivudine, HBV DNA increased and returned to the pretreatment level in 1 patient, while it stayed lower than the pretreatment level in the other. In contrast, ALT decreased and remained low after the withdrawal of lamivudine in both patients.

Figure 3 shows the changes in HBV DNA and ALT of the 6 patients positive for HBeAg who received lamivudine for 6 months and in whom ALT and HBV DNA lev-

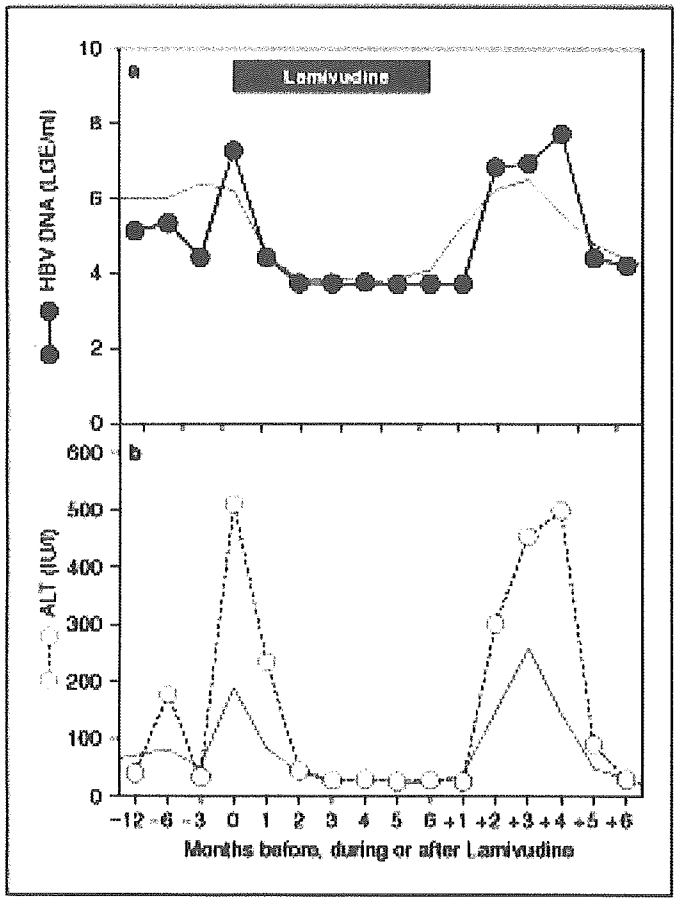


Fig. 3. Changes in HBV DNA (a) and ALT (b) in the representative responder who was positive for HBeAg and received lamivudine for 6 months. The shaded areas show the mean values of the 4 patients. Their ALT levels were kept within normal limits for 6 months after withdrawal when the levels of HBV DNA were less than 10^5 genome equivalents/ml.

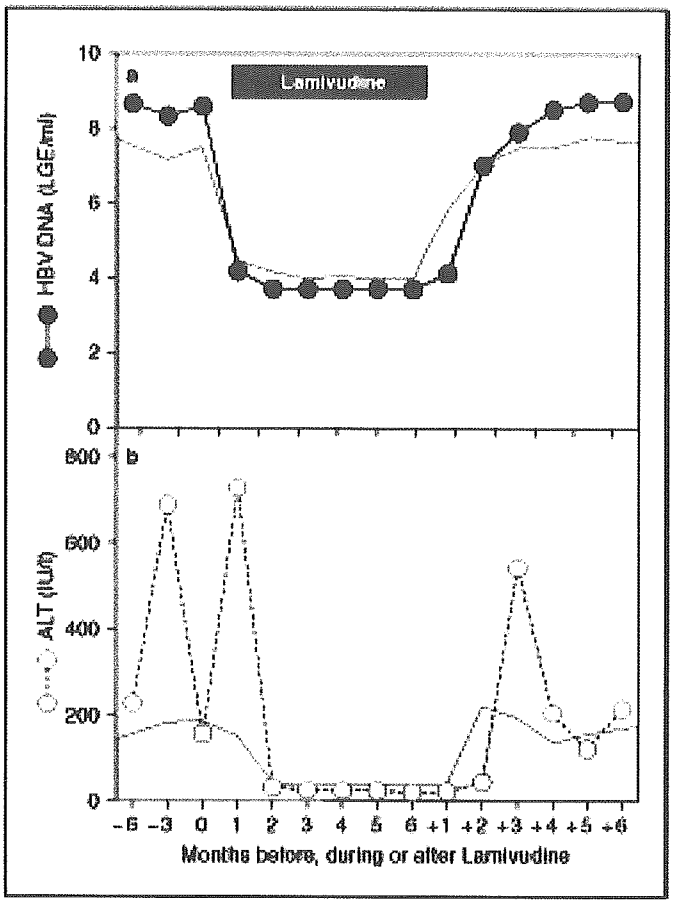


Fig. 4. Changes in HBV DNA (a) and ALT (b) in the representative nonresponder who was positive for HBeAg and given lamivudine for 6 months. The shaded areas show the mean values of the 8 nonresponders. Their HBV DNA and ALT levels decreased during the treatment period. After the withdrawal of lamivudine, all patients had flare-ups of HBV DNA, whereas only 4 (50%) of them had flare-ups of ALT. Levels of ALT 6 months posttreatment were elevated even after PTF in 6 patients. Levels of HBV DNA 6 months after treatment were above 10^5 genome equivalents/ml in all the patients.

els decreased after PTF. The mean pretreatment levels of ALT and HBV DNA were 187 ± 212 IU/l and 6.2 ± 1.6 LGE/ml, respectively, and the maximum ALT level after withdrawal of the drug was 1,191 IU/l. Their ALT levels decreased to normal values 6 months after withdrawal, and HBV DNA levels at that time point were less than 10^5 genome equivalents/ml. Four of the 12 patients (33%) had such a good clinical course, and they were regarded as responders. Three patients seroconverted from HBeAg to anti-HBe 6 months after withdrawal and the remaining patient turned seronegative for HBeAg at this time point.

Figure 4 shows the changes in HBV DNA and ALT of the HBeAg-positive patients who received lamivudine for 6 months, but in whom HBV DNA and ALT levels did not decrease as remarkably as in the patients shown in figure 3. Eight out of the 12 patients (67%) had such a course, and they were regarded as nonresponders. The mean pretreatment levels of HBV DNA and ALT were 7.6 ± 1.1 LGE/ml and 186 ± 196 IU/l, respectively. After the withdrawal of lamivudine, all the patients had flare-ups of HBV DNA, while only 4 of them (50%) had flare-ups of ALT. In 6 patients, ALT levels were elevated 6 months after withdrawal even though they had gone through PTF. HBV DNA levels at that time point were above 10^6 genome equivalents/ml in all the patients. Two

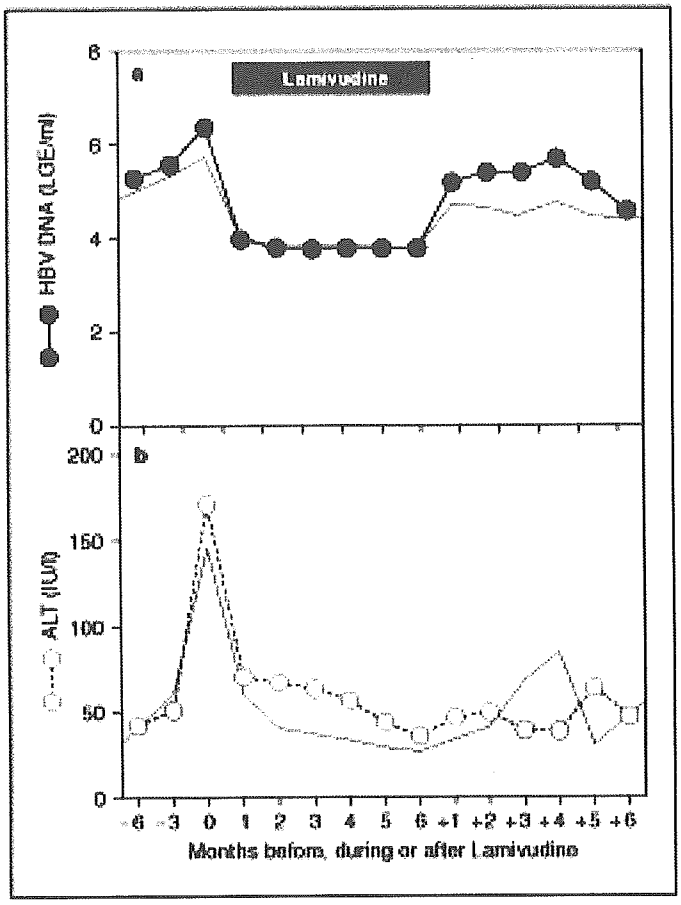


Fig. 5. Changes in HBV DNA (a) and ALT (b) in the representative anti-HBe-positive patients who received lamivudine for 6 months. The shaded areas show the mean values of the 6 patients. Levels of ALT decreased and remained low in 5 patients even after the withdrawal of lamivudine. PTF of ALT as well as HBV DNA was observed in only 1 patient.

Table 1. Demographic, clinical and virological features of responders and nonresponders to short-term lamivudine

Features	Responders (n = 4)	Nonresponders (n = 8)	Differences
Age, years	43 ± 2.9	44 ± 8.7	NS
Females	3 (75)	1 (13)	NS
Treatment naive	2 (50)	6 (75)	NS
ALT, IU/l	187 ± 212	186 ± 196	NS
HBV DNA, LGE/ml	6.2 ± 1.6	7.6 ± 1.1	NS
Hyaluronic acid	52.2 ± 21.4	46.9 ± 32.2	NS
Platelets, $\times 10^4$/mm^3	17.1 ± 2.8	20.7 ± 6.2	NS

Figures in parentheses represent percentage. NS = Not significant.

patients seroconverted from HBeAg to anti-HBe 6 months after administration, and the remaining 6 patients stayed seropositive for HBeAg at that time.

Table 1 compares clinical parameters, including age, sex, pretreatment levels of ALT, HBV DNA and hyaluronic acid, between the 4 responders and the 8 nonresponders; they were all positive for HBeAg and had received lamivudine for 6 months. No differences were noted between the two groups of patients.

Figure 5 shows changes in HBV DNA and ALT levels of the 6 patients who were negative for HBeAg and given lamivudine for 6 months. ALT levels decreased and remained low in 5 (83%) of them even after the withdrawal of lamivudine. PTF of ALT and HBV DNA was observed in only 1 patient.

Flare-ups of ALT and HBV DNA were not observed during treatment in any of the 6 patients, and none of them developed jaundice or serious side effects.

Discussion

In most of the patients with chronic HBV infection symptoms eventually disappear after the patients have gone through flare-ups of ALT, which reflects immune clearance of hepatocytes infected with HBV [11–13]. Patients with continuous liver injury are considered to lack adequate immune responses to HBV. Upregulation of virus-specific lymphocytes, therefore, is a rational therapeutic approach.

Corticosteroid withdrawal therapy, which causes flare-ups and subsequent remission of hepatitis, has been used for this purpose [14, 15]. Priming with corticosteroid, however, upregulates transcription of HBV via corticosteroid-responsive elements of the virus, and can fan PTF toward hepatic decompensation. If upregulation of immune responses without increasing viral loads is possible, it would offer a safer therapeutic option.

Nucleotide analogues decrease viral loads by the direct inhibition of viral replication. Theoretically, a decrease in viral loads can break the immune tolerance by restoring exhausted lymphocytes. Indeed, lamivudine treatment restores T cell responsiveness to HBV [16, 17], which instigated us to do this pilot study.

Lamivudine treatment for 6 months decreased ALT and HBV DNA levels in 33% of the patients positive for HBeAg. The response rate is comparable to that of interferon therapies for 12–24 weeks [18] and superior to that of 1-year lamivudine treatment [19]. The high rate achieved may be attributable to the low HBV DNA levels

in responders. It is well known that a low pretreatment HBV DNA level is predictive of a virological response to lamivudine [19] as well as interferon treatment [20, 21]. Furthermore, it is uncertain whether the remission induced by lamivudine withdrawal is durable or not. Longer follow-ups are necessary to confirm the therapeutic effect of this pilot study.

A rebound of ALT and HBV DNA occurred in 2 of the 3 HBeAg-positive patients who received lamivudine for 3 months. The decrease in HBV DNA and ALT levels that followed the rebound, however, was not durable. It seems that a 3-month treatment period was too short for these patients.

The optimal length of the period of treatment to induce upregulation of immune responses is still controversial. Recent studies have disclosed the time of maximum HBV-specific CTL response after lamivudine treatment to range from 4 to 24 weeks [16, 17]. In theory, the best time to stop antiviral treatment should coincide with the time of maximal immune response [22]. It is desirable to monitor specific immune responses to HBV and so to assess the timing for the withdrawal of lamivudine. At present, 6 months is deemed an optimal treatment period, because the efficacy of lamivudine for this period is comparable or superior to other treatment options, and because YMDD mutants rarely emerge during this period [2, 19, 23, 24].

As shown in table 1, the clinical backgrounds of responders and nonresponders were not different. However, female patients may have a better clinical outcome than male patients, which has not been reported in other antiviral treatment regimens. These findings may have to do with the fact that liver fibrosis progresses more slowly in female than male patients [25]. Again, larger clinical studies are necessary to examine whether the gender difference influences the efficacy of short-term lamivudine therapy.

In this study, only one of the HBeAg-negative patients had flare-ups of ALT and HBV DNA, unlike in HBeAg-positive patients. It may be accounted for by levels of ALT and HBV DNA which are lower in HBeAg-negative than HBeAg-positive patients as well as a treatment period which is too long to upregulate the immunological reaction. All in all, short-term lamivudine therapy in HBeAg-negative patients is effective for the suppression of ALT and HBV DNA, albeit its durability would require a longer period of observation.

In conclusion, short-term lamivudine treatment is safe and may be used as a therapeutic option for patients with chronic hepatitis B. Further studies are necessary to evaluate the durability of this therapy.

References

1 Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M: A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med 1995;333:1657–1661.

2 Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA: Lamivudine as initial treatment for chronic hepatitis B in the United States. N Engl J Med 1999;341:1256–1263.

3 Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, Fevery J, De Man RA, Thomas HC: Lamivudine therapy for chronic hepatitis B: A six-month randomized dose-ranging study. Gastroenterology 1997;113:1258–1263.

4 Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray DF: Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: A randomised trial. Gut 2000;46:562–568.

5 Honkoop P, de Man RA, Niesters HG, Zondervan PE, Schalm SW: Acute exacerbation of chronic hepatitis B virus infection after withdrawal of lamivudine therapy. Hepatology 2000;32:635–639.

6 Honkoop P, de Man RA, Heijtink RA, Schalm SW: Hepatitis B reactivation after lamivudine. Lancet 1995;346:1156–1157.

7 Lim SG, Wai CT, Rajnakova A, Kajji T, Guan R: Fatal hepatitis B reactivation following discontinuation of nucleoside analogues for chronic hepatitis B. Gut 2002;51:597–599.

8 Kamisango K, Kamegawa C, Suzui M, Gato S, Hirao A, Gonzales F, Yasuda K, Iino S: Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. J Clin Microbiol 1999;37:310–314.

9 Usuda S, Okamoto H, Iwanari H, Baba K, Tsuda F, Miyakawa Y, Mayumi M: Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product. J Virol Methods 1999;80:97–112.

10 Usuda S, Okamoto H, Tanaka T, Kudo-Ijuagaen K, Ho-Tsand PV, Miyakawa Y, Mayumi M: Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods 2000;87:81–89.

11 Liaw YF, Chu CM, Su IJ, Huang MJ, Lin DY, Chang-Chien CS: Clinical and histological events preceding hepatitis B e antigen seroconversion in chronic type B hepatitis. Gastroenterology 1983;84:216–219.

12 Lok AS, Lai CL: Acute exacerbations in Chinese patients with chronic hepatitis B virus (HBV) infection. Incidence, predisposing factors and etiology. J Hepatol 1990;10:29–34.

13 Chu CM, Liaw YF: Immunohistological study of intrahepatic expression of hepatitis B core and E antigens in chronic type B hepatitis. J Clin Pathol 1992;45:791–795.

14 Liaw YF: Hepatitis viruses under immunosuppressive agents. J Gastroenterol Hepatol 1998;13:14–20.

15 Perrillo RP: Acute flares in chronic hepatitis B. The natural and unnatural history of an immunologically mediated liver disease. Gastroenterology 2001;120:1009–1022.

16 Boni C, Bertoletti A, Penna A, Cavalli A, Pilli M, Urbani S, Scognamiglio P, Boehme R, Panebianco R, Fiaccadori F, Ferrari C: Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. J Clin Invest 1998;102:968–975.

17 Boni C, Penna A, Ogg GS, Bertoletti A, Pilli M, Cavallo C, Cavalli A, Urbani S, Boehme R, Panebianco R, Fiaccadori F, Ferrari C: Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: New perspectives for immune therapy. Hepatology 2001;33:963–971.
18 Wong DK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J: Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. Ann Intern Med 1993;119:312–323.
19 Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF: A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61–68.
20 Perrillo RP, Schiff ER, Davis GL, Bodenheimer HC Jr, Lindsay K, Payne J, Dienstag JL, O'Brien C, Tamburro C, Jacobson IM, et al: A randomized, controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. N Engl J Med 1990;323:295–301.
21 Lok AS, Wu PC, Lai CL, Lau JY, Leung EK, Wong LS, Ma OC, Lauder IJ, Ng CP, Chang HT: A controlled trial of interferon with or without prednisone priming for chronic hepatitis B. Gastroenterology 1992;102:2091–2097.
22 Komarova NL, Barnes E, Klenerman P, Wodarz D: Boosting immunity by antiviral drug therapy: A simple relationship among timing, efficacy, and success. Proc Natl Acad Sci USA 2003;100:1855–1860.
23 Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM: Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. Hepatology 1999;30:567–572.
24 Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL: Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Gastroenterology 2000;119:172–180.
25 Huo T, Wu JC, Hwang SJ, Lai CR, Lee PC, Tsay SH, Chang FY, Lee SD: Factors predictive of liver cirrhosis in patients with chronic hepatitis B: A multivariate analysis in a long-itudinal study. Eur J Gastroenterol Hepatol 2000;12:687–693.

ELSEVIER

Identification of a New Autoantibody in Patients With Chronic Hepatitis

Yasunobu Fukuda, Hiroshi Yotsuyanagi, Seido Ooka, Taichi Sekine, Junki Koike, Toshifumi Takano, Michihiro Suzuki, Fumio Itoh, Kusuki Nishioka, and Tomohiro Kato

ABSTRACT: To comprehensively study autoantibodies in patients with chronic hepatitis (CH), especially those with hepatitis C virus (HCV) infection, proteins extracted from HepG2 cells were separated by two-dimensional electrophoresis. Spots reacting with sera from 15 patients with CH-C were detected by Western blotting. Proteins extracted from the spots were subjected to mass spectroscopy for identification by mass fingerprinting. Antigenicity of the proteins identified was confirmed by Western blotting and enzyme-linked immunoabsorbent assay. The localization of the autoantigens so detected was investigated by immunohistochemistry. Among 20 protein spots detected, four were identified as actin, heat shock protein (HSP) 70, HSP60, and a novel protein (hepalaminin). Hepalaminin consists of two domains of laminin β-2 and a specific domain. Autoantibodies against the specific domain were detected in 60.8% of patients with CH-C, 37.7% of those with CH-B, 42.3% of those with autoimmune CH, 28.6% of nonalcoholic steatohepatitis, 10.0% of asymptomatic HCV carriers, but in no healthy volunteers. Antihepalaminin positivity in CH-C and CH-B was related to histologic grading. Immunohistochemical staining demonstrated that hepalaminin is present in the cytoplasm of hepatocytes and cholangiocytes but not of fibroblasts or the vascular epithelium. Hepalaminin is a novel protein expressed in hepatocytes and cholangiocytes. Autoimmunity to this protein may exacerbate inflammation in chronic viral hepatitis. *Human Immunology 65, 1530–1538 (2004).* © American Society for Histocompatibility and Immunogenetics, 2004. Published by Elsevier Inc.

KEYWORDS: Chronic hepatitis C; autoimmunity; autoantibody; laminin; hepatitis C virus; interferon

ABBREVIATIONS

2DE-WB	two-dimensional electrophoresis and Western blotting
AA	amino acid
AIH	autoimmune hepatitis
AsC-C	asymptomatic HCV carrier
BSA	bovine serum albumin
CH-B	chronic hepatitis B
CH-C	chronic hepatitis C
ELISA	enzyme-linked immunosorbent assay
HCV	hepatitis C virus
HL	hepalaminin
HSP	heat shock protein
MBP	maltose binding protein
NASH	nonalcoholic steatohepatitis
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate

INTRODUCTION

Chronic hepatitis C (CH-C) is accompanied by continuous liver injury, which is considered to be the result of an insufficient immune response to the virus [1–8]. Indeed, cytotoxic T cell (CTL) responses to viral proteins are reduced in CH compared with acute, self-limited viral hepatitis [9–12]. The mechanisms of immune insufficiency to the virus are unknown.

Infection with hepatitis C virus (HCV) may lead to autoantibody production. Several kinds of autoantibodies—antinuclear antibody [13, 14], anti–smooth muscle antibody [13, 14], antithyroid antibody [15, 16], antilymphocyte antibody [17], and type 1 liver-kidney microsomal antibody [18, 19]—are detected in some patients. However, these autoantibodies are not specific

From the Rheumatology, Immunology and Genetics Program, Institute of Medical Science (Y.F., H.Y., S.O. T.S., K.N., T.K.), Division of Gastroenterology and Hepatology, and Departments of Internal Medicine (Y.F., H.Y. M.S., F.I.), and Pathology (J.K., T.T.), St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan.

Address reprint requests to: H. Yotsuyanagi, Department of Internal Medicine, Division of Gastroenterology and Hepatology, St. Marianna University School of Medicine, Miyamae-ku, Kawasaki, 216-8512, Japan; Tel.: +81-44-977-8111; Fax: +81-44-977-2696; E-mail: hyotsu@marianna-u.ac.jp.

Received February 24, 2004; revised August 11, 2004; accepted August 31, 2004.

Human Immunology 65, 1530–1538 (2004)
© American Society for Histocompatibility and Immunogenetics, 2004
Published by Elsevier Inc.

0198-8859/04/$–see front matter
doi:10.1016/j.humimm.2004.08.186

against the liver. Therefore, a systematic investigation of liver-derived proteins is useful to understand autoimmunity against the liver. However, comprehensive profiles of autoantibodies against the liver have not been studied so far.

To systematically survey autoantibodies against hepatocytes, we used a proteomic approach and identified a novel autoantibody in the sera of patients with CH-C and subsequently investigated the prevalence of the autoantibody in chronic hepatitis.

PATIENTS AND METHODS

Patients, Liver Specimens, and Serum Samples

Serum samples used for screening for autoantigens, using 2-dimensional electrophoresis and Western blotting (2DE-WB), were collected from 15 patients with chronic HCV infection (three asymptomatic carriers and 12 patients with CH) who were selected randomly from the patients who visited our institution in 2002. They were negative for antinuclear antibody and the levels of immunoglobulin (Ig) G were within the normal range.

Serum samples from 207 individuals were used for enzyme-linked immunosorbent assay (ELISA) and WB with recombinant proteins. The study comprised 185 patients (79 with CH-C, 53 with chronic hepatitis B [CH-B], 26 with autoimmune chronic active hepatitis [AIH], seven with nonalcoholic steatohepatitis [NASH] and 20 asymptomatic HCV carriers [AsC-C]) and 21 healthy volunteers. All the patients except the AsC-C had abnormal alanine aminotransferase levels and were considered to have CH. Asymptomatic HCV carrier was defined by positive serum HCV RNA with continuously normal alanine aminotransferase concentrations in repeated blood examinations for longer than 1 year. The 79 patients with CH-C and 20 AsC-Cs did not include any of the 15 patients whose sera were used for the original screening. Hepatitis C virus genotypes were determined for 75 of 79 patients, 33 with genotype 1 and 42 with genotype 2. A total of 133 of the 186 patients underwent liver biopsy and 27 of the 79 patients with CH-C were treated with interferon-α alone (6 MU 6 times/week for 2 weeks and 6 MU three times/week for 24 weeks; 504 MU total) after their biopsy. The histologic activity of hepatitis was evaluated independently by two pathologists (J.K., T.T.) using the liver biopsies according to the scoring system described by Knodell *et al.* [20].

Serum samples from age- and sex-matched healthy donors were used as controls for each disease category. The research was carried out in accordance with the human experimentation guidelines of our institution and the Declaration of Helsinki.

Preparation of Cell Samples

A whole-cell lysate was prepared from cultured HepG2 cells by the freeze-thaw method [21]. The cell pellet, resuspended in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS), was frozen in liquid nitrogen and thawed at 37°C, five times. After centrifugation at 4°C for 30 minutes, the supernatant was collected and its protein concentration was determined using Bradford's method. The supernatant was stored at −80°C until use.

2DE-WB

The extracted protein samples were diluted in a dehydration buffer (8 M urea, 2% CHAPS, 2.8 mg/ml DTT, and a trace of bromophenol blue) containing 0.5% immobilized pH gradient buffer (pH range 3–10; Amersham Pharmacia Biotech [APB], Sweden) and loaded onto 7-cm Immobiline Drystrips (APB) in an immobilized pH gradient reswelling tray (APB) at room temperature overnight, as described elsewhere [22]. From 200 to 1000 μg of the extracted proteins were applied to the drystrip gels. Isoelectric focusing was performed in a horizontal electrofocusing apparatus (MultiPhor II, APB) according to the manufacturer's instructions. After isoelectric focusing, the immobilized pH gradient strips were equilibrated in the first equilibration solution (10 mg dithiothreitol per 1 ml sodium dodecyl sulfate [SDS] equilibration buffer [1.5 M Tris-Cl, pH 8.8; 6 M urea, 30% glycerol, 2% SDS]), and then in the second equilibration solution (25 mg iodoacetamide per 1 ml SDS equilibration buffer). The equilibrated strips were placed on top of 12.5% SDS polyacrylamide gel electrophoresis (PAGE) slab gels and sealed with 0.5% lower melt gel, and then the second electrophoresis was performed at 20°C with a constant current of 40 mA.

For the 1-dimensional electrophoresis, approximately 3 μg of a purified recombinant fusion protein and fusion partner protein of a negative control, as described in the following section, were separated by 12.5% SDS-PAGE.

After electrophoresis, the SDS-PAGE gels were stained with Coomassie brilliant blue or used for protein transfer onto nitrocellulose membranes. For WB, the membranes were blocked in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% Tween 20 for 1 hour, washed in PBS with 0.1% Tween 20 for 30 minutes, and then incubated with serum samples diluted in PBS with 0.1% Tween 20 containing 1% BSA for 1 hour. For the 2DE-WB, a mixture of 15 serum samples was used, in which each sample was diluted at 1:500. In the 1-DEWB, serum samples diluted at 1:500 were used separately. After five washings in PBS with 0.1% Tween 20, the bound antibodies were reacted with horseradish peroxidase–conjugated goat anti-human IgG (Zymed Laboratories, San