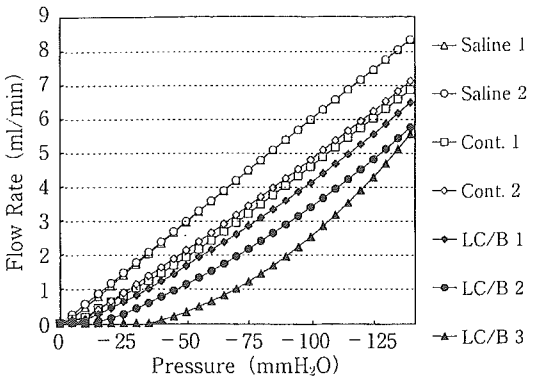


Fig. 3 Representative pressure-flow rate (P-Q) relationships. P-Q relationships show 2 HBS (saline: △, ○), 2 normal controls (Cont: □, △), and 3 representative patients in the LC groups in grade B (LC/B: ◆, ●, ▲), respectively. The hematocrit value of the erythrocyte suspension is 3%.

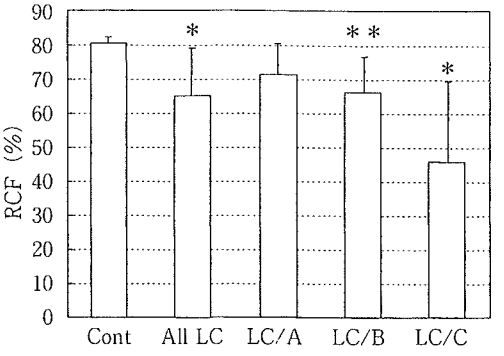


Fig. 4 Erythrocyte filterability in patients with LC. Bars show normal controls (Cont, n=5), the entire LC patients (All LC, n=31), the LC groups in grade A (LC/A, n=8), the LC groups in grade B (LC/B, n=19), and the LC groups in grade C (LC/C, n=4), respectively. The ratio (%) of the flow rate of the erythrocyte suspension to that of the suspending medium at 100 $mmH_2O$ was used as an index of erythrocyte (red cell) filterability (RCF). Values are expressed as means + standard deviation (*: $P<0.05$, **: $P<0.01$).

patients is lower than that in normal controls at any given pressure, indicating that the erythrocyte filterability in the patients is obviously decreased relative to that in the controls.

### 3. 3 Erythrocyte filterability in patients with LC

Fig. 4 shows the mean erythrocyte filterability in normal controls and that in patients with LC. The ratio (%) of the flow rate of the erythrocyte suspension to that of suspending medium at 100 $mmH_2O$ was used as an index of erythrocyte filterability. The mean erythrocyte filterability in 5 normal controls was 80.5 ± 1.7%, whereas that in the entire 31 LC patients was 64.6 ± 14.5% ($p < 0.03$). The erythrocyte filterability in the LC groups in grade A (70.9 ± 9.6%) was lower than that in the controls, but there was no statistical difference between the two at the significant level of 5% ($p=0.053$, n=8). However, the filterability in the LC groups in grade B (65.9 ± 11.0%: $p < 0.01$, n=19) and in grade C (45.9 ± 23.8%: $p < 0.02$, n=4) were significantly decreased compared with that in the controls.

### 3. 4 Correlation between erythrocyte filterability and clinical examination data

In the entire 31 patients with LC, the erythrocyte filterability was not correlated with the representative hematological parameters; that is, the hemoglobin content of blood (Hb), the hematocrit value (Ht), the mean corpuscular hemoglobin (MCH) and the mean corpuscular hemoglobin concentration (MCHC) of erythrocytes, and the platelet content of blood. However, the mean corpuscular volume (MCV) of erythrocytes showed a weak, inverse correlation with the filterability ($r= -0.41$), reflecting macrocytic anemia in the advanced state of the clinical severity (Table 1). Also, the filterability was not correlated with values of serum albumin, total bilirubin, total cholesterol, cholinesterase, prothrombin time, total bile acid, AST, ALT, lactate dehydrogenase (LDH), γ-glutamyl transpeptidase (γ GTP) and LCAT.

While, in 19 patients in the LC groups in grade B, the erythrocyte filterability showed a positive

correlation with the Hb content of blood (r= 0.53) and with the Ht value (r=0.55), as shown in Fig. 5, suggesting that the filterability is roughly proportional to the severity of anemia in the LC groups in grade B. Furthermore, the erythrocyte filterability showed a weak, inverse correlation with LDH (r= − 0.46) and total bile acid (r= − 0.39), and a weak, positive correlation with cholinesterase (r=0.42) and γ GTP (r=0.40).

## 4. Discussion

Erythrocyte filterability plays a pivotal role in maintaining the fluent microcirculation. In particular, it is important in patients with LC showing hemodynamic derangements such as portal hypertension and numerous collateral circulations, including esophageal varices. Previous investigations using the nuclepore filtration technique[11, 12] or ektacytometry[12] presented that the erythrocyte deformability was impaired in LC. However, these findings were not quantified according to the clinical severity of LC, and those techniques are not good in reproducibility or sensitivity, as mentioned above. In this study, using the newly developed, sensitive and reproducible filtration apparatus (Fig. 2), we have clearly demonstrated that the erythrocyte filterability is impaired in cirrhotic patients, and that this rheological abnormality revealed a good correlation with the Child-Pugh classification that is considered to closely reflect the clinical severity of LC.

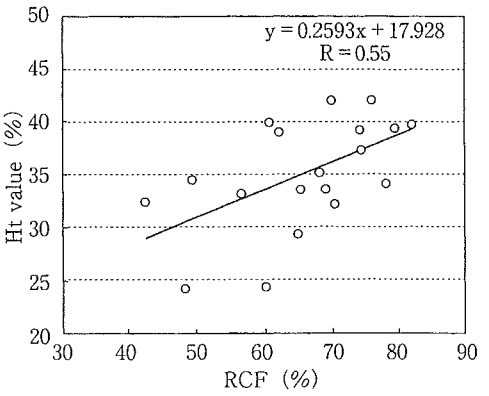


Fig. 5 Positive correlation between the erythrocyte filterability (RCF: %) and Ht value (%) in 19 patients in the LC groups in grade B.

Here, it is to be noted that the pressure-flow rate relationships obtained by the decreasing, negative pressure-based filtration were similar to those of a gravity-based filtration[1, 2], as shown in Fig. 3. Nowadays, the physiological significance and clinical benefit of the gravity-based nickel mesh filtration is evident[1, 2, 14, 17-19]. However, in the performance of the gravity-based filtration, great care must be taken to avoid contamination from emboli, formed at times such as when filling the test materials into the vertical tube and installing the nickel mesh filter in the holder. In the negative pressure-based filtration, there are no such handling problems; accordingly, the performance of the new filtration technique is very easy, rapid, and reproducible. The other methods that sucked test cells into a filter using a constant pressure or a constant rate as a driving force, continuously suck even when the cells are plugging the filter, thereby leading sometimes to cell breakage; therefore, such methods are not physiological tests. The driving force used in our new method, i.e., decreasing negative pressure, does not cause such cell rupture, only causing physiological and/or pathophysiological cell plugging in the filter, similar to gravity. Thus, we have quantitatively showed the impaired filterability of erythrocytes from patients with LC using the new physiological technique.

It is generally accepted that the erythrocyte filterability is mainly determined by 1) the membrane properties, 2) the cellular internal viscosity that is reflected in the MCHC, and 3) the geometric factors of the erythrocytes that are reflected in the surface area to volume ratio, i.e., MCV, and shape changes[1, 2, 20]. In the present study, the erythrocyte filterability in LC patients was not correlated with MCHC. In addition, the

filterability showed a weak, inverse correlation with MCV ($r = -0.41$), and no discernible shape change was observed. Also, it is noteworthy here that abnormal membrane lipid composition of erythrocytes in LC resulted in a high cholesterol/phospholipid ratio mainly due to a decrease in phosphatidylethanolamine and sphingomyelin[10, 11]. Such erythrocyte membrane lipid alteration is considered to cause a reduced membrane fluidity[7, 10] and a reduced erythrocyte deformability[11, 12]. Therefore, these reports and our results support that the impaired filterability in this study is attributable mainly to the altered erythrocyte membrane properties and partly to the changes in surface area to volume ratio of erythrocytes.

The lipid composition of the mature erythrocyte membrane is highly dependent on the surrounding plasma lipid profile, and LCAT plays a key role in the membrane lipid turnover. In the case of chronic cholestasis and a rise in bile acid associated with LC, LCAT activity is reduced[4]. LCAT deficiency typically causes an imbalance in the cholesterol/phospholipid ratio, and leads to an increase in the membrane rigidity and the formation of target cells[6] or spur cells[7], which are susceptible to one of the causes of hemolysis due to the hypersplenism[5]. Although no discernible erythrocyte shape changes were found in this study, LCAT activity was apparently decreased in accordance with the clinical severity of LC (Table 2). Therefore, it is possible that alterations of lipid composition and the subsequent decrease in the membrane fluidity cause a decrease in the erythrocyte filterability.

Furthermore, portal hypertension and splenomegaly are a common manifestation of LC, in which the hepatic sinusoidal microstructure is rearranged under the progressive hepatic fibrosis. Persistent elevation in the portal vein pressure induces numerous collateral microcirculations and reactive hepatospletic reticuloendothelial hyperplasia[21]. Moreover, augmented portal blood volume[22] and increased blood viscosity due to hypergammaglobulinemia accelerate the portal hypertension[23]. Portal resistance is elevated in the LC patients, and this elevation mainly occurs at the intrahepatic sinusoidal area[22], suggesting the existence of injured intrahepatic microcirculation. It is well known that several kinds of mechanical stress cause impairments in erythrocyte filterability through the mechanism of the action of $Ca^{2+}$– mediated signal transduction[19, 24, 25] It is, therefore, likely that the accelerated portal hypertension in the LC patients mechanically stressed the erythrocytes and impaired the erythrocyte filterability. The impaired erythrocytes could be easily trapped by the sinusoidal reticuloendothelial system and subsequently sequestered, thereby leading to hemolysis and further development of anemia.

It is well known that the severity of the LC groups in grade A (in this study, n=8) is not so severe, and that of the LC groups in grade C (n=4) is very severe and sometimes associated with many complications. As a result, it is considered that the LC groups in grade B (n=19) show typical signs and symptoms of LC. Therefore, we discuss the correlations between the erythrocyte filterability and clinical examination data focused in the LC groups in grade B. The present study revealed that the erythrocyte filterability in the LC groups in grade B correlated with the Ht value ($r=0.55$) and with the Hb concentration of blood ($r=0.53$) ; that is, the filterability decreased as the anemia advanced, suggesting that the filterability is roughly proportional to the severity of anemia. Also, a weak, inverse correlation between the filterability and values of LDH ($r = -0.46$) was observed. Because the LDH value increases as liver dysfunction and hemolysis are progress, this result indicates that the filterability reflects the severity of both liver dysfunction and anemia. Moreover, there is a weak, inverse correlation between the filterability and values of bile acid

($r = -0.39$). Because bile acid increases as liver dysfunction is advances and because taurocholic acid, a representative bile acid, impairs erythrocyte filterability[1], the result suggests that the filterability is related to liver dysfunction through the action of bile acid. In addition, we found a weak correlation between the filterability and cholinesterase ($r = 0.42$). Because the production of cholinesterase decreases as liver dysfunction is advanced, this result further suggests that the filterability reflects liver dysfunction through the production of cholinesterase. These results strongly support an idea that the impaired filterability and subsequent hemolysis may be the causes of anemia.

In conclusion, the present rheological study quantitatively demonstrated that the erythrocyte filterability in cirrhotic patients is impaired in relation to the clinical severity of LC; to our knowledge, this is the first quantitative document concerning these factors. Furthermore, the present study suggests that the impaired filterability arises from the altered erythrocyte membrane properties and a possible mechanical stress due to the accelerated portal hypertension accompanied with the rearranged hepatic sinusoidal microstructure, thus reflecting disorders of the microcirculation.

(Part of this research has been presented at the 27th meeting of the Japanese Society of Biorheology on June 10, 2004, in Tokyo).

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### References

1) Arai, K., Iino, M., Shio, H. and Uyesaka, N.: Further investigations of red cell deformability with nickel mesh. *Biorheology* **27**: 47-65, 1990.

2) Nakamura, T., Hasegawa, S., Shio, H. and Uyesaka, N.: Rheologic and pathophysiologic significance of red cell passage through narrow pores. *Blood Cells* **20**: 151-165, 1994.

3) Koyama, T., Maruyama, T., Seki, R., O'Rear, E., Shio, H. and Uyesaka, N.: New, quantitative nickel mesh filtration apparatus based on continuously decreasing negative pressure. *Jpn. J. Physiol.* **53**: 481-486, 2003.

4) Sherlock, S. and Dooley, J.: The hematology of liver disease. *In Disease of the Liver and Biliary System*, S. Sherlock and J. Dooley (Eds), 10th ed. Chap. 4, Blackwell, London, 1997, pp. 43-62.

5) Morse, E.E.: Mechanisms of hemolysis in liver disease. *Ann. Clin. Lab. Sci.* **20**: 169-174, 1990.

6) Cooper, R.A.: Lipid of the red memberane: normal composition and variability in disease. *Semin. Hematol.* **7**: 296-310, 1970.

7) Owen, J.S., Brown, D.J.C., Harry, D.S., N Mulmtyre., GH Beaven., H Lsenberg., and WB Gratzee.: Erythrocyte echinocytosis in liver disease: role of abnormal plasma high density lipoproteins. *J. Clin. Invest.* **76**: 2275-2284, 1985.

8) Yang, Y.Y., Lin, H.C., Lee, W.C., Huang, Y.T., Hou, M.C., Lee, F.Y., Chang, F.Y. and Lee, S.D.: Plasma erythropoietin level in patients with cirrhosis and its relationship to the severity of cirrhosis and renal function. *J. Gastroenterol. Hepatol.* **18**: 1156-1161, 2003.

9) Tacke, F., Schoffski, P., Luedde, T., Meier, P.N., Ganser, A., Manns, M.P. and Trautwein, C.: Analysis of factors contributing to higher erythropoietin levels in patients with chronic liver disease. *Scand. J. Gastroenterol.* **39**: 259-266, 2004.

10) Owen, J.S., Bruckdorfer, K.R., Day, R.C. and Mcintyre, N.: Decreased erythrocyte membrane fluidity and altered lipid composition

in human liver disease. *J Lipid Res.* **23**: 124-132:1999.

11) Garnier, M., Hanss, M. and Paraf, A.: Erythrocyte filterability reduction and membrane lipids in liver cirrhosis. *Clin Hemorheol Micricirc.* **3**: 45-52, 1983.

12) Barefoed, D., Stine, P.C.W., Caldwell, N.M. and Stuart, J.: Erythrocyte morphology as a determinant of abnormal erythrocyte deformability in liver disease. *Clin Hemorheol Micricirc.*, **5**: 473-481: 1985.

13) Balls, S. K., Dover, J. G., and Charache, S.: Effect of hydroxyurea on the rheologic properties of sickle erythrocytes in vivo. *Am. J. Hemat.*, **32**, 104-111, 1989.

14) Rodgers, G.P., Dover, G.J., Uyesaka, N., Noguchi, C.T., Schechter, A.N. and Nienhuis, A.W.: Augmentation by erythropoietin of the fetal-hemoglobin response to hydroxyurea in sickle cell disease. *N. Engl. J. Med.*, **328**, 73-80, 1993.

15) Child, C.G.: The liver and portal hypertension. *Major Probl. Clin. Surg.* **1**: 1-85,1964.

16) Pugh, R.N.H., Murray-Lyon, I.M., Dawson, J.L., Pietroni, M.C. and Williams, R.: Transection of the oesophagus for bleeding oesophageal varices. *Br. J. Surg.* **60**: 649-649, 1973.

17) Ejima, J., Ijuchi, T., Ohnishi, Y., Maruyama, T., Kaji, Y., Kanaya, S., Fujino, T., Uyesaka, N. and Ohmura, T.: Relationship of high-density lipoprotein cholesterol and red blood cell filterability: cross-sectional study of healthy subjects. *Clin. Hemorheol. Micricirc.* **22**: 1-7 2000.

18) Okamoto, K., Maruyama, T., Kaji, Y., Harada, M., Mawatari, S., Fujino, T. and Uyesaka, N.: Verepamil prevents impairment in filterability of human erythrocytes exposed to oxidative stress. *Jpn. J. Physiol.* **54**: 39-46, 2004.

19) Oonishi, T., Sakashita, K. and Uyesaka, N.: Regulation of red blood cell filterability by $Ca^{2+}$ influx and cAMP-mediated signaling pathways. *Am. J. Physiol.* **273**: C1828-C1834, 1997.

20) Mohandas, N. and Chasis, J.A.: Red cell deformability, membrane material properties and shape: regulation by transmembrane, skeletal and cytosolic proteins and lipids. *Semin. Hematol.* **30**: 171-192, 1993.

21) Shah, S.H., Hayes, P.C., Allan, P.L., Nicoll, J. and Finlayson, N.D.: Measurement of spleen size and its relation to hypersplenism and portal hemodynamics in portal hypertension due to hepatic cirrhosis. *Am. J. Gastroenterol.* **91**: 2580-2583, 1996.

22) Moriyasu, F., Nishida, O., Ban, N., Nakamura, T., Miura, K., Sakai, M., Miyake, T. and Uhino, H.: Measurement of portal vascular resistance in patients with portal hypertension. *Gasteroenterol.* **90**: 710-717, 1986.

23) Pries, A.R., Reglin, B. and Secomb, T.W.: Structural adaptation of microvascular networks: functional roles of adaptive responses. *Am. J. Physiol.* **281**: H1015-H1025, 2001.

24) O' Rear, E.A., Udden, M.M., Farmer, J.A., McIntire, L.V. and Lynch, E. C.: Increased intracellar calcium and decreased deformability of erythrocytes from prosthetic hart valve patients. *Clin. Hemorheol.* **4**: 461-471, 1984.

25) Johnson, R.M. and Tang, K.: Induction of $Ca^{2+}$-activated $K^{+}$ channel in human erythrocytes by mechanical stress. *Biochim. Biophys. Acta* **1107**: 314-318, 1992.

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# Timing of interferon therapy and sources of infection in patients with acute hepatitis C

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**Abstract**

*Background/Aims:* Controversy over the selection of patients and optimum therapeutic method for acute hepatitis C has continued. The aims of this study were to investigate the source of infection, and to evaluate the timing of interferon (IFN) therapy in patients with acute hepatitis C in Japan.
*Methods:* The records of 102 patients from 12 facilities in Japan who developed acute hepatitis C after 1990 were investigated. In the patients treated with IFN, we performed multivariate analysis to investigate factors related to sustained virological response (SVR).
*Results:* Medical procedure was the most common source of infection, accounting for 32.4% in the 102 patients (33/102). Of 81 patients treated with IFN, 71 patients were followed after IFN therapy, and 57/71 (80.3%) had SVR. The SVR rate was significantly higher in patients treated with IFN within 24 weeks from onset of symptoms than the SVR rate in those treated after 25 weeks ($P = 0.0016$). Multivariate analysis revealed that only the duration between onset of symptoms and initiation of IFN therapy (within 24 weeks) was related to SVR.

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*Abbreviations:* HCV, hepatitis C virus; IFN, interferon; ALT, alanine aminotransferase; SVR, sustained virological response; Peg-IFN, pegylated interferon
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*Conclusions:* Our multicenter cooperative survey revealed that medical procedure was the most frequent source of infection in acute hepatitis C. As concerns the therapy, interferon treatment should be initiated within 24 weeks after onset of symptoms.


*Keywords:* Hepatitis C virus (HCV); Acute hepatitis; Medical procedure; Interferon

## 1. Introduction

There are about 170 million people infected with the hepatitis C virus (HCV) worldwide [1], and the infection progresses to hepatic cirrhosis in 10–30% [1,2]. Since patients often lack subjective symptoms even in acute hepatitis C [3], infection is often realized by patients when the pathology progresses to hepatic cirrhosis and hepatocellular carcinoma. There are a variety of sources of infection, such as medical procedure, intravenous drug use, and sexual behavior [4,5]. In addition, vertical transmission of HCV has been reported, and it seems that maternal viral load is significant for infection to fetus [6]. On the other hand, as a therapy for acute hepatitis C, interferon (IFN) administration has been established to be effective [4,5,7–13].

Although the initial prevention of hepatitis C virus (HCV) infection is ideal, the most effective method of preventing progression to the chronic hepatitis C is still controversial in the acute phase. In Japan, the development of acute hepatitis C due to blood transfusion has markedly decreased after introduction of the HCV antibody test for screening of blood donors [14]. However, infection from intravenous (i.v.) drug use and incidences due to accidental contamination of medical staff are still important problems [15,16]. Investigation for the sources of infection in acute hepatitis C is very important for the prevention. In this study, we investigated a national survey on the route of infection of acute hepatitis C and the therapeutic effectiveness according to the timing of IFN therapy. This survey consists of the largest number of case reports and may reflect the current situation of acute hepatitis C in Japan.

## 2. Patients and methods

### 2.1. Patients

A retrospective study was performed in patients of 12 facilities nationwide who developed acute hepatitis C after 1990. The total number of patients at the facilities was 102. Informed written consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Age, gender, source of infection, HCV serotype or genotype, HCV-RNA level, histology of liver biopsy, fluctuation in alanine aminotransferase (ALT) level, presence or absence of IFN therapy, course when not treated with IFN, duration between onset of symptoms and IFN therapy, type of IFN, total dose of IFN, administration method, total duration of administration, and therapeutic results were investigated in each patient.

### 2.2. Diagnosis of acute hepatitis C

The diagnostic criteria of acute hepatitis C were HCV-RNA detectable at the time of an elevated ALT level, followed by development conversion of HCV antibody. Patients in whom HCV antibody was already positive at the onset were excluded.

### 2.3. Natural course

In patients who followed the natural course without any treatments, the chronic hepatitis was defined as persistence of HCV-RNA positivity for 6 months or longer, and resolution was defined as a disappearance of serum HCV-RNA within 6 months followed by persistent negativity for 6 months or longer.

### 2.4. Definition of fluctuation of ALT

In patients diagnosed with acute hepatitis C, when one peak of the serum ALT level was observed, the fluctuation was designated as monophasic, and when two or more peaks were observed, the fluctuation was designated as bi- or multiphasic.

### 2.5. Serologic tests

Anti-HCV antibody was determined using a second-generation or third-generation enzyme-linked immunosorbent assay (Ortho Diagnostics Systems, Tokyo, Japan). Hepatitis C virus RNA was quantified by using the bDNA signal amplification assay (Chiron Corp.) or the Cobas Amplicor HCV Monitor test ver1.0 or 2.0 (Roche Diagnostic Systems, Tokyo, Japan). The data were represented as Meq/ml, K copies/ml, and KIU/ml, respectively. Detection of HCV-RNA to determine the response of IFN treatment was used by Amplicor HCV (Roche Diagnostics K.K., Japan). Hepatitis C virus serotype was determined using the genotyping enzyme-linked immunosorbent assay (International Reagents Corporation, Tokyo, Japan) to be type 1 or 2 [17].

### 2.6. IFN therapy

For IFN, IFN-α (natural form, gene recombinant, or consensus IFN), or IFN-β was used (Table 4). No concurrent treatment with IFN and ribavirin was administered to any patient. Among patients treated with IFN, the sustained

virological response (SVR) was defined undetectable HCV-RNA in serum at least 6 months after cessation of therapy. Non-response was defined as detectable HCV-RNA for 6 months after cessation of therapy.

### 2.7. Statistical analysis

Data were expressed as the mean ± standard deviation for continuous variables and as counts for categorical variables. The results were compared using the Chi-square test, Fisher's exact probability test, or Mann–Whitney *U*-test, depending upon the type of data analysed. Logistic regression was used to analyse the factors contributing to SVR with IFN therapy. *P* values <0.05 were considered significant. Statistical analyses were performed by using Stat View software (version 5.0; SAS Institute Inc., Cary, NC).

## 3. Results

### 3.1. Patient characteristics

The baseline characteristics of the 102 patients in this study are shown in Table 1. The distribution of patients by gender and age is shown in Table 2.

### 3.2. Natural course

The natural course of the disease was followed in 21 patients, and the course could be followed to the outcome in 18 patients (the prognosis was unknown in three patients) (Table 3). The disease progressed to chronic hepatitis C in 61.1% of the patients and resolved spontaneously in 38.9% of the patients. The age and the fluctuation pattern of the ALT level were significantly different between the two groups. As for gender, serum HCV-RNA level, and serogroup, no correlation with spontaneous resolution or chronic hepatitis C was observed.

Table 1
Base-line characteristics of 102 patients

| | |
|---|---|
| Age | 38.6 ± 16.2 (16–84) |
| Male/female (mean age) | 46 (39.2 ± 16.0)/56 (38.2 ± 16.5) |
| Source of infection (%) | |
| Medical procedure | 33 (32.4) |
| Accidental needle stick | 21 (20.6) |
| Sexual behavior | 8 (7.8) |
| Drug abuse | 6 (5.9) |
| Tattoo | 3 (2.9) |
| Unknown | 31(30.4) |
| Viral load (high[a]/low/N.D.) | 46/45/11 |
| HCVserotype(1/2/N.D.) | 54/23/25 |
| IFN/without IFN | 81/21 |

N.D., not determined; IFN, interferon. Details of the routes in medical procedure: surgery 14, blood transfusion 5, endoscopy 3, intravenous injection 4, invasivc proccdurc 3, dcntal thcrapy 3, dialysis 1.
[a] Viral load (high): more than 100 KIU/ml or 1 Meq/ml.

### 3.3. IFN therapy

Table 4 shows the backgrounds of the 81 patients treated with IFN. Of 71 patients in whom the effect was clarified,

Table 2
Distribution of patients according to gender and age

| Age (years) | Number of patients | | | | | |
|---|---|---|---|---|---|---|
| | Medical procedure (M/F) | Accidental needlestick (M/F) | Sexual behavior (M/F) | Drug abuse (M/F) | Tattoo (M/F) | Unknown (M/F) |
| <19 | 0/1 | 0/0 | 0/0 | 0/1 | 0/0 | 0/1 |
| 20–29 | 5/1 | 3/8 | 1/3 | 2/1 | 3/0 | 2/6 |
| 30–39 | 4/3 | 3/3 | 2/1 | 0/1 | 0/0 | 3/3 |
| 40–49 | 2/4 | 0/4 | 1/0 | 0/1 | 0/0 | 2/3 |
| 50–59 | 4/3 | 0/0 | 0/0 | 0/0 | 0/0 | 2/3 |
| 60–69 | 4/1 | 0/0 | 0/0 | 0/0 | 0/0 | 2/0 |
| 70–79 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 1/1 |
| >80 | 0/1 | 0/0 | 0/0 | 0/0 | 0/0 | 0/2 |
| Total | 19/14 | 6/15 | 4/4 | 2/4 | 3/0 | 12/19 |

M, male, F, female.

Table 3
Base-line characteristics of 18 untreated patients

| | Resolved group (seven cases) | Chronic group (11 cases) | *P* value |
|---|---|---|---|
| Age | 64.4 ± 15.2 | 45.6 ± 14.3 | 0.0331[a] |
| Gender (male/female) | 2/5 | 4/7 | >0.9999 |
| HCV RNA level (high[b]/low/N.D.) | 2/4/1 | 6/4/1 | 0.6084 |
| Serogroup (1/2/N.D.) | 4/0/3 | 4/2/5 | 0.4667 |
| Fluctuation of ALT level (monophasic/bi- or multiphasic/N.D.) | 5/0/2 | 0/8/3 | 0.0008[a] |

N.D., not determined; ALT, alanine aminotransferase. Fluctuation of ALT level: monophasic; one peak of the serum ALT was observed, bi- or multiphasic; two or more peaks of the serum ALT were observed (N.D. was excluded from statistical comparisons).
[a] Statistically significant.
[b] Viral load (high): more than 100 KIU/ml or 1 Meq/ml.

Table 4
Base-line characteristics of 81 patients treated with interferon

| | |
|---|---|
| Age | 38.6 ± 16.2 |
| Gender (male/female) | 43/38 |
| HCV RNA level (high[a]/low/N.D.) | 38/36/7 |
| HCV serogroup (1/2/N.D.) | 46/21/14 |
| Fluctuation of ALT level (monophasic/bi-or multiphasic/N.D.) | 21/53/7 |
| Type of IFN (α/β) | 63/18 |
| Total IFN dose (MU) | 470 ± 228.1 (52–972) |
| Duration of IFN administration (w) | 17.6 ± 8.9 (4.0–42.0) |
| Outcome (SVR[b]/NR/N.D.) | 57/14/10 |

N.D., not determined; ALT, alanine aminotransferase; IFN, interferon; MU, million units; SVR, sustained virological response; NR, non-response: detectable HCV RNA in serum for 6 months after cessation of therapy.
[a] HCV RNA level (high): more than 100 KIU/ml or 1 Meq/ml.
[b] Sustained virological response: undetectable HCV RNA in serum at least 6 months after cessation of therapy.

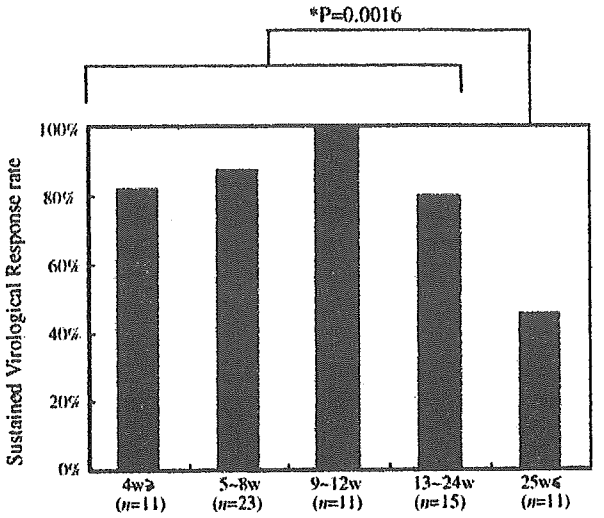


Fig. 1. Sustained virological response rate according to duration between onset of symptoms and initiation of IFN therapy. The groups treated with IFN 0–24 weeks after onset of symptoms and treated after 25 weeks were compared. Comparison by the Chi-square test. (*) Statistically significant; w, week.

57 patients (80.3%) had SVR. Table 5 shows the results of the logistic regression analysis of SVR-related factors. Age, gender, serogroup, HCV-RNA level, fluctuation of ALT, duration between onset and initiation of IFN, type of IFN, total IFN dose, and duration of IFN administration were evaluated statistically by univariate and multivariate analysis. On multivariate analysis as well as univariate analysis, the duration between onset of symptoms and initiation of IFN therapy was the only factor related to SVR.

The SVR rate according to the duration before initiation of IFN therapy was investigated (Fig. 1), and the SVR rate was found to be significantly higher in patients treated before 24 weeks than in patients treated after 25 weeks. However, immediate administration has not been associated with higher SVR rate (0–8 weeks versus 9–24 weeks).

On comparison of the SVR rate by the source of infection, the SVR rate was 100% in the patients infected by accidental needlestick (19/19) (the prognosis was unknown in two of 21 patients infected by needlestick). This was significantly higher than that in patients infected via other routes (19/19 versus 38/52, $P < 0.05$). The duration between onset of symptoms and initiation of IFN therapy was investigated according to the source of infection, and the duration was shortest in the needlestick group (9.7 ± 5.3 weeks).

## 4. Discussion

We examined the source of infection and optimal timing of therapy in patients with acute hepatitis C at 12 facilities in Japan. Since there has been no study performed in more than 100 patients with acute hepatitis C in Japan, this study may reflect the current situation in Japan. HCV serogroup of 25 patients were not determined (Table 1). Several reasons are considered. Firstly, the study is retrospective. Secondly,

Table 5
Logistic regression analysis of odds ratio for sustained virological response

| Variable | Odds ratio | 95% CI | *P* value |
|---|---|---|---|
| Univanate | | | |
| Age(40>/40≤) | 2.48 | 0.73–8.46 | 0.147 |
| Gender (female/male) | 2.48 | 0.74–8.33 | 0.143 |
| Serogroup (1/2) | 1.03 | 0.23–4.54 | 0.969 |
| HCV RNA level (high[a]/low) | 1.75 | 0.46–6.68 | 0.413 |
| Fluctuation of ALT (monophasic/bi-or multiphasic) | 1.57 | 0.38–6.45 | 0.531 |
| Duration between onset and initiation of IFN (≤24w/≥25w) | 7.50 | 1.85–30.48 | 0.005[b] |
| Type of IFN (alpha/beta) | 4.33 | 0.52–36.18 | 0.176 |
| Total IFN dose (>300MU/≤300MU) | 2.27 | 0.63–8.15 | 0.208 |
| Duration of IFN administration (≥24w/<24w) | 1.43 | 0.44–4.67 | 0.551 |
| Multivariate | | | |
| Duration between onset and initiation of IFN (≤24w/≥25w) | 15.78 | 1.37–181.61 | 0.027[b] |

ALT, alanine aminotransferase; IFN, interferon; MU, million units; 95% CI, 95% confidence interval.
[a] HCV RNA level high: More than 100 KIU/ml or 1 Meq/ml.
[b] Statistically significant.

titer of anti-HCV is often low in early phase of acute hepatitis C. Many patients were considered to be infected during a medical procedure. Studies on risk of surgery for the development of acute hepatitis C have been reported previously [18]. Alfonso et al. performed a large-scale surveillance in Italy and found that 25.5% of patients (261/1023) with acute hepatitis C had undergone an invasive procedure. Therefore, medical care should be recognized as an important source of infection in the sporadic incidence of acute hepatitis C. On the other hand, in blood donors of Western Mexico, the most frequent risk factors for HCV transmission were transfusion (42%) and household exposure (14.8%) [19]. Therefore, the main risk factors for infection may differ with countries.

Since IFN therapy for acute hepatitis C is not covered by the health care insurance, the therapy could not be administered to all patients. The progression to the chronic hepatitis C in the 18 patients with natural courses without IFN therapy was almost consistent with previous reports [20,21]. As shown in Table 3, a significant difference was observed in age, but this may have been due to the two patients in their 80s in the spontaneous resolution group (data not shown). The important point is that the ALT fluctuation was monophasic in all patients in the spontaneous resolution group. In contrast, the fluctuation was bi- or multiphasic in patients who progressed to chronic hepatitis C. As a characteristic of acute hepatitis C in which spontaneous elimination of the virus is likely to occur, it has been reported that many cases are accompanied by subjective symptoms, such as jaundice and influenza-like symptoms [22,23]. Subjective symptoms are sometimes influenced by the patient's subjective sense. In contrast, the fluctuation of the ALT level may be a more objective index. Hofer et al. observed the natural course for at least 30 days after onset, and when serum HCV-RNA became negative during this period, the disease was resolved at a high rate, suggesting that IFN therapy should be administered to patients in whom negative conversion of HCV-RNA did not occur within 30 days [22]. Combined with our results, it might be likely that the disease resolves spontaneously in patients in whom the ALT level followed the monophasic course, as well as in those in whom the disease is symptomatic and negative conversion of HCV-RNA occurs in the early stage.

As the results of IFN therapy, the SVR rate was 80.3% (57/71) as shown in Table 4. Our present study, albeit retrospective analysis, revealed that therapy initiated within 24 weeks was the only factor related to the SVR in both univariate and multivariate analysis (Table 5). In the randomized controlled study by Hwang et al., the factor related to SVR was the HCV-RNA level before initiation of therapy [9]. However, there were only 33 patients, which may have led to a result different from our results. On the other hand, Nomura et al. recently performed a randomized controlled trial in patients with acute hepatitis C, and their results demonstrate that the SVR rate was significantly higher in the early-intervention group (IFN therapy was initiated 8 weeks after the onset) than in the late-intervention group (IFN therapy was initiated after 1 year observation from the onset) (87% versus 40%) [24]. Otherwise, Gruner et al. prospectively investigated the T-cell dynamics in patients with acute hepatitis C, and found that activity of HCV-specific IFN-γ-producing T cells started to decrease 24 weeks after onset [25]. In addition, T cell actions have been reported to be important for elimination of HCV in the early stage of infection [26–30], and the defective functions of HCV-specific T cells might contribute to viral persistence in chronically infected patients [31]. It is interesting that our results support their reports.

Next, we evaluated the optimal timing of initiation of therapy within 24 weeks. In our previous study, we administered therapy after observation of the course for about 4 weeks when signs of the chronic hepatitis began to appear, not immediately after the onset, and obtained good results [32,33]. Licata et al. investigated the optimum timing of IFN therapy by meta-analysis [34]. Their analysis shows that delaying therapy 2 months after the onset of the disease does not affect the efficacy of treatment, therefore, they suggest that patients should be treated within 60 days from the onset to avoid the unnecessary treatment of affected patients who would spontaneously recover. In our study, the highest SVR rate was obtained in the group treated 9–12 weeks after onset of symptoms as shown in Fig. 1, which was consistent with their analytical results.

The SVR rate obtained by combination therapy with Pegylated-IFN (Peg-IFN) and ribavirin for chronic hepatitis C was 30–54% [35–37], but for acute hepatitis C, the therapeutic result was good even when IFN was administered alone. To elucidate this difference, it may be important to investigate not only the T-cell dynamics but also viral genome in various aspects [7]. In our present study, no patients were treated with Peg-IFN. Recently, the efficacy of Peg-IFN monotherapy with acute hepatitis C has been reported. Santantonio et al. evaluated the delaying Peg-IFN therapy, targeting sixteen patients who failed to spontaneously clear the virus within 12 weeks from the onset. They reported that 15/16 patients (94%) showed SVR [38]. Since the highest SVR was obtained in the group treated 9–12 weeks after onset in our study, it is important to start the IFN therapy in optimal timing regardless of the kind of IFN. The high SVR has been obtained by IFN monotherapy, so that, it is necessary to investigate whether ribavirin should be administered concurrently with IFN.

In conclusion, the major sources of infection of acute hepatitis C in Japan were the medical procedure and accidental needlestick. The disease may be likely to resolve spontaneously in patients in whom fluctuation of the ALT level follows the monophasic course. The SVR rate was significantly higher in the group treated with IFN within 24 weeks after the onset of symptoms than in the group treated after 25 weeks. In cases of acute hepatitis C, it is desirable to administer IFN at least within 24 weeks when the ALT level starts to follow a multiphasic course.

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## References

[1] Alter HJ, Seeff LB. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin Liver Dis 2000;20:17–35.
[2] Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. Lancet 1997;349:825–32.
[3] Alberti A, Boccato S, Vario A, Benvegnu L. Therapy of acute hepatitis C. Hepatology 2002;36(Suppl.):195–200.
[4] Jaeckel E, Cornberg M, Wedemeyer H, et al. Treatment of acute hepatitis C with interferon alfa-2b. N Engl J Med 2001;345:1452–7.
[5] Komine F, Yamaguchi T, Moriyama M, et al. A case of sexually transmitted acute hepatitis C: confirmation by analysis of viral genome. Hepatol Res 1999;14:84–91.
[6] Kowala-Piaskowska A, Figlerowicz M, Mozer-Lisewska I, et al. Vertical transmission of hepatitis C virus infection. Hepatol Res 2004;30:137–40.
[7] Saito T, Watanabe H, Shao L, et al. Transmission of hepatitis C virus quasi species between human adults. Hepatol Res 2004;30:57–62.
[8] Omata M, Yokosuka O, Takano S, et al. Resolution of acute hepatitis C after therapy with natural beta interferon. Lancet 1991;338:914–5.
[9] Hwang SJ, Lee SD, Chan CY, Lu RH, Lo KJ. A randomized controlled trial of recombinant interferon alpha-2b in the treatment of Chinese patients with acute post-transfusion hepatitis C. J Hepatol 1994;21:831–6.
[10] Takano S, Satomura Y, Omata M. Effects of interferon beta on non-A, non-B acute hepatitis: a prospective, randomized, controlled-dose study. Gastroenterology 1994;107:805–11.
[11] Lampertico P, Rumi M, Romeo R, et al. A multicenter randomized controlled trial of recombinant interferon-alpha 2b in patients with acute transfusion-associated hepatitis C. Hepatology 1994;19:19–22.
[12] Vogel W, Graziadei I, Umlauft F, et al. High-dose interferon-α2b treatment prevents chronicity in acute hepatitis C. A pilot study. Dig Dis Sci 1996;41(Suppl.):81–5.
[13] Camma C, Almasio P, Craxi A. Interferon as treatment for acute hepatitis C. A meta-analysis. Dig Dis Sci 1996;41:1248–55.
[14] Japanese Red Cross non A, non B-hepatitis Research Group. Effect of screening for hepatitis C virus antibody and hepatitis B virus core antibody on incidence of post-transfusion hepatitis. Lancet 1991;338:1040–1.
[15] Noguchi S, Sata M, Suzuki H, Ohba K, Mizokami M, Tanikawa K. GB virus C (GBV-C)/hepatitis G virus (HGV) infection among intravenous drug users in Japan. Virus Res 1997;49:155–62.
[16] Kiyosawa K, Sodeyama T, Tanaka E, et al. Hepatitis C in hospital employees with needlestick injuries. Ann Intern Med 1991;115:367–9.
[17] Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, et al. Significance of specific antibody assay for genotyping of hepatitis C virus. Hepatology 1994;19:1347–53.
[18] Mele A, Spada E, Sagliocca L, et al. Risk of parenterally transmitted hepatitis following exposure to surgery or other invasive procedures: results from the hepatitis surveillance system in Italy. J Hepatol 2001;35:284–9.
[19] Vivas-Arceo C, Benavides SA, De Jesus Trujillo J, et al. Hepatitis C virus: prevalence and routes of infection among blood donors of West Mexico. Hepatol Res 2003;25:115–23.
[20] Alter MJ, Margolis HS, Krawczynski K, et al. The natural history of community-acquired hepatitis C in the United States. N Engl J Med 1992;327:1899–905.
[21] Tanaka E, Kiyosawa K. Natural history of acute hepatitis C. J Gastroenterol Hepatol 2000;15(Suppl.):97–104.
[22] Hofer H, Watkins-Riedel T, Janata O, et al. Spontaneous viral clearance in patients with acute hepatitis C can be predicted by repeated measurements of serum viral load. Hepatology 2003;37:60–4.
[23] Gerlach JT, Diepolder HM, Zachoval R, et al. Acute hepatitis C: high rate of both spontaneous and treatment-induced viral clearance. Gastroenterology 2003;125:80–8.
[24] Nomura H, Sou S, Tanimoto H, et al. Short-term interferon-alfa therapy for acute hepatitis C: a randomized controlled trial. Hepatology 2004;39:1213–9.
[25] Gruner NH, Gerlach TJ, Jung MC, et al. Association of hepatitis C virus specific CD8+ T cells with viral clearance in acute hepatitis C. J Infect Dis 2000;181:1528–36.
[26] Diepolder HM, Zachoval R, Hoffmann RM, et al. Possible mechanism involving T-lymphocyte response to non-structural protein 3 in viral clearance in acute hepatitis C virus infection. Lancet 1995;346:1006–7.
[27] Missale G, Bertoni R, Lamonaca V, et al. 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 1996;98:706–14.
[28] Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. J Exp Med 2001;194:1395–406.
[29] Thimme R, Bukh J, Spangenburg HC, et al. Viral and immunological determinants of hepatitis C virus clearance, persistence, and disease. Proc Natl Acad Sci USA 2002;99:15661–8.
[30] Ulsenheimer A, Gerlach JT, Gruener NH, et al. Detection of functionally altered hepatitis C virus-specific CD4+ T cells in acute and chronic hepatitis C. Hepatology 2003;37:1189–98.
[31] Wedemeyer H, He XS, Nascimbeni M, et al. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J Immunol 2002;169(6):3447–58.
[32] Sata M, Hashimoto O, Noguchi S, et al. Transmission routes and clinical course in sporadic acute hepatitis C. J Viral Hepat 1997;4:273–8.
[33] Sata M, Ide T, Noguchi S, et al. Timing of IFN therapy initiation for acute hepatitis C after accidental needlestick. J Hepatol 1997;27(2):425–6.
[34] Licata A, Bona DD, Schepis F, Shahied L, Craxi A, Camma C. When and how to treat acute hepatitis C? J Hepatol 2003;39:1056–62.
[35] Heathcote EJ, Schiffman ML, Cooksley WG, et al. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. N Engl J Med 2000;343:1673–80.
[36] Zeuzem S, Feinman SV, Rasenack J, et al. Peginterferon alfa-2a in patients with chronic hepatitis C. N Engl J Med 2000;343:1666–72.
[37] 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;358:958–65.
[38] Santantonio T, Fasano M, Sinisi E, et al. Efficacy of a 24-week course of PEG-interferon alpha-2b monotherapy in patients with acute hepatitis C after failure of spontaneous clearance. J Hepatol 2005;42:329–33.

研究紹介

# C型肝炎ウイルス持続感染者に対する薬物療法
## ―インターフェロン療法の普及とその現状―

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優れた新しい医薬品や薬物療法は、広く医療現場に普及し患者に用いられることによって、初めて大きな価値を持つ。しかし、それらが普及していくのは、必ずしも容易なことではない。

人口動態統計によれば、2003年時点で日本人の死因のトップは悪性新生物（がん）によるもので、その数は年々増加の傾向にある。1990年頃までは胃がんがトップを占めていたが、最近では肺がんや肝がんの増加が目立っている。わが国では、2003年に第3次対がん10ヵ年総合戦略が策定されるなど、これらのがんの予防や治療法の開発・普及が唱えられている。

### 肝がんと肝炎ウイルス

日本における肝がん患者は3万人を超えている。その死亡率は他のアジア諸国とほぼ同等だが、欧米と比較するとかなり高い水準にある[1)]。この主な原因はB型あるいはC型肝炎ウイルス（それぞれHBV、HCV）といわれており、これらのウイルスに感染した患者は、将来肝がん発症のリスクを背負うことになる。

日本では肝がんの原因の約80%はHCVの持続感染に起因するものである。HCVに感染した場合、そのまま放置すると70%前後が持続感染の状態となり、さらに慢性肝炎へと進展する。その後20～30年を経て、さらにその半数が肝硬変、肝がんへと進展する。

かつては、持続感染したHCVを排除する手段はなく、慢性肝炎へ進行すると殆どの場合は自然治癒が望めず、肝がんへの進行を阻止することもできなかった。しかし、1980年代後半になり、HCVを患者の身体から排除し、肝炎の治癒、あるいは肝硬変への進展や肝がん発症に対しての抑制効果をもたらす画期的な薬剤として、インターフェロン（IFN）が使われはじめた。当初は、B型慢性肝炎だけの適応であったが、1989年にHCVが発見され、1992年にはC型肝炎の治療薬としても使用されるようになった。また、2001年には経口抗ウイルス薬リバビリンとIFNの併用、2003年には効果の持続性に優れるペグインターフェロンなど新たな治療法が保険適用とされており、これらの治療によるウイルス駆除例や肝炎鎮静例では、肝線維化の改善、肝がん発生の抑止、さらに生命予後の改善が明らかにされるなど治療成果をあげている。

現在わが国では、HCVに持続感染している人は、150万人以上存在すると推測されている。HCV持続感染者（HCVキャリア）は、自覚症状がない

1）国立がんセンター「がんの統計'05」における2001年の肝および肝内胆管の悪性新生物による死亡率（人口10万人当たり）は、日本で男性38.3、女性16.7、以下それぞれ米国：5.8、3.4、イギリス：2.0、0.8、フランス：13.9、3.1、ドイツ8.2、5.0、中国35.7、14.9、韓国32.5、10.0となっている。

ことが多いため、感染していることを自覚しないままに慢性肝炎から肝硬変や肝がんに進展する例が多くみられ、適切な時期に治療を受ける機会のない感染者が存在することが問題となっている[2,3]。そのため、厚生労働省では、平成14年度より「C型肝炎等緊急総合対策」の一環として、地域住民を対象とした「肝炎ウイルス検診」(HCV並びにHBV)を開始した。この検診には、40歳から70歳までの老人保健法に基づく健康診査の受診者に対し5歳刻みに実施する節目検診と、過去に肝機能異常を指摘されたなど、早期に検査を受ける必要のある人を対象とした節目外検診[4]がある。

## 普及していないインターフェロン(IFN)療法

IFNがC型肝炎の治療薬として承認されてから13年が経過している。IFN療法は、インフルエンザ様症状やうつ症状が発現しやすいなどの副作用が伴うことによる制約があるものの、徐々に製剤の改良や治療法の改善が図られ、有効性が向上し、使用し易くなった結果、現在ではC型慢性肝炎の第一選択薬として肝臓専門医では高く評価されるに至っている。

ところが、平成15年度のアンケート調査から得られた全国調査(節目検診、節目外検診)によると、HCV陽性で要精密検者数20,364名のうち二次医療機関へ受診した者7,769名の中で、何らかの治療を受けた者の割合は24%であり、このうちIFN治療が行われた割合は13%であったと報告されている。約半数は専門医への受診をしておらず、専門医への受診率向上が課題と考えられている[5]。

現在われわれが行っている調査では、ある地域のHCV感染患者と医師を対象にアンケートを実施し、IFN療法が医療現場にどの程度普及しているのか、患者と医師とのIFN療法に対する認識はどの程度違いがあるのか、そしてあるべき治療と現実の間にギャップを生んでいる要因は何かを明らかにしようとしている。

本アンケート調査は現在実施中の段階にあるが、2006年1月6日までに回答の得られた患者および医師108例分のデータから、医療現場におけるIFN療法の実状の一部を紹介したい。

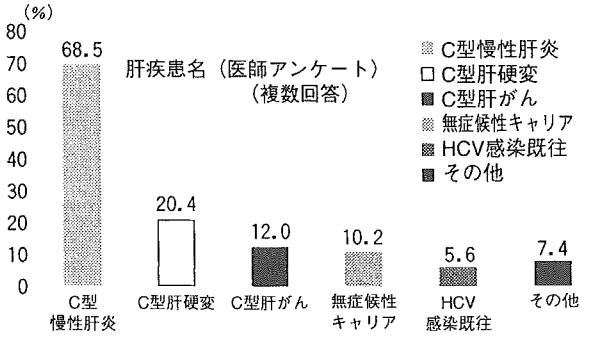
## 調査の対象と方法

ある地域の医療機関8施設(診療所7、病院1)において、通院患者の中でHCV感染者とその患者を診療している医師に対してそれぞれアンケート調査を実施した。

調査項目としては、患者、医師それぞれに、①患者背景、②IFN療法について、③IFN療法を行わなかった場合はその理由、などを尋ねている。

この度集計した段階では、対象患者108名の通院先は、診療所102名、病院6名であり、年代別では、約7割が60歳以上であった。図1に示すように、診断名はC型慢性肝炎が7割近くを占めていた。

図1　対象患者の診断名



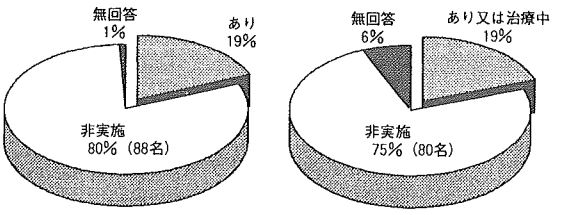

2)厚生労働省「C型肝炎について(一般的なQ&A)」(2003年8月)(改訂V版)
3)厚生労働省「C型肝炎対策等の一層の推進について」(2005年8月2日)C型肝炎対策等に関する専門家会議
4)節目外検診:上記以外の節目検診の対象とならない者のうち、以下の人に対して実施する検診
・過去に肝機能異常を指摘されたことのある者
・広範な外科的処置を受けたことのある者又は妊娠・分娩時に多量に出血したことのある者であって定期的に肝機能検査を受けていない者
・基本健康診査の結果、ALT(GPT)値により要指導とされた者
5)沖田極「厚生科学研究費補助金肝炎等克服緊急対策研究事業(肝炎分野)肝炎ウイルス検診要精検者の二次医療機関への受診状況に関する全国調査」

## これまでの集計結果

(1) IFN 療法の実施状況

IFN 療法については、図2に示されるように、多くの患者で実施されていなかった。

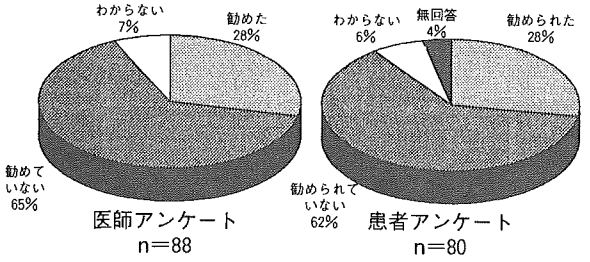
図2　IFN 療法の実施状況




(2) IFN 療法の推奨について

多くの患者に IFN 療法は実施されていなかったが、背景にどのような経緯があったのだろうか。図3は、IFN 非実施患者に対する、医師から患者への IFN 療法の推奨状況を示したものである。これによると、6割以上の患者に対し、医師は IFN 療法を勧めていなかった。

図3　IFN 療法非実施患者への IFN 推奨状況




また、図4には、IFN 療法を受けていない患者が、IFN 療法を推奨された際にどのような判断をしたかを示している。ここでは、IFN 療法が勧められているにも拘らず、8割を超える患者が受療を断っていた。なお、IFN 療法を受けた患者については医師からの推奨の有無を尋ねていないが、これらの患者は推奨を受けこの治療を受けるに至ったと考えられ、これを併せて集計すると、IFN 療法を断った患者は、それを勧められた患者全体の4割強であった（図5）。

図4　IFN 療法推奨に対する患者の判断
（IFN 療法非実施患者のみ）

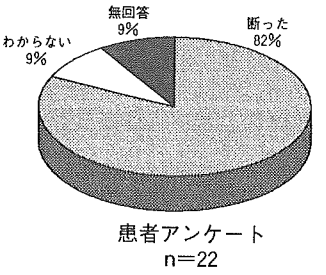
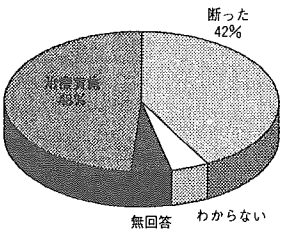



図5　IFN 療法推奨に対する患者の判断
（IFN 療法実施者を含めた場合）




## 今後の集計・解析に向けて

以上はあくまで途中経過であり、今後回収が進む予定の病院（専門医）による回答があまり反映されていない。しかし、現時点まで得られたデータからも IFN 療法を実施された患者は少ないこと、また多くの場合、医師からも勧められていないことが明らかになっている。今後さらに集計を進め、何故医師が IFN 療法を勧めなかったのか、何故患者は勧められても断ったのか、IFN 療法についての理解が医師と患者の双方において進んでいるのか、などについて、医師が専門医か一般医か、患者の診断名は何か、といった様々な属性別分析も加えることにより、解析していく。新しい医薬品や薬物療法が普及しにくい原因を考察し、その対応策について何らかの示唆が得られるのではないかと考える。

# Nucleotide Mutations Associated With Hepatitis B e Antigen Negativity

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One hundrded and forty four patients with chronic hepatitis B were tested to identify new mutations associated with hepatitis B e antigen (HBeAg) negativity, using a full genome sequence analysis. All the patients were Chinese and had hepatitis B virus infection of genotype C. Patients with none of the pre-core or core promoter mutations were significantly ($P < 0.001$) less common in the group with anti-HBe (13%) than in the group with HBeAg (56%). The complete nucleotide sequence was determined in four anti-HBe-positive patients who had neither pre-core nor core promoter mutations and in five HBeAg-positive patients who also had neither of these mutations (the groups were matched for age and sex). Six mutations were found to be significantly more common in the former group than in the latter: G529A (3/4 vs. 0/5), C934A (4/4 vs. 1/5), A1053G (4/4 vs. 1/5), G1915T/A (4/4 vs. 0/5), T2005C/A (4/4 vs. 0/5), and C3026T (3/4 vs. 0/5). Three of the six mutations were significantly more common in the four anti-HBe-positive patients who had neither pre-core nor core promoter mutations, compared to 11 HBeAg-positive patients who had pre-core and core promoter mutations, and also compared to 15 anti-HBe-positive patients who had pre-core and core promoter mutations, suggesting further the specificity of these mutations. Of the six mutations, two resulted in amino acid substitution in the polymerase protein, and one is located near the enhancer I region. The results suggest that the six newly discovered mutations are associated with HBeAg negativity. ***J. Med. Virol. 76:170–175, 2005.*** © 2005 Wiley-Liss, Inc.

**KEY WORDS:** hepatitis B e antigen (HBeAg); genotype; nucleotide mutation



## INTRODUCTION

Approximately 350 million people are chronic carriers of hepatitis B virus (HBV) worldwide [Maynard, 1990; Maddrey, 2000]. Chronic HBV infection is the cause of up to 50% of cirrhosis and 70–90% of hepatocellular carcinomas (HCC) in China, South-East Asia, and Africa [Lok, 1992; Fattovich, 1998], and in Asian countries, almost all patients with chronic HBV infection have been infected perinatally from hepatitis B e antigen (HBeAg)-positive mothers [Okada et al., 1976]. HBeAg is considered to be a marker for viral replication, but some HBeAg-negative patients remain viremic and continue to have active liver disease [Hadziyannis et al., 1983; Lok et al., 1984; Bonino et al., 1986]. Many of these patients are found to have a G to A change at nucleotide 1896, which creates a stop codon (TAG) in the precore (Pre-C) open reading frame, which in turn prevents translation of the Pre-C protein and aborts HBeAg production [Carman et al., 1989]. Other patients have mutations in the core promoter (CP) region, including an A to T mutation at nucleotide 1762 and a G to A mutation at nucleotide 1764 [Okamoto et al., 1994]. In vitro studies of this double mutation show decreased transcription of Pre-C messenger RNA and hence a resultant decrease in HBeAg production by 70% [Buckwold et al., 1996; Chan et al., 1999]. A recent follow-up study on Pre-C and CP mutations has also

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shown that the presence of these mutations is useful for predicting seroconversion [Yamaura et al., 2003].

Besides the G1896A mutation and the A1762T/G1764A mutation, a number of point mutations, as well as deletions and insertions of nucleotides, have been detected in the Pre-C region and CP region that could correlate with seroconversion [Okamoto et al., 1990; De Castro et al., 2001]. In the present study, the complete HBV genome was examined for other nucleotide mutations associated with HBeAg negativity, in addition to mutations in the Pre-C and CP regions.

## MATERIALS AND METHODS

### Patients

A cohort of 193 Chinese patients with chronic HBV infection who visited the Liver Disease Clinic of the Second Hospital of HeBei Medical University in Shijiazhuang city, North China, between June and August 2001 were enrolled in the study. These patients comprised 124 men and 69 women and had a median age of 29.1 years old (range: 5–73 years old). Patients who were co-infected with hepatitis C or D virus or with the human immunodeficiency virus and patients with other concomitant causes of chronic liver disease were excluded. According to the consensus diagnostic criteria for HBV infection, 182 patients were diagnosed with chronic hepatitis B. The remaining 11 patients had persistently normal alanine aminotransferase (ALT: normal range 10–21 IU/L) levels, suggesting an inactive HBV carrier stage. None of the 193 patients were treated with antiviral agents such as interferon or lamivudine. Of the 193 patients, 169 (87.6%) were of genotype C, 21 (10.9%) of genotype B, and 3 (1.5%) of genotype A. For the mutation analysis, 144 patients who were positive for either HBeAg or anti-HBe were selected from the 169 genotype C patients. Informed consent was obtained from each patient.

### Conventional HBV Markers and Genotyping of HBV

Hepatitis B surface antigen (HBsAg), HBeAg and anti-HBe were measured using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan). Serum concentration of HBV DNA was measured using the AMPLICOR HBV Monitor test (Roche Diagnostics K.K., Tokyo, Japan), which has a quantitative range of 2.6–7.6 log copies/ml. When the concentration to be tested was beyond this range, the actual concentration was determined using a serum sample diluted 100-fold with normal human serum. The HBV genotype was determined using the restriction fragment length polymorphism (RFLP) method on an S-gene sequence amplified by polymerase chain reaction (PCR) with nested primers [Mizokami et al., 1999].

### Determination of Pre-C and CP Mutations

The 1,896th nucleotide in the Pre-C region of G or A was detected with an enzyme-linked mini-sequence assay kit (Roche Diagnostics), and the results were expressed as the percentage mutation rate, as defined by Aritomi et al. [1998]. If the mutation rate was 0%, the strain was considered to be Pre-C mutation-negative, while a Pre-C mutation-positive strain was recorded when the mutation rate exceeded 0%. The double mutation in the CP region (A1762/T1764) was detected using an HBV CP mutation detection kit (Smitest: Genome Science Laboratories, Tokyo, Japan), and the results were classified into three categories: wild, mixed, and mutant types. A wild type strain was considered to be CP mutation-negative, while mixed and mutant types were recorded as CP mutation-positive strains. The detection limits of the pre-C and the CP mutation detection kits are both 1,000 copies/ml.

### Determination of Nucleotide Sequence

The complete genome sequence was determined according to the method described by Rokuhara et al. [2000]. Briefly, nucleic acids were extracted from a serum sample of 100 μl with a DNA/RNA extraction kit (Smitest EX-R&D: Genome Science Laboratories Co., Ltd.). Two microliters of each DNA solution were used for amplification by PCR. The reaction was carried out in 25 μl of PCR-mixture containing 250 μmol/L of each dNTP, 1× PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 0.001% gelatin], 0.25 U EX-Taq DNA polymerase (TaKaRa, Tokyo), and 0.25 μM of a primer pair. The PCR was initiated using the hot-start technique.

To determine the full-length nucleotide sequence of HBV, two fragments (fragments A and B) were amplified by PCR, using the primers shown in Table I. Fragment A (1,498 bases in length; nt 457–nt 1954) was amplified with nested pairs of outer (SB1 and CB2) and inner primers (SB3 and CB4), while fragment B (2162 bases in length; nt 1611–nt 557), was amplified with nested pairs of outer primers (es2 and PS4) and inner primers (is2 and PS3). The first round of PCR was performed with an outer primer set for 40 cycles (94°C for 1.5 min, 55°C for 1 min, and 72°C for 2 min), and was followed by an extension reaction at 72°C for 7 min. The second round was undertaken with an inner primer set for 30 cycles, and was also followed by an extension reaction. PCR products were subjected to electrophoresis on a 1.0% agarose gel with ethidium bromide staining and visualization with an UV transilluminator. The band containing the target sequence was removed and DNA was isolated using GFX™ PCR DNA and a Gel Band Purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The nucleotide sequence was directly determined by the dideoxy method, using the sequencing primers shown in Table I. The accuracy of the sequence was ensured by comparison of the sequence data for the complete genome obtained with sense-sequencing primers and that obtained with anti-sense-sequencing primers.

### Statistical Analysis

Mann–Whitney's *U* test was utilized for quantitative data, and Fisher's exact test and a Chi-square test were

TABLE I. Primers Used for PCR and Sequencing of HBV DNA

| Primer | | Sequence | nt position |
|---|---|---|---|
| Primers for PCR of fragment A | | | |
| SB1 | Sense | 5-TGCTGCTATGCCTCATCTTC | (414–433) |
| CB2 | Anti-sense | 5-GGAAAGAAGTCAGAAGGCAA | (1974–1955) |
| SB3 | Sense | 5-AGGTATGTTGCCCGTTTCTC | (457–476) |
| CB4 | Anti-sense | 5-AAAAGAGAGTAACTCCACAG | (1954–1935) |
| Primers for PCR of fragment B | | | |
| es2 | Sense | 5-ACGTCGCATGGAGACCACCG | (1601–1620) |
| PS4 | Anti-sense | 5-CAGTTTCCGTCCGAAGGTTTTG | (594–573) |
| is2 | Sense | 5-GAGACCACCGTGAACGCCCA | (1611–1630) |
| PS3 | Anti-sense | 5-GAAACATAGAGGTGCCTTGAGCAG | (557–534) |
| Primers for sequencing | | | |
| SB3 | Sense | 5-AGGTATGTTGCCCGTTTCTC | (457–476) |
| as1 | Anti-sense | 5-TGCGAAAGCCCAGGATGATG | (631–612) |
| s2 | Sense | 5-TGCGAAAGCCCAGGATGATG | (760–783) |
| as2 | Anti-sense | 5-AGTTGGCGAGAAAGTGAAAGCCTG | (1107–1084) |
| s3 | Sense | 5-CTCTGCCGATCCATACTGCGGAA | (1256–1278) |
| as3 | Anti-sense | 5-CGGGACGTAGACAAAGGACGT | (1434–1414) |
| is2 | Sense | 5-GAGACCACCGTGAACGCCCA | (1611–1630) |
| ea1 | Anti-sense | 5-TGAAAAAGTTGCATGGTGCTGGTG | (1827–1804) |
| s4 | Sense | 5-TATCGGGAGGCCTTAGAGTCTCCG | (2012–2035) |
| as4 | Anti-sense | 5-ATAGGGGCATTGGTCT | (2314–2298) |
| s5 | Sense | 5-CGCAGAAGATCTCAATCTCGG | (2417–2437) |
| as5 | Anti-sense | 5-GGATAGAACCTAGCAGGCAT | (2654–2635) |
| s6 | Sense | 5-GGGTCACCATATTCTTGGGAA | (2814–2834) |
| as6 | Anti-sense | 5-GGGTTGAAGTCCCAATCTGGATT | (2987–2965) |
| is1 | Sense | 5-AAGCTCTGCTAGATCCCAGAGT | (18–39) |
| ea2 | Anti-sense | 5-TAGAAAATTGAGAGAAGTCCACCA | (280–257) |
| s1 | Sense | 5-CATCCTGCTGCTATGCCTCATC | (409–430) |
| as1 | Anti-sense | 5-TGCGAAAGCCCAGGATGATG | (631–612) |

Nucleotides are numbered from the unique *Eco*RI site of HBV.

used for qualitative data. $P$ values less than 0.05 were considered significant. Analyses were carried out using SPSS version 10.0J (SPSS Inc., Chicago, IL).

## RESULTS

Of the 144 patients selected for mutation analysis, 90 (62.5%) were HBeAg-positive and the remaining 54 (37.5%) were anti-HBe-positive. The clinical and virological backgrounds of the two groups of patients are compared in Table II. The 90 HBeAg-positive patients tended to be younger and have a higher concentration of HBV DNA than the 54 anti-HBe patients. Patients with none of the Pre-C and CP mutations were significantly ($P < 0.001$) more common in the HBeAg-positive patients (56%) than in the anti-HBe-positive patients (13%).

A comparison of the clinical background of seven anti-HBe-positive patients who had neither Pre-C nor CP mutations and 47 anti-HBe-positive patients who had at least one of the mutations is shown in Table III. Distributions of age, gender, ALT level, and HBV DNA concentration did not differ between the two groups.

Nucleotide sequences of the complete genome were determined in four out of seven anti-HBe-positive patients who had neither Pre-C nor CP mutations and in 5 out of 50 HBeAg-positive patients who also had neither mutation. All nine of the genome sequences

TABLE II. Comparison of Clinical and Virological Backgrounds of Patients With HBeAg and Those With Anti-HBe

| | HBeAg-positive n = 90 | Anti-HBe-positive n = 54 | $P$ |
|---|---|---|---|
| Age[a] | 25 (5–53) | 36 (11–73) | <0.001[b] |
| Gender (M:F) | 58:32 | 30:24 | >0.2[c] |
| ALT[a] | 89 (11–2100) | 62 (13–458) | >0.2[b] |
| HBV DNA (log copies/mL)[a] | 8.3 (4.4–7.9) | 5.0 (3.2–8.8) | <0.001[b] |
| Pre-C/CP mutations | | | |
| Both negative | 50 (56%) | 7 (13%) | <0.001[c] |
| Pre-C mutation only | 13 (14%) | 20 (37%) | |
| CP mutation only | 12 (13%) | 5 (9%) | |
| Both positive | 15 (17%) | 22 (41%) | |

[a]Data are expressed as median values (range).
[b]Mann–Whitney test.
[c]Chi-square test.

TABLE III. Comparison of Clinical and Virological Backgrounds of Anti-HBe-Positive Patients With Neither Pre-C nor CP Mutations and Anti-HBe Patients With at Least one of These Mutations

| | Pre-C and CP mutation-negative n = 7 | Pre-C and/or CP mutation-positive n = 47 | P |
|---|---|---|---|
| Age[a] | 37 (18–60) | 36 (11–73) | >0.2[b] |
| Gender (M:F) | 4:3 | 26:21 | >0.2[c] |
| ALT[a] | 44 (18–86) | 65 (13-458) | 0.17[b] |
| HBV DNA (log copies/ml)* | 4.7 (3.3–5.5) | 5.0 (3.2–8.8) | >0.2[b] |

[a]Data are expressed as median values (range).
[b]Mann–Whitney test.
[c]Chi-square test.

determined had nucleotide lengths of 3,215 bases, and thus there were no insertions or deletions. When the full genome sequences were compared, the six mutations shown in Table IV were significantly more common in the four anti-HBe-positive patients than in the five HBeAg-positive patients. The positions of the six mutations in the HBV genome are shown in Figure 1. Of the four mutations located in the polymerase gene, the G529A and C934A mutations cause amino acid substitutions in the polymerase protein. The C3026T mutation does not cause an amino acid substitution in the polymerase, but rather in the pre-S1 protein, while the A1053G mutation does not lead to an amino acid substitution, but the mutation is located near the enhancer I region. The G1915T/A and T2005C/A mutations are located in the core gene, but do not result in an amino acid substitution. Patients with at least one of the three mutations (G529A, C934A, and A1053G) which might affect HBV replication had a significantly ($P = 0.029$) lower level of HBV DNA (n = 22, median 5.3 copies/ml, range 3.8–8.9) than those patients who had no mutations (n = 13, median 8.5 copies/ml, range 3.8–8.9).

To examine further the specificity of the six mutations, these mutations were also determined in 11 HBeAg-positive patients who were positive for Pre-C and CP mutations and in 15 anti-HBe-positive patients who were also positive for Pre-C and CP mutations. The frequencies of the six mutations were compared between groups of patients classified according to their HBeAg/anti-HBe and Pre-C/CP mutation status. Three (G1915T/A, T2005C/A, and C3026T) of the six mutations were found to be significantly more common in anti-HBe-positive patients who had neither a Pre-C nor a CP mutation than in the two groups of patients with Pre-C and CP mutations, as shown in Table V.

The nucleotide sequence data reported in this paper have been registered in the DDBJ/EMBL/GenBank nucleotide sequence databases, with the accession numbers AB198076-84.

## DISCUSSION

Studies to date have shown that the stop codon mutation in the Pre-C region (G1896A) and the double mutation in the CP region (A1762T/G1764A) are independently associated with the seroconversion of HBeAg, and that the Pre-C mutation is more directly associated with seroconversion than the core promoter mutation [Okamoto et al., 1994; Yamaura et al., 2003]. Only a small number of anti-HBe-positive patients (13%) were both negative for the Pre-C and CP mutations, and in the present study this rate was significantly lower than that (56%) in HBeAg-positive patients. These results are consistent with previous reports, suggesting that the two mutations are the main causes of seroconversion. However, there are also patients in whom HBeAg secretion discontinues without

TABLE IV. Comparison of Full Nucleotide Sequences of HBV With Neither Pre-C nor CP Mutations for HBeAg-Positive and Anti-HBe-Positive Patients

| Nucleotide mutation | Amino acid substitution (viral protein) | HBeAg Pre-C and CP mutation-negative n = 5 | Anti-HBe Pre-C and CP mutation-negative n = 4 | P |
|---|---|---|---|---|
| G529A | D480N (P)<br>None (S) | 0 | 3 | 0.048 |
| C934A | L615I (P) | 1 | 4 | 0.040 |
| A1053G | None (P) | 1 | 4 | 0.040 |
| G1915T/A | None (C) | 0 | 4 | 0.008 |
| T2005C/A | None (C) | 0 | 4 | 0.008 |
| C3026T | A60V (Pre-S1)<br>None (P) | 0 | 3 | 0.048 |

Six mutation sites with significant differences are shown. Data are expressed as the number of positives. Statistical analysis was performed with a chi-square test. P, polymerase protein; S, surface protein; C, core protein; Pre-S1, pre-surface 1 protein.

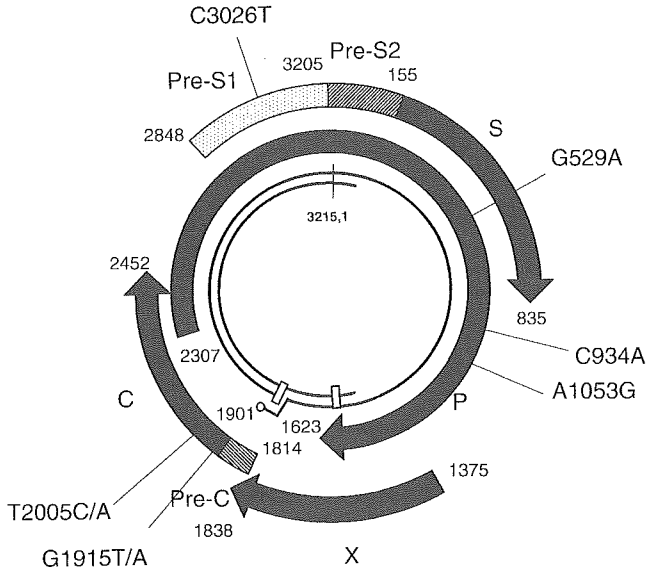


Fig. 1. Organization of the hepatitis B virus genome (genotype C) and the approximate positions of the six nucleotide mutations in the HBV genome. The inner circles represent the minus and plus DNA strands of the viral genome. The different open reading frames encoded by the genome, designated as S, C, P, and X, are indicated by the arrows. Abbreviations: S, surface antigen; C, core; P, polymerase; Pre-C, precore.

appearance of Pre-C and/or CP mutations. Thus, we speculated that some other mutations might be associated with HBeAg seroconversion. A variety of other mutations in the CP and Pre-C regions have been detected in previous studies [Carman et al., 1989; Tillmann et al., 1995; Baumert et al., 1996; Laras et al., 1998; Chan et al., 2000; De Castro et al., 2001; Yoo et al., 2003], but other regions of the HBV genome have not been analyzed sufficiently for mutations associated with HBeAg seroconversion.

When the full nucleotide sequences of HBV genomes of HBeAg-positive and anti-HBe-positive patients with neither Pre-C nor CP mutations were compared, six mutations (G529A, C934A, A1053G, G1915T/A, T2005C/A, C3026T) were found to be significantly more common in the anti-HBe-positive patients. The six mutations were also more common in anti-HBe-positive patients who had neither Pre-C nor CP mutations than in HBeAg-positive patients or in anti-HBe-positive patients who had Pre-C and CP mutations, with the results being statistically significant for three (G1915T/A, T2005C/A, C3026T) of the six mutations. These results suggest that the six mutations are associated with HBeAg negativity.

The mechanisms through which the six mutations facilitate HBeAg negativity were not investigated in the present study. However, some possible mechanisms can be considered, based on the locations of these mutations in the HBV genome. The G529A and C934A mutations cause amino acid substitutions in the polymerase protein. Thus, these two mutations may attenuate HBV replication through changes in the enzymatic activity of the polymerase. The A1053G mutation is located near the enhancer I region, which may affect the replication of HBV [Bock et al., 2000]. Patients who had at least one of the three mutations associated with HBV replication tended to have a lower level of HBV DNA than those who had none of these mutations, providing further support for a replication-associated mechanism. It has been reported that amino acid substitutions in immunogenic epitopes in the core protein are found most frequently during or after seroconversion from HBeAg to anti-HBe [Akarca and Lok, 1995; Carman et al., 1995]. We found two mutations in the core gene, but these mutations did not cause amino acid substitutions. Thus, the mechanisms through which the G1915T/A and T2005C/A mutations exert their effects remains unclear.

In anti-HBe-positive patients, the clinical background, including the mean age, gender distribution, ALT level and HBV DNA level, were similar in patients with and without Pre-C and/or CP mutations. Although these comparisons were cross-sectional, the results suggest that mutations other than those in the Pre-C and CP regions have a similar impact in patients in whom seroconversion occurs, compared to Pre-C and CP mutations.

The six mutations identified in the present study have not been described previously. These mutations

TABLE V. Comparison of Six Mutations Among Three Groups Classified According to Their HBeAg/anti-HBe and Pre-C/CP Mutation Status

| Mutation site | Anti-HBe Pre-C and CP mutation-negative n = 4 | HBeAg Pre-C and/or CP mutation-positive n = 11 | Anti-HBe Pre-C and/or CP mutation-positive n = 15 |
|---|---|---|---|
| G529A | 3 | 3 | 3 |
| C934A | 4 | 6 | 10 |
| A1053G | 4 | 4 | 9 |
| G1915T/A | 4[a] | 3 | 1 |
| T2005C/A | 4[b] | 3 | 4 |
| C3026T | 3[c] | 0 | 1 |

Data are expressed as the number of positives. Statistical analysis was performed with Fisher's exact test. Other comparisons were not statistically significant.
[a]$P = 0.026$ versus 11 patients with HBeAg, and $P = 0.001$ versus 15 patients with anti-HBe.
[b]$P = 0.026$ versus 11 patients with HBeAg, and $P = 0.018$ versus 15 patients with anti-HBe.
[c]$P = 0.009$ versus 11 patients with HBeAg, and $P = 0.016$ versus 15 patients with anti-HBe.

are thought to be associated with HBeAg negativity because they were found specifically in anti-HBe-positive patients with neither a Pre-C nor a CP mutation. However, several issues remain to be resolved to clarify the real significance of the six mutations, including the mechanisms through which they facilitate HBeAg negativity, their universality in genotypes other than genotype C, and their clinical relevance. Furthermore, it is possible that immune-based selection pressures that cause loss of HBeAg are responsible for the selection of the mutations identified in the present study [Locarnini, 2004]. Therefore, it is not possible to conclude that the new mutations are definitely associated with seroconversion, but they do provide new clues regarding the nature of seroconversion.

## ACKNOWLEDGMENTS

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## REFERENCES

Akarca US, Lok AS. 1995. Naturally occurring core-gene-defective hepatitis B viruses. J Gen Virol 76:1821–1826.

Aritomi T, Yatsuhashi H, Fujino T, Yamasaki K, Inoue O, Koga M, Kato Y, Yano M, 1998. Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan. J Gastroenterol Hepatol 13:1125–1132.

Baumert TF, Rogers SA, Hasegawa K, Liang TJ. 1996. Two core promoter mutations in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest 98:2268–2276.

Bock CT, Malek NP, Tillmann HL, Manns MP, Trautwein C. 2000. The enhancer I core region contributes to the replication level of hepatitis B virus in vivo and in vitro. J Virol 74:2193–2202.

Bonino F, Rosina F, Rizzetto M, Rizzi R, Chiaberge E, Tardanico R, Callea F, Verme G. 1986. Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. Gastroenterology 90:1268–1273.

Buckwold VE, Xu Z, Chen M, Ou JH. 1996. Effects of a naturally occurring mutation in the hepatitis virus basal core promoter on precore gene expression and viral replication. J Viral 70:5845–5851.

Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet ii:588–590.

Carman WF, Thursz M, Hadziyannis S, McIntyre G, Colman K, Gioustoz A, Fattovich G, Alberti A. 1995. Hepatitis B e antigen negative chronic active hepatitis: Hepatitis B virus core mutations occur predominantly in known antigenic determinants. J Viral Hepat 2:77–84.

Chan HLY, Hussain M, Lok ASF. 1999. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 29:976–984.

Chan HLY, Leung NW, Hussain M, Wong ML, Lok AS. 2000. Hepatitis B e antigen-negative chronic hepatitis B in Hong Kong. Hepatology 31:763–768.

De Castro L, Niel C, Gomes SA. 2001. Low frequency of mutations in the core promoter and precore regions of hepatitis B virus in anti-HBe positive Brazilian carriers. BMC Microbiol 1:10.

Fattovich G. 1998. Progression of hepatitis B and C to hepatocellular carcinoma in Western countries. Hepatogastroenterology 45:1206–1213.

Hadziyannis SJ, Lieberman HM, Karvountzis GG, Shafritz DA. 1983. Analysis of liver disease, nuclear HBcAg, DNA viral replication, and hepatitis B virus DNA in liver and serum of HBeAg vs. anti-HBe positive carriers of hepatitis B virus. Hepatology 3:656–662.

Laras A, Koskinas J, Avgidis K, Hadziyannis SJ. 1998. Incidence and clinical significance of hepatitis B virus precore gene translation initiation mutations in e antigen-negative patients. J Viral Hepatitis 5:241–248.

Locarnini S. 2004. Molecular virology of hepatitis B virus. Semin Liver Dis 24:3–10.

Lok ASF. 1992. Natural history and control of perinatally acquired hepatitis B virus infection. Dif Sis 10:46–52.

Lok ASF, Hadziyannis S, Weller IVD, Karvountzis MV, Monjardino J, Karayiannis P, Montano L, Thomas HC. 1984. Contribution of low level HBV replication to continuing inflammatory activity in patients with anti-HBe positive chronic hepatitis B virus infection. Gut 25:1283–1287.

Maddrey WC. 2000. Hepatitis B: An important public health issue. J Med Virol 61:362–366.

Maynard JE. 1990. Hepatitis B: Global importance and need for control. Vaccine 8:S18–20.

Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. 1999. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS lett 450:66–71.

Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y. 1976. E antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N Engl J Med 294:746–749.

Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. 1990. Hepatitis B viruses hosts along with seroconversion to the antibody against e antigen. J Virol 64:1298–1303.

Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol 68:8102–8110.

Rokuhara A, Tanaka E, Yagi S, Mizokami M, Hashikura Y, Kawasaki S, Kiyosawa K. 2000. De novo infection of hepatitis B virus in patients with orthotopic liver transplantation: Analysis by determining complete sequence of the genome. J Med Virol 62:471–478.

Tillmann H, Trautwein C, Walker D, Michitaka K, Kubicka S, Boker K, Manns M. 1995. Clinical relevance of mutations in the precore genome of the hepatitis B virus. Gut 37:568–573.

Yamaura T, Tanaka E, Matsumoto A, Rokuhara A, Orii K, Yoshizawa K, Miyakawa Y, Kiyosawa K. 2003. A case-control study for early prediction of hepatitis B e antigen seroconversion by hepatitis B virus DNA levels and mutations in the precore region and core promoter. J Med Virol 70:545–552.

Yoo BC, Park JW, Kim HJ, Lee DH, Cha YJ, Park SM. 2003. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. J Hepatol 38:98–103.