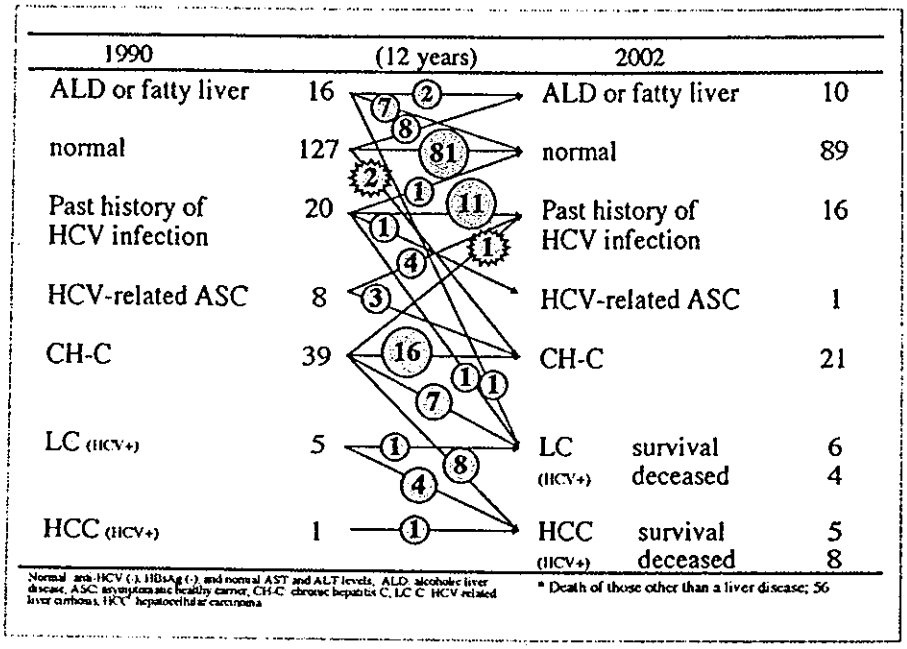

Figure 5. Outcome of 216 persons with liver disease (HBV carriers are omitted) identified among the inhabitants a follow-up after 12 years.

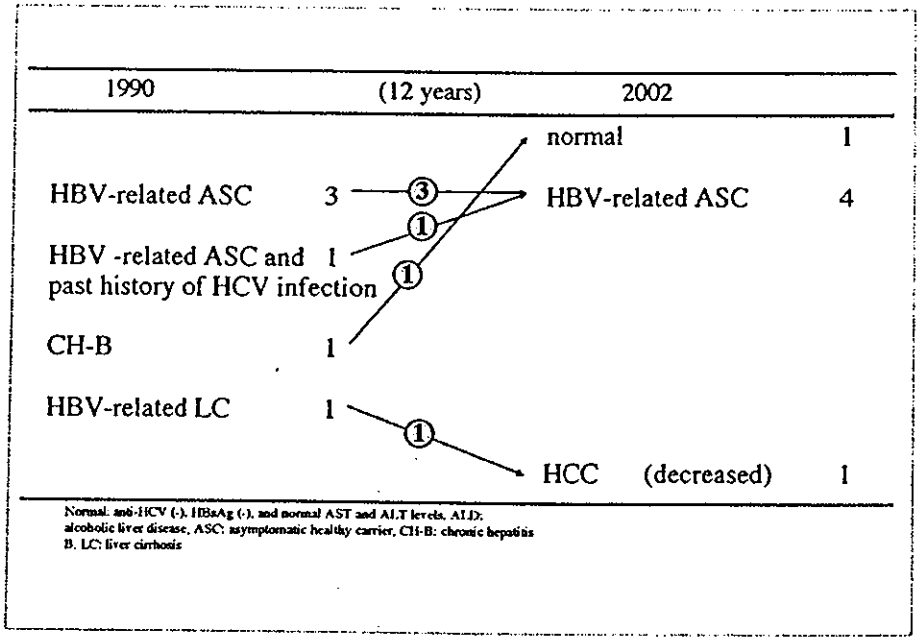

Figure 6. Outcome of 6 HBV carriers identified among the inhabitants in a follow-up after 12 years.

also an HBV-related asymptomatic healthy carrier in 2002. The other person who was an HBV- and HCV-related asymptomatic healthy carrier as well as the remaining person with chronic hepatitis B and C in 1990 had unknown liver disease in 2002.

Discussion

Hepatitis C is a global health problem caused by HCV infection. However, there is a great geographic difference in the prevalence of HCV infection. Memon and Memon reviewed the literature on the epidemiology of HCV infection from 1991 to 2000 (14). In healthy volunteer blood donors, the incidence of anti-HCV is 0.05% in Germany (15), 0.01% in Northern Ireland (16), 0.03% in Italy (17), 0.088% in Scotland (18), 0.35% in the UK (19), 0.17-0.5% in USA (20-22), 0.78% in Australia (23), 0.93-1.2% in Spain (24,25), 0.19-2.2% in Japan (26-29), and up to 24.8% in Egypt (30). The incidence of infection as determined by anti-HCV in healthcare professionals dealing with blood and blood

products is ever higher in each country (31-41). Furthermore, certain groups of individuals such as intravenous drug users have an increased risk of acquiring HCV infection irrespective of their geographical location (14). However, in Japan, it had been reported that there were some areas where the general incidence of HCV infection is extremely high in persons who are not intravenous drug users (7-11,42-47). In Japan the incidence of anti-HCV in local residents is 19.7% (403/2,046) in H village in Fukuoka prefecture (42), 14.1% (158/1,122) in Iki Island in Nagasaki prefecture (43), 17.7% (77/435) in Area-O of town T in Akita prefecture (44), 20.7% (493/2,382) in N city in Yamagata prefecture (45), 22% (671/3,117) in the southern part of Japan (46), 23.6% (120/509) in H town in Fukuoka prefecture (7), and 28.5% (82/288) in Y town in the Setonaikai sea (47). The most likely reason for the high prevalence of HCV infection in these areas is considered to be iatrogenic infection through insufficient sterilization of needles and/or syringes for treatment in the same clinic (7,42,44).

We conducted a follow-up survey after 12 years for the 509 inhabitants who were examined for liver disease in an HCV hyperendemic area (7). In terms of the area (H town), we previously reported that medical treatment was considered to be a causative route of HCV transmission (8), that most HCV carriers died from HCC or liver cirrhosis (11), and that the prevalence of various extrahepatic manifestations in HCV carriers was higher than in those without HCV (12,13). As shown in Fig. 5, after 12 years, of the 39 residents with chronic hepatitis C, 7 suffered from liver cirrhosis and 8 developed HCC. That is, in 12 years, the incidence of HCC that originated from chronic hepatitis C was 20.5% (1.7% annual rate). Four out of 5 persons with liver cirrhosis who were diagnosed in 1990 had died from HCC during these 12 years. In other words, the incidence of HCC that originated from liver cirrhosis was 80% (6.7% annual rate). The 1.7% annual rate of HCC developing from chronic hepatitis C and the 6.7% annual rate of HCC developing from liver cirrhosis in H town's general population closely resemble the rate of HCC that develops in HCV-infected patients who consulted the hospital, which has been reported up to the present time. The incidence of HCC that originates from chronic hepatitis C and liver cirrhosis in examination of patients who consulted the hospital is reported to be 2 and 7-8% respectively, in Japan (3,4). Ikeda *et al* reported that the appearance rate of HCC in 349 patients only with positive anti-HCV was 21.5% in the 5th year, 53.2% in the 10th year, and 75.2% in the 15th year (4). This is the first report of the natural course of HCV infection in 'the general population'.

Of the 69 persons who died, the mortality rate caused by HCC or liver cirrhosis was 44 and 53%, respectively, among 25 persons with positive anti-HCV, and 19 with positive HCV RNA. The HCV or HBV carriers had significantly higher mortality rates from liver cirrhosis and HCC than those who were not carriers ($P<0.00001$). We reported that the ALT value and HCV RNA were associated with deaths due to HCC or liver cirrhosis by multivariate analyses (11). In other words, eradicating persistent HCV infection or normalizing the ALT value would lead to controlling the development of HCC.

On the other hand, during the 12-year observation period, 2 (1.4%) of the 143 inhabitants without HCV infection became infected with HCV. The appearance rate of new HCV-infected residents was 0.12%/year in this area. In our previous report of the same subjects from 1990 to 1995, the seroconversion rate of HCV was 0.28%/year (8). Of 287 subjects negative at the first examination in 1990, 4 became positive by 1995 (8). Medical treatment is considered the causative route of HCV transmission. We consider that not only medical treatment for HCV carriers but education of medical staff who work in H town, such as doctors, dentists, nurses, and dialysis staff, is required in order to prevent new infection and to acquire correct knowledge of liver disease.

Recently, IFN therapy in patients with chronic hepatitis and liver cirrhosis was shown to be associated with a reduced incidence of HCC (48). Yoshida *et al* reported on data from a large surveillance program of patients with chronic hepatitis C (49). In multivariate analysis, IFN therapy was associated with a reduced risk for HCC compared with untreated controls (adjusted risk ratio, 0.516).

We prospectively studied 509 consecutive residents in a HCV hyperendemic area of Japan for 12 years. In conclusion, the early detection and treatment for HCC should be carried out as HCV carriers are aging. As Yoshizawa reported (2), persistent HCV carriers should receive aggressive IFN therapy to eradicate HCV, or anti-inflammatory therapies to suppress the development of HCC, or HCC should be diagnosed at the early stage for early treatment and prolonged life span. We should eradicate of HCC as a national goal.

Acknowledgments

This study was supported, in part, by grants as a project for establishing new high technology research centers, by a Grant-in-Aid for Scientific Research (C) (No. 11670548), by a Grant-in-Aid for Young Scientists (B) (No. 14770256) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Research on Hepatitis and BSE (2001-2003) under the auspices of the Ministry of Health, Labor and Welfare.

References

1. Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J Viral Hepatitis 6: 35-47, 1999.
2. Yoshizawa H: Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: projection to other countries in the foreseeable future. Oncology 62 (Suppl. 1): 8-17, 2002.
3. Higuchi M, Tanaka E and Kiyosawa K: Epidemiology and clinical aspects on hepatitis C. Jpn J Infect Dis 55: 69-77, 2000.
4. Ikeda K, Saitoh S, Koida I, *et al*: A multivariate analysis of risk factors for hepatocellular carcinogenesis: a prospective observation of 795 patients with viral and alcoholic cirrhosis. Hepatology 18: 47-53, 1993.
5. Niederau C, Lange S, Heintges T, *et al*: Prognosis of chronic hepatitis C: results of a large, prospective cohort study. Hepatology 28: 1687-1695, 1998.
6. Hayashi J, Yoshimura E, Nabeshima A, *et al*: Seroepidemiology of hepatitis C virus infection in hemodialysis patients and the general population in Fukuoka and Okinawa, Japan. J Gastroentol 29: 276-281, 1994.
7. Sata M, Nakano H, Suzuki H, *et al*: Sero-epidemiologic study of hepatitis C virus infection in Fukuoka, Japan. J Gastroenterol 33: 218-222, 1998.
8. Fukuizumi K, Sata M, Suzuki H, Nakano H and Tanikawa K: Hepatitis C virus seroconversion rate in a hyper-endemic area of HCV in Japan: a prospective study. Scand J Infect Dis 29: 345-347, 1997.

9. Fukuizumi K, Sata M, Suzuki H, Kumashiro R and Tanikawa K: Natural disappearance of serum HCV RNA; prospective study in a hyperendemic area. Hepatol Res 9: 144-151, 1997.
10. Noguchi S, Sata M, Suzuki H, Mizokami M and Tanikawa K: Routes of transmission of hepatitis C virus in an endemic rural area of Japan. Molecular epidemiologic study of hepatitis C virus infection. Scand J Infect Dis 29: 23-28, 1997.
11. Nagao Y, Fukuizumi K, Tanaka K, Kumashiro R and Sata M: The prognosis for life in an HCV hyperendemic area. Gastroenterology 125: 628-629, 2003.
12. Nagao Y, Sata M, Fukuizumi K, Tanikawa K and Kameyama T: High incidence of oral precancerous lesions in a hyperendemic area of hepatitis C virus infection. Hepatol Res 8: 173-177, 1997.
13. Nagao Y, Sata M, Fukuizumi K, Ryu F and Ueno T: High incidence of oral lichen planus in an HCV hyperendemic area. Gastroenterology 119: 882-883, 2000.
14. Memon MI and Memon MA: Hepatitis C: an epidemiological review. J Viral Hepatitis 9: 84-100, 2002.
15. Schottstedt V, Tuma W, Bunger G and Lefevre H: PCR for HBV, HCV and HIV-1 experiences and first results from a routine screening programme in a large blood transfusion service. Biologicals 26: 101-104, 1998.
16. Morris K and Bharucha C: Completed hepatitis C lookback in Northern Ireland. Transfus Med 7: 269-275, 1997.
17. Prati D, Capelli C, Silvani C, *et al*: The incidence and risk factors of community-acquired hepatitis C in a cohort of Italian blood donors. Hepatology 25: 702-704, 1997.
18. Crawford RJ, Gillon J, Yap PL, *et al*: Prevalence and epidemiological characteristics of hepatitis C in Scottish blood donors. Transfus Med 4: 121-124, 1994.
19. Mutimer DJ, Harrison RF, O'Donnell KB, *et al*: Hepatitis C virus infection in the asymptomatic British blood donor. J Viral Hepatitis 2: 47-53, 1995.
20. Anderson SC, Hathaway T, Kuramoto IK, *et al*: Comparison of two second-generation anti-hepatitis C virus ELISA on 21431 US blood donor samples. J Viral Hepatitis 2: 55-61, 1995.
21. Murphy EL, Bryzman S, Williams AE, *et al*: Demographic determinants of hepatitis C virus seroprevalence among blood donors. JAMA 275: 995-1000, 1996.
22. Chuang TY, Brashear R and Lewis C: Porphyria cutanea tarda and hepatitis C virus: a case-control study and meta-analysis of the literature. J Am Acad Dermatol 41: 31-36, 1999.
23. Allain JP, Coghlan PJ, Kenrick KG, *et al*: Prediction of hepatitis C virus infectivity in seropositive Australian blood donors by supplemental immunoassays and detection of viral RNA. Blood 78: 2462-2468, 1991.
24. Lissen E, Alter HJ, Abad MA, *et al*: Hepatitis C virus infection among sexually promiscuous groups and the hetero-sexual partners of hepatitis C virus infected index cases. Eur J Clin Microbiol Infect Dis 12: 827-831, 1993.
25. Munoz-Gomez R, Garcia-Monzon C, Garcia-Buey L, *et al*: Hepatitis C virus infection in Spanish volunteer blood donors: HCV RNA analysis and liver disease. Eur J Gastroenterol Hepatol 8: 273-277, 1996.
26. Tanaka E, Kiyosawa K, Sodeyama T, *et al*: Prevalence of antibody to hepatitis C virus in Japanese schoolchildren: comparison with adult blood donors. Am J Trop Med Hyg 46: 460-464, 1992.
27. Watanabe J, Matsumoto C, Fujimura K, *et al*: Predictive value of screening tests for persistent hepatitis C virus infection evidenced by viraemia. Japanese experience. Vox Sang 65: 199-203, 1993.
28. Sasaki F, Tanaka J, Moriya T, *et al*: Very low incidence rates of community-acquired hepatitis C virus infection in company employees, long-term inpatients, and blood donors in Japan. J Epidemiol 6: 198-203, 1996.
29. Tanaka H, Tsukuma H, Hori Y, *et al*: The risk of hepatitis C virus infection among blood donors in Osaka, Japan. J Epidemiol 8: 292-296, 1998.
30. Arthur RR, Hassan NF, Abdallah MY, *et al*: Hepatitis C anti-body prevalence in blood donors in different governorates in Egypt. Trans R Soc Trop Med Hyg 91: 271-274, 1997.
31. Schlipkoter U, Roggendorf M, Cholmakow K, Weise A and Deinhardt F: Transmission of hepatitis C virus (HCV) from a haemodialysis patient to a medical staff member. Scand J Infect Dis 22: 757-758, 1990.
32. Campello C, Majori S, Poli A, Pacini P, Nicolardi L and Pini F: Prevalence of HCV antibodies in health-care workers from northern Italy. Infection 20: 224-226, 1992.
33. Petrosillo N, Puro V, Ippolito G, *et al*: Hepatitis B virus, hepatitis C virus and human immunodeficiency virus infection in health care workers: a multiple regression analysis of risk factors. J Hosp Infect 30: 273-281, 1995.
34. Herbert AM, Walker DM, Davies KJ and Bagg J: Occupationally acquired hepatitis C virus infection. Lancet 339: 305, 1992.
35. Neal KR, Dornan J and Irving WL: Prevalence of hepatitis C antibodies among healthcare workers of two teaching hospitals. Who is at risk? BMJ 314: 179-180, 1997.
36. Lodi G, Porter SR, Teo CG and Scully C: Prevalence of HCV infection in health care workers of a UK dental hospital. Br Dent J 183: 329-332, 1997.
37. Thomas DL, Factor SH, Kelen GD, Washington AS, Taylor E Jr and Quinn TC: Viral hepatitis in health care personnel at The Johns Hopkins Hospital. The seroprevalence of and risk factors for hepatitis B virus and hepatitis C virus infection. Arch Intern Med 153: 1705-1712, 1993.
38. Goetz AM, Ndimbie OK, Wagener MM and Muder RR: Prevalence of hepatitis C infection in health care workers affiliated with a liver transplant center. Transplantation 59: 990-994, 1995.
39. Tokars JI, Frank M, Alter MJ and Arduino MJ: National surveillance of dialysis-associated diseases in the United States, 2000. Semin Dial 15: 162-171, 2002.
40. Nakashima K, Kashiwagi S, Hayashi J, *et al*: Low prevalence of hepatitis C virus infection among hospital staff and acupuncturists in Kyushu, Japan. J Infect 26: 17-25, 1993.
41. Fujiyama S, Kawano S, Sato S, *et al*: Changes in prevalence of anti-HCV antibodies associated with preventive measures among hemodialysis patients and dialysis staff. Hepatogastroenterology 42: 162-165, 1995.
42. Hayashi J, Kishihara Y, Yamaji K, *et al*: Transmission of hepatitis C virus by health care workers in a rural area of Japan. Am J Gastroenterol 90: 794-799, 1995.
43. Nakashima K, Ikematsu H, Hayashi J, Kishihara Y, Mutsutake A and Kashiwagi S: Intrafamilial transmission of hepatitis-C virus among the population of an endemic area of Japan. JAMA 274: 1459-1461, 1995.
44. Ohshima S, Komatsu M, Nakane K, *et al*: Iatrogenic GB virus C/ hepatitis G virus infection in an area endemic for hepatitis C virus. J Hosp Infect 44: 179-185, 2000.
45. Kuboki M, Shinzawa H, Shao L, *et al*: A cohort study of hepatitis C virus (HCV) infection in an HCV epidemic area of Japan: age and sex-related seroprevalence of anti-HCV antibody, frequency of viremia, biochemical abnormality and histological changes. Liver 19: 88-96, 1999.
46. Inoue G, Horiike N, Michitaka K and Onji M: Hepatitis C virus clearance is prominent in women in an endemic area. J Gastroenterol Hepatol 15: 1054-1058, 2000.
47. Kitano M, Sakaguchi K, Miyashita M, *et al*: Prevalence of hepatitis G virus (HGV) infection in an endemic area of hepatitis C virus (HCV) infection. Hepatogastroenterology 47: 1340-1342, 2000.
48. Nishiguchi S, Kuroki T, Nakatani S, *et al*: Randomised trial of effects of interferon-alpha on incidence of hepato-cellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 346: 1051-1055, 1995.
49. Yoshida H, Shiratori Y, Moriyama M, *et al*: Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. Ann Intern Med 131: 174-181, 1999.

J Gastroenterol 2004; 39:570–574
DOI 10.1007/s00535-003-1344-z

Journal of Gastroenterology

A significant reduction in serum alanine aminotransferase levels after 3-month iron reduction therapy for chronic hepatitis C: a multicenter, prospective, randomized, controlled trial in Japan

Motoyoshi Yano[1], Hisao Hayashi[9], Kentaro Yoshioka[1], Yutaka Kohgo[2], Hiroyuki Saito[2], Yoshiro Niitsu[3], Junji Kato[3], Shiro Iino[4], Hiroshi Yotsuyanagi[5], Yoshimasa Kobayashi[6], Kinya Kawamura[6], Shinichi Kakumu[7], Masahiko Kaito[8], Jiro Ikoma[8], Shinya Wakusawa[9], Takeshi Okanoue[10], Yoshio Sumida[10], Fumiaki Kimura[11], Eiji Kajiwara[12], Michio Sata[13], and Kei Ogata[13]

[1]Third Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan
[2]Third Department of Internal Medicine, Asahikawa Medical College, Asahikawa, Japan
[3]Fourth Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan
[4]Department of Medicine, Seizankai Kiyokawa Hospital, Tokyo, Japan
[5]Department of Gastroenterology-Hepatology, St. Marianna Medical College, Kawasaki, Japan
[6]Second Department of Internal Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan
[7]Division of Gastroenterology, Department of Medicine, Aichi Medical University School of Medicine, Aichi, Japan
[8]Third Department of Internal Medicine, Mie University School of Medicine, Tsu, Japan
[9]Department of Medicine, Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa, Japan
[10]Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medicine, Kyoto, Japan
[11]Department of Internal Medicine, Tamano Municipal Hospital, Tamano, Japan
[12]Department of Gastroenterology, Nippon Steel Yawata Memorial Hospital, Kitakyushu, Japan
[13]Second Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan

Editorial on page 601

Background. Increasing evidence indicates that iron cytotoxicity plays an important role in the pathogenesis of chronic hepatitis C (CHC). However, the biochemical effects of iron reduction therapy on CHC remain to be confirmed in a controlled study. This study aimed to test whether iron removal by repeated phlebotomy improves serum alanine aminotransferase (ALT) levels in patients with CHC. ***Methods.*** Patients were randomly assigned to an iron reduction therapy or control group. The patients in the treatment group received 3-month iron reduction therapy by biweekly phlebotomy, while the patients in the control group were followed up for 3 months with regular blood tests alone. ***Results.*** Thirty-three patients completed the 3-month treatment, while 29 patients received the complete follow-up. The serum ALT levels were reduced from 118 ± 79 to 73 ± 39 IU/L in the treatment group, but did not change in the control group (106 ± 45 versus 107 ± 48 IU/L). Post-treatment enzyme activity was decreased significantly from the baseline. Furthermore, it was significantly lower than the 3-month control level. Although 5 patients withdrew from the study, none was affected by any side effects of repeated phlebotomy that required them to discontinue the treatment. ***Conclusions.*** This short-term controlled trial demonstrated the biochemical efficacy and safety of iron reduction therapy for patients with CHC.

Key words: oxidative stress, free radicals, phlebotomy

Received: May 22, 2003 / Accepted: November 7, 2003
Reprint requests to: M. Yano

Introduction

Without treatment, chronic hepatitis C (CHC) may result in hepatic cirrhosis or may be complicated by hepatocellular carcinoma. At present, interferon (IFN) is the only antiviral agent known to clear hepatitis C virus (HCV) RNA. A number of factors contribute to predicting the response to IFN, including the gender and age of the patient, the disease stage, viral load, and HCV genotype.[1] Ribavirin, an antiviral nucleotide analogue, reduces the viral load of patients, resulting in an enhanced response with IFN when combined.[2] Recently, it was reported that the use of pegylated IFN improved the response rate of CHC.[3–6] When pegylated IFN is combined with ribavirin, the elimination rate might be increased to 50%.[7,8] However, IFN monotherapy or combination therapy is not completely effective and the majority of patients with CHC do not receive any benefit from these antiviral agents. Therefore, alternative therapies are required.

Iron reduction therapy for CHC was first introduced in practice based on the histochemical detection of iron deposits in the liver,[9] and then on the detection of lyso-

somal iron stores in hepatocytes.[10] The remarkable reduction in the serum levels of ALT activity by phlebotomy suggests that iron-induced oxidative stress is involved in the pathogenesis of CHC. Over the last few years, evidence has accumulated indicating that free radicals and subsequent lipid peroxidation play an important role in CHC.[11-14] Individuals infected with HCV may be sensitive to iron-induced oxidative stress, which is regulated qualitatively by the ferrous ion. Since a report from Japan,[15] trials have been performed in Europe and the United States to test whether iron reduction therapy enhances the IFN effects on viral clearance.[16-20] It is generally agreed that iron reduction improves the biochemical parameters of CHC, but there is some controversy as to whether IFN treatment following iron reduction therapy enhances viral clearance. Unfortunately, phlebotomy for CHC is not authorized in Japan because of the lack of control studies on Japanese patients. Therefore, this multicenter, prospective, randomized, controlled trial was performed on Japanese patients nationwide.

Materials and methods

Laboratory tests

Laboratory tests included serum ALT activities, albumin concentration, and complete blood counts, including hemoglobin, using standard automated analyzers. The serum ferritin levels were measured by enzyme immunoassay. Quantitative detection of HCV-RNA was performed by Amplicor monitor HCV assay (Roche Diagnostic Systems, Basel, Switzerland). Blood tests were performed monthly in the control group and biweekly in the phlebotomy group. Blood for tests was drawn just before phlebotomy was performed each time.

Patients

All patients were positive for circulating HCV-RNA and had abnormal serum ALT activities at entry. Exclusion criteria at entry were (1) previous antiviral or immunosuppression therapy within 6 months; (2) decompensated liver cirrhosis; (3) laboratory values below the following: (a) hemoglobin concentration 13.0 g/dl for males and 11.0 g/dl for females, (b) platelet count 70000/mm^3, (c) serum albumin 3.7 g/dl; (4) severe complications such as renal, cardiac, pulmonary, or hematological disease; and (5) pregnancy. From October 2000 to March 2002, 67 patients (44 males and 23 females) who consented to the phlebotomy protocol entered the study. They were randomized into phlebotomy and control groups.

Iron reduction protocol

In the phlebotomy group, 22 patients underwent treatment by standard methods, i.e., ursodeoxycholic acid (UDCA) and/or Stronger Neo-Minophagen C (SNMC) for more than 3 months before entry, 10 by UDCA, 4 by SNMC, and 8 by both. Similarly, in the control group, 19 patients underwent treatment by these methods for more than 3 months, 10 by UDCA, 1 by SNMC, and 8 by both. These treatments were continued during the study period with the same doses and intervals as during the prestudy period. The patients in the treatment group received 3-month iron reduction therapy by phlebotomy, while those in the control group were followed up for 3 months with monthly blood tests. Phlebotomy of 200 ml in volume for the female patients less than 50 kg in body weight or 400 ml for other patients was repeated biweekly until the serum ferritin level reached the endpoint of 10 ng/ml, which is recognized as the level indicating a subclinical iron-deficient state. When the hemoglobin level fell below 10 g/dl, phlebotomy was discontinued until recovery of the anemia via erythropoiesis.

Ethics

This study was approved by the ethics committees of each institute and all patients provided written informed consent to participate.

Statistics

Data are expressed as means ± SD. Statistical analyses were performed using StatView 4.0 (Abacus Concepts, Berkeley, CA, USA). $P < 0.05$ was taken to indicate significance. Continuous variables were analyzed using the unpaired or paired two-tailed t test. Binary variables were analyzed using the χ^2 test and Fisher's exact test.

Results

Effects

Thirty-three patients completed the 3-month treatment, while 29 patients underwent the complete follow-up for 3 months. There were no differences in patient background between the treatment and control groups (Table 1). The blood volume drawn from the treated patients over 3 months was 1780 ± 680 ml. The serum ALT levels were reduced from 118 ± 79 to 73 ± 39 IU/l in the treatment group, but did not change in the control group (106 ± 45 versus 107 ± 48 IU/l). Post-treatment enzyme activity was decreased significantly from the baseline (paired t test, $P < 0.01$). Furthermore, it was lower than the 3-month control level (unpaired t

test, $P < 0.01$). HCV-RNA levels did not change in either group (557 ± 413 versus 608 ± 443, treatment group; 434 ± 314 versus 471 ± 336, control group).

The endpoint of a serum ferritin level of 10 ng/ml, which is recognized as a level indicating a subclinical iron-deficient state, was reached within 3 months in 8 of the 33 treated patients. A comparison between the 8 patients who achieved iron reduction to the subclinical iron-deficient state and the 25 patients who did not is summarized in Table 2. Low serum ferritin concentration was the only predictor of achieving iron depletion in 3 months. The pretreatment serum ferritin levels of patients who achieved iron reduction were lower than those of patients who did not achieve iron reduction (129 ± 78 versus 309 ± 173 ng/ml, unpaired t test, $P < 0.01$). Similar to the posttreatment serum ferritin levels, the posttreatment serum ALT activity and hemoglobin concentration and the blood volume removed differed between the two groups (51 ± 17 versus 81 ± 41 IU/l for ALT, $P < 0.01$; 11.4 ± 1.5 versus 13.3 ± 1.3 g/dl for hemoglobin, $P < 0.05$; 1300 ± 500 versus 1900 ± 700 ml for blood, $P < 0.05$).

Table 1. Clinical parameters in the phlebotomy and control groups

	Phlebotomy	Control	*P* value
Number of patients	33	29	
Age (years)	56 ± 11	57 ± 12	0.50
Gender (M/F)	21/12	20/9	0.66
Hb (g/dl)	14.7 ± 1.2	14.4 ± 1.2	0.55
ALT (IU/l)	116 ± 79	106 ± 45	0.61
Ferritin (ng/ml)	256 ± 173	290 ± 214	0.35
HCV-RNA (KIU/ml)	557 ± 413	434 ± 314	0.43

There were no differences between the two groups for any parameter
ALT, alanine aminotransferase; HCV, hepatitis C virus

Side effects

Five patients withdrew from the study, three in the treatment group (all males; 37, 61, and 72 years old) and two in the control group (both females, 51 and 78 years old), because of the patient's noncompliance, namely, failure to regularly visit the hospital. None of them was affected by any side effect of repeated phlebotomy that required them to discontinue the study. One patient experienced vagal reflex with transient faintness, bradycardia, and systolic blood pressure of 80 mmHg that responded promptly to a drip infusion of saline.

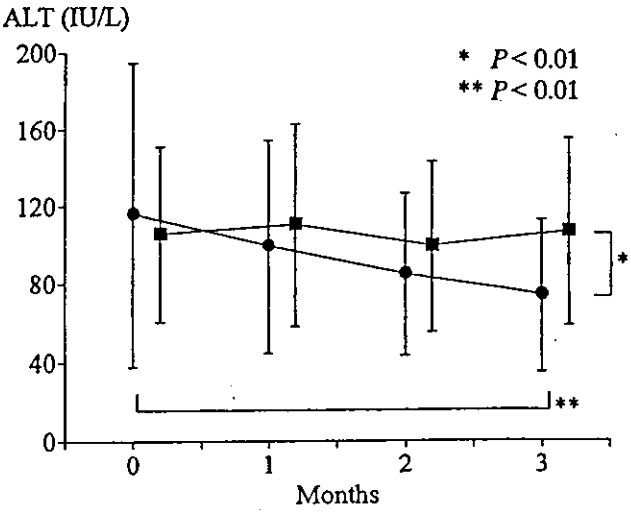

Fig. 1. Changes in serum alanine aminotransferase (*ALT*) levels in the 3-month treatment. The serum levels of ALT were reduced significantly by the 3-month iron reduction therapy (●—●; 118 ± 79 to 73 ± 39 IU/l), but did not change throughout the observation period of 3 months (■—■; 106 ± 45 vs. 107 ± 48 IU/l). The post-treatment level differed not only from the baseline level (*, paired *t* test, $P < 0.01$), but also from the post-3-month level of the control group (**, unpaired *t* test, $P < 0.01$)

Table 2. Comparisons between the groups of treated patients that achieved or did not achieve iron reduction

	Achieved	Did not achieve	*P* value
Number of patients	8	25	
Age (years)	56 ± 12	57 ± 11	0.83
Gender (M/F)	4/4	17/8	0.36
Pretreatment			
Hemoglobin (g/dl)	14.0 ± 1.2	14.9 ± 1.1	0.13
ALT (IU/l)	97 ± 79	122 ± 79	0.45
Ferritin (ng/ml)	129 ± 78	309 ± 173	0.009
After 3 months of treatment			
Hemoglobin (g/dl)	11.4 ± 1.5	13.3 ± 1.3	0.003
ALT (IU/l)	51 ± 17	81 ± 41	0.049
Ferritin (ng/ml)	8.0 ± 1.7	53 ± 51	0.019
Blood removed (ml)	1300 ± 500	1900 ± 700	0.043

Discussion

This multicenter, prospective, controlled trial in Japanese patients confirmed the results of pilot studies showing that body iron removal by phlebotomy induces an improvement in the serum ALT activity in CHC.[9,10] In this study, many patients had preexisting treatments, i.e., UDCA and/or SNMC. The proportion of such patients was not different in the two groups, and the treatments were continued during the study period with the same doses and intervals as during the prestudy period in both groups. The likelihood that these treatments modified the serum ALT levels in the phlebotomy group was very low because the treatments were also continued in the control group, in which ALT levels did not change.

Since the initial report from Japan,[15] there have been many other reports demonstrating that iron removal from patients with CHC as a premedication before IFN treatment provides biochemical benefits.[16–20] Most of the studies, however, were on Caucasian patients, who have different genetic and nutritional factors than Asian patients. For example, 5%–10% of Caucasian individuals are heterozygous for C282Y, a major mutation of HFE, the primary hemochromatosis gene.[21] In contrast, the mutation is very rare in Asian populations, including Japanese.[22–24] Furthermore, in studies in which the effect of IFN alone was compared with that of postiron reduction IFN, the first phase of iron reduction before IFN was not controlled sufficiently. Considering the fact that most patients with CHC have fatty metamorphosis[25] and are highly predisposed to diabetes mellitus,[26,27] a prospectively designed controlled study may be required to evaluate iron reduction therapy. In fact, dietary iron restriction alone or combined nutritional therapy with a low-calorie diet improves the biochemical parameters of CHC patients.[28–30] The present, prospective, controlled trial definitively showed that iron reduction therapy per se may have significant potential to improve the biochemical parameters of CHC patients, regardless of the genetic background of iron overload disorders or calorie intake.

This study also demonstrated that the treatment period is critical for obtaining the maximum effect of iron reduction therapy. An endpoint of 10 ng/ml of serum ferritin induces mild iron deficiency anemia in most patients with CHC, in whom high iron bioavailability is highly required for stimulated erythropoiesis, resulting in effective elimination of the available precursor element for free radical generation. Higher posttreatment levels of serum ALT were found in the subgroup of patients with higher ferritin levels and higher hemoglobin concentrations. We designated these patients as a group that did not achieve iron reduction: they did not suffer intolerable side effects, but could not attain an iron-deficient state due to the short-term treatment period and a larger amount of iron accumulation in the liver before the treatment period. We expect that a longer treatment period for this group would result in a similar effect to that in the group that achieved iron reduction.

A 5-years iron-reduction treatment induced an apparent slowdown in the histological progression of 14 patients with CHC.[31] Another long-term combination therapy consisting of iron reduction and a low-iron diet not only suppressed the histological progression but also normalized the hepatic 8-hydroxy-2′-deoxyguanosine levels, which might be related to suppression of the malignant change to hepatocellular carcinoma in treated patients.[29] In these long-term studies of iron reduction therapy there were no major side effects requiring special medical attention. Long-term phlebotomy in elderly patients without disorders of other organs is generally considered to be safe based on these results and the experience with therapies for hemochromatosis.

The repeated phlebotomy applied here for CHC has been a standard treatment for hemochromatosis.[32] In hemochromatosis, a large amount of iron can be removed safely by phlebotomy through the removal of iron-rich hemoglobin, provided that the procedure is performed postprandially in a supine position. This posture avoids either faintness in a vagotonic individual or peripheral circulation collapse. Almost all our patients tolerated the iron reduction therapy as well as hemochromatosis patients. Although not noted in similarly treated of hemochromatosis patients, iron depletion in CHC patients induced a remarkable reduction in the serum ALT levels. Therefore, when a patient is found to have an active form of CHC, iron reduction therapy should be recommended as an alternative to antiviral agents.[33] Once iron reduction therapy for CHC is authorized in Japan, great benefit might be provided for patients who need adjuvant therapy other than IFN.

During this study, a nurse was accidentally pricked with a needle while drawing HCV-RNA-positive blood. Six months follow-up failed to detect any biochemical abnormality suggesting the onset of acute hepatitis. HCV-positive blood is infectious, and caution is required during all medical procedures. Medical staff should be fully aware of the potential risks.

References

1. National Institutes of Health. National Institutes of Health Consensus Development Conference Panel statement: management of hepatitis C. Hepatology 1997;26(Suppl 1):2S–10S.
2. McHutchison JG, Poynard T. Combination therapy with interferon plus ribavirin for the initial treatment of chronic hepatitis C. Semin Liver Dis 1999;19:57–65.

3. Reddy KR, Wright TL, Pockros PJ, Shiffman M, Everson G, Reindollar R, et al. Efficacy and safety of pegylated (400kd) interferon alpha-2a compared with interferon alpha-2a in non-cirrhotic patients with chronic hepatitis C. Hepatology 2001;33: 433–8.
4. Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, et al. Peginterferon alfa-2a in patients with chronic hepatitis C. N Engl J Med 2000;343:1666–72.
5. Heathcote EJ, Shiffman ML, Cooksley WG, Dusheiko GM, Lee SS, Balart L, et al. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. N Engl J Med 2000;343:1673–80.
6. Lindsay KL, Trepo C, Heintges T, Shiffman ML, Gordon SC, Hoefs JC, et al. A randomized, double blind trial comparing pegylated interferon alfa-2b to interferon alfa-2b as initial treatment for chronic hepatitis C. Hepatology 2001;34:395–403.
7. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet 2001;358:958–65.
8. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–82.
9. Hayashi H, Takikawa T, Nishimura N, Yano M, Isomura T, Sakamoto N. Improvement of serum aminotransferase levels after phlebotomy in patients with chronic active hepatitis C and excess hepatic iron. Am J Gastroenterol 1994;89:986–8.
10. Hayashi H, Takikawa T, Nishimura N, Yano M. Serum aminotransferase levels as an indicator of the effectiveness of venesection for chronic hepatitis C. J Hepatol 1995;22:268–71.
11. Farinati F, Cardin R, De Maria N, Della Libera G, Marafin C, Lecis E, et al. Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. J Hepatol 1995; 22:449–56.
12. Kageyama F, Kobayashi Y, Kawasaki T, Toyokuni S, Uchida K, Nakamura H. Successful interferon therapy reverses enhanced hepatic iron accumulation and lipid peroxidation in chronic hepatitis C. Am J Gastroenterol 2000;95:1041–50.
13. Sumida Y, Nakashima T, Yoh T, Nakajima Y, Ishukawa H, Mitsuyoshi H, et al. Serum thioredoxin levels as an indicator of oxidative stress in patients with hepatitis C virus infection. J Hepatol 2000;33:616–22.
14. Sumida Y, Nakashima T, Yoh T, Kakisaka Y, Nakajima Y, Ishikawa H, et al. Serum thioredoxin elucidates the significance of serum ferritin as a marker of oxidative stress in chronic liver diseases. Liver 2001;21:295–9.
15. Yano M, Kakumu S, Hayashi H, Takikawa T, Nishimura N. Phlebotomy followed by alpha interferon therapy in patients with chronic hepatitis C (abstract). Hepatology 1995;22:474A.
16. Piperno A, Sampietro M, D'Alba R, Roffi L, Fargion S, Parma S, et al. Iron stores, response to alpha-interferon therapy and effects of iron depletion in chronic hepatitis C. Liver 1996;16:248–54.
17. Fong TL, Han SH, Tsai NC, Morgan TR, Mizokami M, Qian D, et al. A pilot randomized, controlled trial of the effect of iron depletion on long-term response to alpha-interferon in patients with chronic hepatitis C. J Hepatol 1998;28:369–74.
18. Herrera JL. Iron depletion is not effective in inducing a virologic response in patients with chronic hepatitis C who failed to respond to interferon therapy. Am J Gastroenterol 1999;94:3571–5.
19. Fontana RJ, Israel J, LeClair P, Banner BF, Tortorelli K, Grace N, et al. Iron reduction before and during interferon therapy of chronic hepatitis C: results of a multicenter, randomized, controlled trial. Hepatology 2000;31:730–6.
20. Di Bisceglie AM, Bonkovsky HL, Chopra S, Flamm S, Reddy RK, Grace N, et al. Iron reduction as an adjuvant to interferon therapy in patients with chronic hepatitis C who previously not responded to interferon: a multicenter, prospective randomized, controlled trail. Hepatology 2000;32:135–8.
21. Merryweather-Clarke AT, Pointon JJ, Jouanolle J, Rochette J, Robson KJ. Geography of HFE C282Y and H63D mutations. Genet Test 2000;4:183–98.
22. Sohda T, Yanai J, Soejima H, Tamura K. Frequencies in the Japanese populations of the HFE gene. Biochem Genet 1999;37: 63–8.
23. Sohda T, Okubo R, Kamimura S, Ohkawa T. Hemochromatosis with HFE mutation in a Japanese patient. Am J Gastroenterol 2001;96:2487–8.
24. Shiono Y, Ikeda R, Hayashi H, Wakusawa S, Sanae F, Takikawa T, et al. C282Y and H63D mutations in the HFE gene have no effect on iron overload disorders in Japan. Inter Med 2001;40: 852–6.
25. Bach N, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 1992;15:572–7.
26. Mason AL, Lau JY, Hoang N, Qian K, Alexander GJ, Xu L, et al. Association of diabetes mellitus and chronic hepatitis C virus infection. Hepatology 1999;29:328–33.
27. Caronia S, Taylor K, Pagliaro L, Carr C, Palazzo U, Petrik J, et al. Further evidence for an association between non-insulin-dependent diabetes mellitus and chronic hepatitis C virus infection. Hepatology 1999;30:1059–63.
28. Matsumoto M, Imanuma S, Sato H, Takamasu M, Okanoue T, Kashima K. Dietary iron reduction on patients with chronic hepatitis C (in Japanese with English abstract). Acta Hepatol Jpn 1999;40:436–44.
29. Kato J, Kobune M, Nakamura. T, Kuroiwa G, Takada K, Takimoto R, et al. Normalization of elevated hepatic 8-hydroxy-2'-deoxyguanousine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 2001;61:8697–702.
30. Iwasa M, Kaito M, Ikoma J, Kobayashi Y, Tanaka Y, Highchu K, et al. Dietary iron restriction improves aminotransferase levels in chronic hepatitis C patients. Hepato-Gastroenterology 2002;49: 537–9.
31. Yano M, Hayashi H, Wakusawa S, Sanae F, Takikawa T, Shiono Y, et al. Long term effect of phlebotomy on biochemical and histological parameters of chronic hepatitis C. Am J Gastroenterol 2002;97:133–7.
32. Vautier G, Murray M, Olynyk JK. Hereditary haemochromatosis: detection and management. Med J Aust 2001;175:418–21.
33. Bonkovsky HL. Therapy of hepatitis: other options. Hepatology 1997;26(Suppl 1):143S–51S.

J Gastroenterol 2004; 39:769–775
DOI 10.1007/s00535-003-1387-1

Journal of Gastroenterology

Prognostic indicators of breakthrough hepatitis during lamivudine monotherapy for chronic hepatitis B virus infection

YASUHITO TANAKA[1], ANTHONY E.T. YEO[2], ETSURO ORITO[3], KIYOAKI ITO[4], NOBORU HIRASHIMA[4], TATSUYA IDE[5], MICHIO SATA[5], and MASASHI MIZOKAMI[1]

[1]Department of Clinical Molecular Informative Medicine, Nagoya City University Medical School Graduate School of Sciences, Kawasumi 1, Mizuho, Nagoya 467-8601, Japan
[2]Jacobus Pharmaceutical Company, Princeton, New Jersey, USA
[3]Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[4]Department of Gastroenterology, Chukyo Hospital, Nagoya, Japan
[5]Second Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan

Editorial on page 813

Background. Breakthrough hepatitis (BTH), defined as a flare of transaminases alanine aminotransferase [ALT]) can occur during lamivudine monotherapy for hepatitis B virus (HBV) infection. There have been many reports of lamivudine-resistant mutations within the C domain of the viral reverse transcriptase; however, the appearance of these mutants is not necessarily correlated with BTH during lamivudine therapy. ***Methods and Results.*** Entire serial HBV genomic sequences before and during lamivudine therapy for 4 patients with BTH and 1 patient without BTH were analyzed and showed changes in the pre-S region. These changes may be associated with ALT flares. Further investigation in a cohort of 36 patients with a median treatment period of 25 months showed that 21 patients had a rise in HBV-DNA titer, of whom 18 had BTH. Univariate statistical analyses showed that possible prognostic indicators for the occurrence of BTH were pre-S deletions ($P = 0.03$) and L180M/M204L mutations ($P = 0.04$). By multivariate Cox regression analyses, significant variables were pre-S deletions (hazard ratio, 0.17; 95% confidence internal (CI), 0.044–0.66) and precore mutations (hazard ratio, 5.70; 95% CI, 1.74–18.71) prior to the commencement of lamivudine monotherapy. Interestingly, BTH occurred after the selection of the wild-type species in the pre-S region during lamivudine monotherapy. ***Conclusions.*** These results suggest that patients with HBV pre-S deletion mutants should be monitored carefully during lamivudine therapy.

Key words: hepatitis B virus, lamivudine, breakthrough hepatitis, pre-S deletion mutation, precore mutation

Received: September 17, 2003 / Accepted: December 16, 2003
Reprint requests to: M. Mizokami

Introduction

A current therapeutic approach for chronic hepatitis B virus (HBV) infection focuses on inhibiting the reverse transcriptase enzyme, using lamivudine. Unfortunately, lamivudine-resistant strains frequently occur during therapy, varying from 16% to 43% of patients after 1 year of treatment.[1] Resistance involves the selection of major mutations in the tyrosine-methionine-aspartate-aspartate-aspartate (YMDD) motif within the C domain of the viral reverse transcriptase, and in the conserved B domain. These mutants have been classified into two groups: group I, with B and C domain mutations (L180M + M204V); and group II, with a C domain mutation (M204L).[2] The appearance of these mutants is not necessarily correlated with alanine aminotransferase (ALT) flares during lamivudine therapy.

There is little information on the prevalence of the mutations in patients with lamivudine resistance in relation to changes in the pre-S region of the HBV genome and other areas during a flare of serum ALT levels while they are on treatment (the flare indicates breakthrough hepatitis [BTH]). In this study, the molecular events associated with the emergence of drug-resistant strains in patients treated with lamivudine were investigated. Sequential serum samples from 36 patients were analyzed to determine which variables, e.g., HBV DNA levels, pre-S mutations, and precore mutations were prognostic indicators for BTH.

Patients and methods

Patients

Thirty-six Japanese patients (26 men and 10 women; median age, 46 years; range, 20–70 years), chronically infected with HBV and treated with lamivudine were

examined. The patients were recruited from Nagoya City University Hospital, Chukyo Hospital, and Kurume University Hospital between 1996 and 2002. The subjects provided their written informed consent. Only HBV genotype C was examined; 17 patients (47%) had liver cirrhosis, with the remainder of the patients having chronic hepatitis. Patients who were coinfected with human immunodeficiency virus type 1 were excluded. Median pretreatment ALT and HBV DNA levels were 116IU/l (range, 20–1034IU/l) and 7.2IU/l (range, 4.1–8.8IU/l), respectively.

Follow up and definitions

All patients were followed up with monthly clinical examination and routine laboratory tests. The upper limit of normal for ALT activity was 40IU/l. Virological breakthrough was considered to have occurred when there was re-elevation of detectable serum HBV DNA. Breakthrough hepatitis (BTH) was defined as the re-elevation of HBV DNA and a rise in ALT levels of more than 80IU/l (two times the upper limit of normal) during lamivudine therapy.

Methods

Lamivudine was administered at a dosage of 100mg/day. The median duration of lamivudine therapy was 25 months (range, 16–44 months). Serum hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), anti-HBe (as measured by chemiluminescence enzyme immuno assay (CLEIA), HBV DNA levels, and ALT were tested monthly. HBV DNA was measured by a highly reproducible and sensitive HBV real-time detection direct test (HBV RTD-Direct test; SRL Inc., Tokyo, Japan), which combines the use of a DNA extraction system based on magnetic beads coated with polyclonal anti-HBs.[3] This procedure has a dynamic range of 0.7–8.0 log international units (IU) per ml.

Nucleic acids were extracted from 100µl of serum by using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Fourteen complete genomes from five patients (four patients with BTH during treatment) were amplified by polymerase chain reaction (PCR) with several primer sets, as previously described.[4] PCR products were sequenced directly by the dideoxy method, using a BigDye Terminator cycle sequencing kit in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Pre-S deletion mutants were distinguished by sizes smaller than that expected (amplicons of 658bp encompassing the entire *pre-S1/S2* gene) on electrophoresis, and confirmed as not being products of artifacts in PCR.[5] Pre-S deletion mutants were considered to be present when the size of amplicons was shorter by more than 2% than that expected for wild-type HBV.[6] In some cases, various small-size amplicons were detected in the presence or absence of full-sized amplicons. For the 14 patients with changes suggestive of pre-S deletions (or smaller amplicons), a fragment of 1.4kbp from the pre-S region, including the YMDD motif, was cloned into the TOPO TA cloning vector (Invitrogen Corp., Carlsbad, CA, USA). Plasmids were amplified in *Escherichia coli* TOP10 (Promega, Madison, WI, USA) and purified by the Qiagen procedure. All sequencing reactions were performed with the above protocol.

Liver cirrhosis was determined by ultrasonography (coarse liver architecture, nodular liver surface, and blunt liver edges) and evidence of hypersplenism (splenomegaly on ultrasonography), and a platelet count of less than 100000/mm^3. Confirmation by a fine-needle biopsy of the liver was performed as required.

Statistical analyses

Data were analyzed with the SAS 8.02 (SAS, Cary, NC, USA) and BMDP 7.0 (SPSS; Chicago, IL, USA). Univariate tests included Fishers' exact test, the χ^2 with Yates' correction, and log-rank ratios. Multivariate analysis, using Cox's regression, with the outcome variable being BTH, examined the dependent variables, which included sex; age; liver histology; HBeAg; pretreatment ALT levels; HBV DNA levels; HBsAg levels; and HBV mutations in the polymerase gene, precore, core promoter, and pre-S regions. Regression diagnostics were performed to ensure the integrity of the models constructed. It was found that a maximum of two dependent variables could be modeled.

Results

Virological and biochemical responses

The levels of HBV DNA and ALT decreased in all patients during lamivudine therapy. Serum ALT levels were normalized in 33 patients (92%) within 12 weeks of therapy. Of the 28 HBeAg-positive patients, 8 lost HBeAg. Among these 8 patients, 4 acquired anti-HBe. During follow-up for a median treatment period of 25 months, a rise in HBV-DNA titer occurred in the 21 patients with mutations in the YMDD motif during treatment, and BTH was observed in 18 of these patients (Table 1). Interestingly, loss of HBV DNA (<0.7 log IU/ml) in the group without virological breakthrough was significantly higher than that in those with breakthrough ($P < 0.0001$). This means that both pre-S wild and deletion clones were suppressed during treatment.

Table 1. Results of lamivudine therapy in patients

Variables	Patients (n = 36)
HBeAg seronegative; number (%)	8/28 (29%)
HBeAg seroconversion; number (%)	4/28 (14%)
Virological breakthrough (BT)	21 (58%)
Biochemical breakthrough hepatitis (BTH)	18 (50%)
M204I/V mutations	19 (53%)
L180M/M204I/V mutations	10 (28%)
Loss of HBV DNA, number (%)[a]	
Without reappearance during treatment	12/15 (80%)*
With reappearance during treatment	1/21 (5%)*
Pre-S deletion mutations[b]	14 (39%)
Basic core promoter mutations (T1762, A1764)[b]	21 (58%)
Precore mutation (A1896)[b]	7 (19%)

*$P < 0.0001$
[a]Detection limit of as low as 0.7 $\log_{10}$ IU/ml
[b]At baseline

For four patients with BTH and one patient without BTH, the entire HBV genome, at three time points, pretreatment, appearance of YMDD mutants, and BTH, was determined. Interestingly, of the four BTH patients, three had pre-S deletions, which included the S promoter region, which is related to HBsAg expression before treatment; before ALT elevation, the pre-S deletion clones changed to wild-type (no deletion; Fig. 1). Figure 2 shows the sequential changes in serum ALT and HBV DNA levels, HBsAg, HBeAg, and precore (nucleotide [nt] 1896)/core promoter sequences (nt 1762, 1764) and pre-S deletions during the emergence of YMDD variants in case 1 (Fig. 2A, B) and case 3 (Fig. 2C, D). In both cases, after the selection of pre-S wild clones, HBsAg expression and HBV DNA increased, followed by ALT elevation and BTH. Interestingly, when lamivudine therapy was replaced by interferon (IFN) therapy (at month 13), ALT re-elevation occurred, with both pre-S and YMDD wild clones appearing at month 17 in case 1. The wild clones were then suppressed by adding IFN to lamivudine (Fig. 2A, B). In

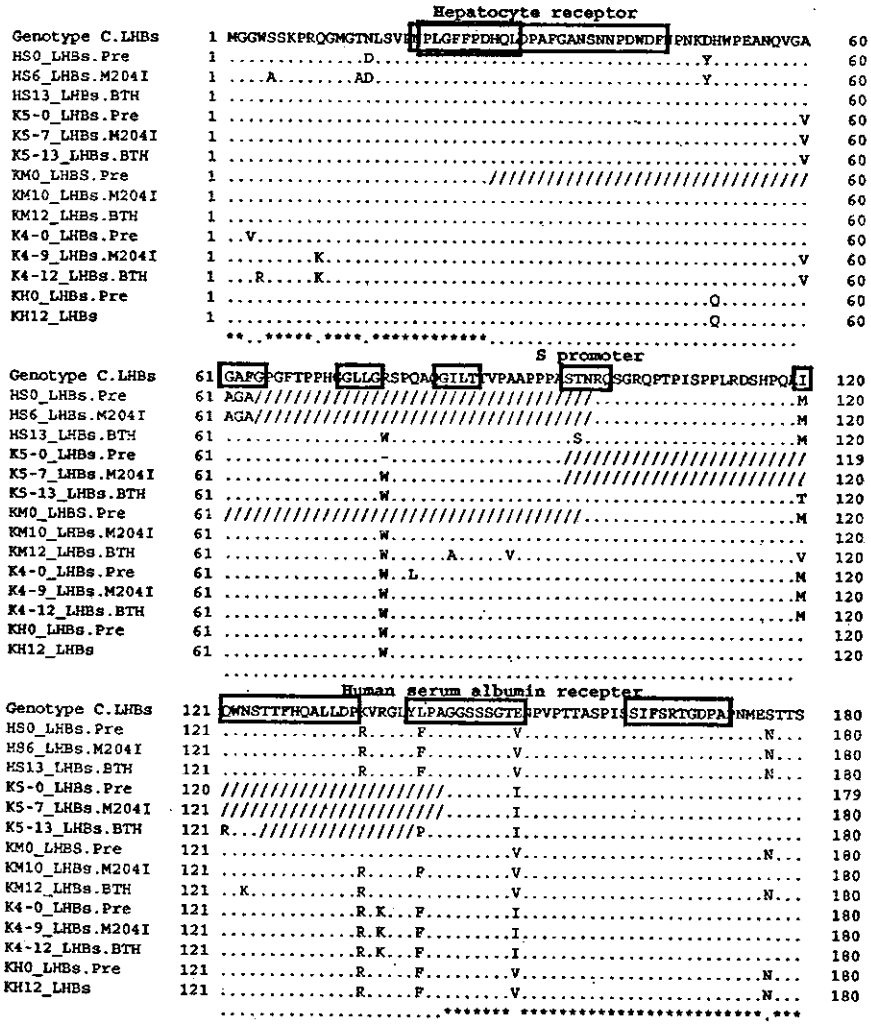

Fig. 1. Changes of serial amino-acid sequences in large S regions during treatment. Four patients with breakthrough hepatitis (*BTH*), and one patient without BTH, had their hepatitis B virus (HBV) full genomes determined at three points: pretreatment, appearance of YMDD mutants, and BTH (or endpoint). Of the four BTH patients, three had pre-S deletions, including the S promoter region, which is related to hepatitis B surface antigen (HBsAg) expression. Prior to alanine aminotransferase (ALT) elevation, their pre-S deletion clones changed to wild-type (no deletion). For one patient without BTH (control), there was no change in the pre-S region during treatment. Putative T-cell epitopes are indicated in *gray boxes*

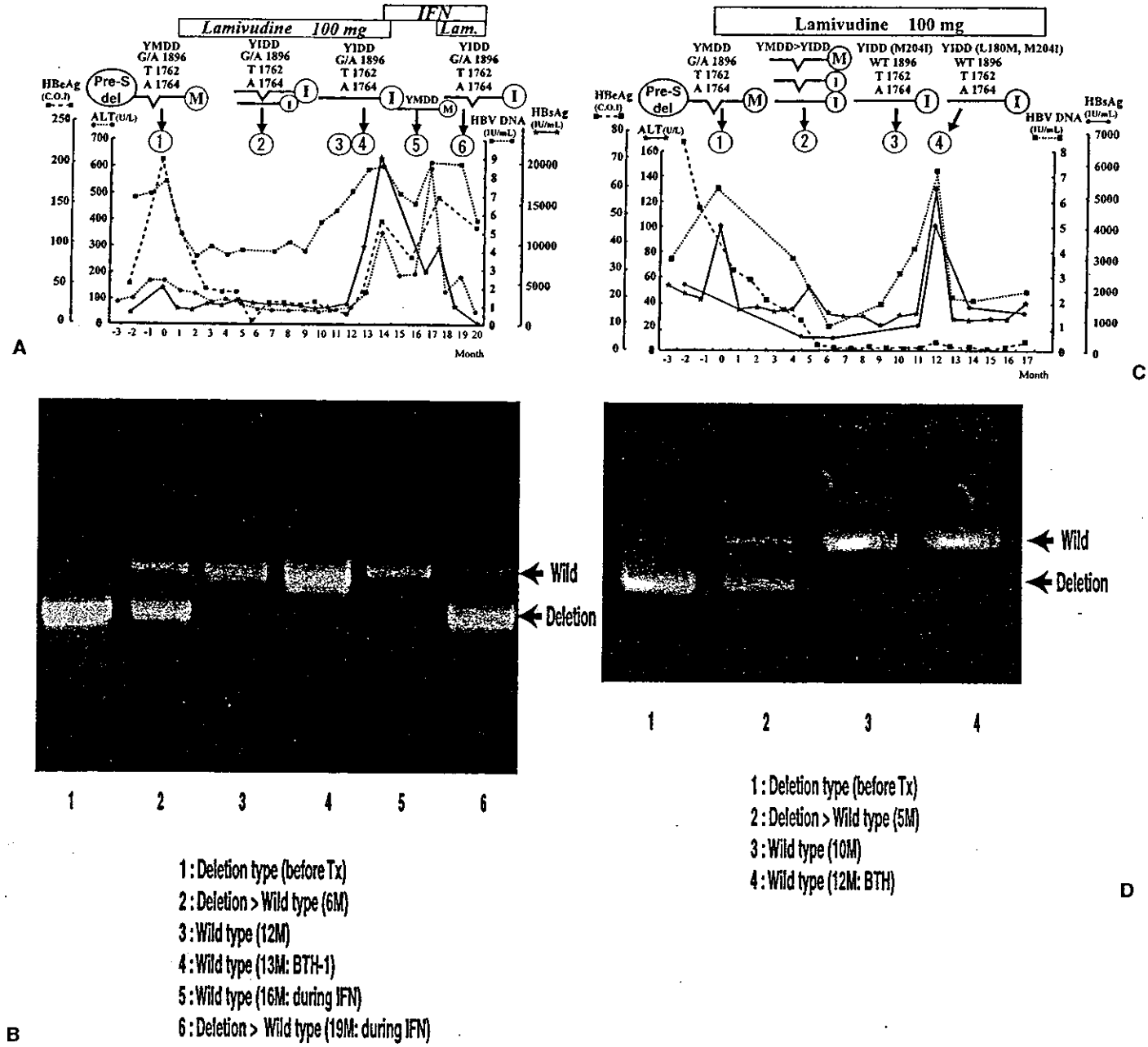

Fig. 2A–D. The sequential changes in serum ALT and HBV DNA levels, HBsAg, hepatitis B envelope antigen (*HBeAg*), and precore (nucleotide [nt] 1896)/core promoter sequences (nt 1762, 1764) and pre-S deletions (*pre-S del*) during the emergence of YMDD variants in (**A**, **B**) case 1 and (**C**, **D**) case 3. ALT elevation (*BTH*) occurred during treatment. The *X axis* represents the months after the start of lamivudine treatment. The *arrows* indicate the time points when sera were obtained for analyzing full-length amplification of HBV gonome. *YMDD*, wild-type HBV strain; *YIDD*, HBV variant with isoleucine-to-methionine substitution at amino-acid 204 of the reverse transcriptase domain of the hepatitis B polymerase gene. The electrophoresis gels of polymerase chain reaction (PCR) results are also shown. *IFN*, interferon; *C.O.I.*, cut-off index; *Tx*, lamivudine therapy; *M*, months (in **B** and **D**)

case 3, BTH by pre-S wild clones with YMDD mutants was treated with glycyrrhizin (Stronger Neo-Minophagen C; Minophagen, Tokyo, Japan) with normalization of ALT. Figure 3 shows the sequential changes in the pre-S deletions, YMDD motifs, core promoter, and precore mutations in relation to the clinical course of three patients with pre-S deletions. These three patients with BTH had core promoter mutations before treatment; however, no changes occurred during treatment. The precore sequence (nt 1896) in three patients was wild-type during follow-up. The L180M/M204I (double mutation) of the polymerase region oc-

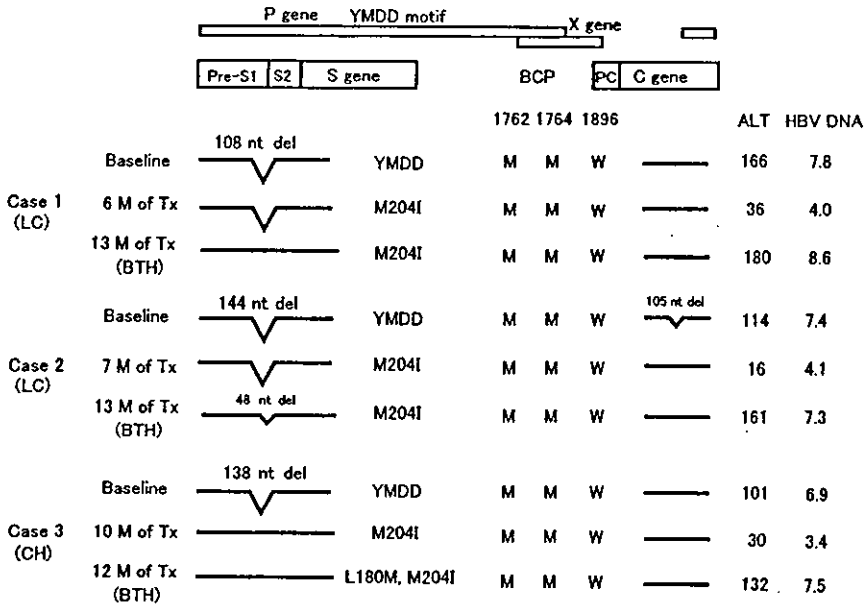

Fig. 3. Sequential changes in pre-S deletions, YMDD motifs, core promoter, and precore mutations in relation to the clinical course of three patients with BTH. *Tx*, lamivudine therapy; *del*, deletions; *M*, mutants, *W*, wild; *LC*, cirrhosis; *CH*, chronic hepatitis; *BCP*, basic core promoter

curred in only one patient at the time of BTH (Fig. 3). In summary, based on serial HBV full genomic analyses in five patients, it appears that the reversion from pre-S deletion mutants to wild types may be associated with BTH, as shown as in Fig. 4. For one patient without BTH (control), no change in the pre-S region was noted during treatment.

Frequency of pre-S deletion mutants

Pre-S deletion mutants were found in the pretreatment sera of 14 out of 36 (39%) patients. The length of pre-S deletions ranged from 31 to 306 bp. There were no smaller deletions (less than 33 bp) in this study. Deletions in frame were present in 12 of the 14 (86%) patients. The deletions in preS1, preS2, and both preS1 and preS2 occurred in 6 (43%), 3 (21%), and 5 (36%), patients, respectively. Deletions of the CCAAT-box in the S-promoter region[7] were found in 8 of the 14 (57%) pre-S deletion mutants. PreS2 deletions were clustered in the 5′-terminal half of the preS2 region.

Figure 5 shows the 14 patients that had pre-S deletion mutants before treatment. In this group, 11 patients had BTH. Interestingly, 9 of the 11 patients with BTH lost the pre-S deletion during treatment, i.e., pre-S wild clone became dominant. Hence, changes to wild type may also be associated with BTH.

Statistical analyses

Univariate analyses indicated that BTH was significantly associated with pre-S deletions ($P = 0.03$) and L180M/M204L mutations ($P = 0.04$; Table 2). In the

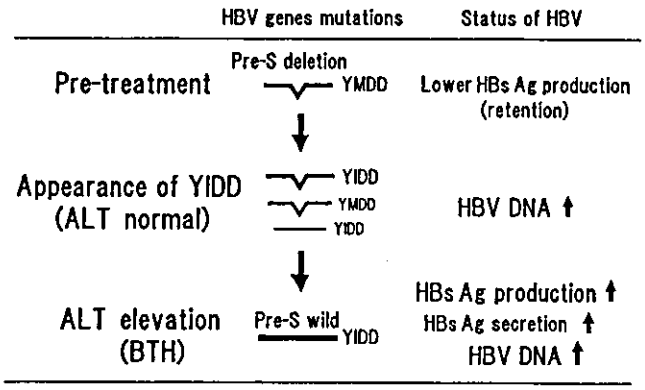

Fig. 4. Schema of BTH. Based on cloning analyses in pre-S and YMDD motif regions, patterns of pre-S and the YMDD motif were changed by BTH during treatment; note replacement of pre-S wild-type, then HBsAg production (or secretion), followed by elevation of HBV DNA and ALT

Table 2. Univariate statistics (log rank test), showing the relationship of various clinical variables with BTH

Variables	*P* Value
L180M/M204I/V mutations	0.04
Hepatitis B e antigen	0.59
Hepatitis B surface antigen	0.22
ALT levels	0.26
Sex	0.77
Age	0.78
HBV DNA levels	0.76
Precore mutations	0.07
Basic core promoter mutations	0.73
Liver cirrhosis	0.18
Pre-S deletion mutations	0.03

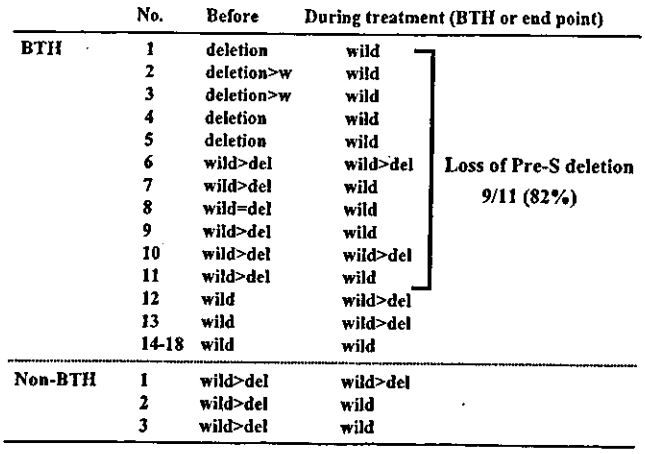

Fig. 5. Loss of pre-S deletion mutants during lamivudine treatment. Fourteen patients had pre-S deletion mutants before treatment (some of them were mixed type). Of these patients, 11 had BTH. Loss of pre-S deletion mutants was found in 9 patients (82%). The changes from pre-S deletion to wild type may be associated with BTH. *del*, deletions; *w*, wild

Table 3. Multivariate model, relating BTH to statistically significant variables

Variables	*P* Value	Hazard ratio (95% confidence interval)
Pre-S deletions in BTH group		1.00 (Referent group)
Pre-S deletions in non-BTH group	0.0035	0.17 (0.044–0.66)
Precore mutations in BTH group		1.00 (Referent group)
Precore mutations in non-BTH group	0.0085	5.70 (1.74–18.71)

multivariate model, the L180/M204L mutation was not statistically significant. However, pre-S deletions remained statistically significant ($P = 0.0035$), and precore mutations acquired significance ($P = 0.0085$; Table 3).

Discussion

YMDD mutants can cause elevation of HBV DNA; however, there is a time lag between the appearance of the YMDD mutants and ALT flares during lamivudine therapy. In this study, the molecular and statistical suggested one mechanism of the ALT flares; the presence of pre-S deletion mutants before treatment, followed by the selection of the wild-type species in the pre-S region.

Patients infected with HBV genotype B, especially Bj, tend to have earlier HBe seroconversion, followed by normalized ALT and a good prognosis.[8,9] Thus, this study focused on the more problematic genotype C. In our cohort, 17 patients (47%) had liver cirrhosis, and pre-S deletion mutants were present in the pretreatment sera of 14 patients (39%). This result is consonant with another recent study in chronic HBV patients, in which those with liver cirrhosis and/or hepatocellular carcinoma ($P < 0.01$) and those with HBV genotype C ($P < 0.001$) had an association with pre-S deletion mutants.[10]

When the wild types were selected by lamivudine monotherapy, serum HBsAg and HBV DNA increased, followed by ALT elevation (BTH) in some cases. This may be caused by the immune response to pre-S wild clones with T-cell and B-cell epitopes. Minami et al.[5] have shown that antibodies against the pre-S2 region were negative; however, the antibody response against the pre-S1 epitopes coincided with the appearance of the variant virus. These findings suggest an activated T-cell and B-cell response during hepatic inflammation. A recent study showed that nonclassical natural killer T (NKT) cells mediated acute hepatitis. This implies that the large S protein may be an immune target of NKT cells.[11]

Kajiya et al.[12] showed that transfection of PCR products containing the pre-S mutant sequences resulted in increased amounts of intracellular replicative intermediates, but decreased secretion of HBsAg and HBeAg, suggesting the accumulation of non-enveloped viral core particles within the cells. The deletion sequence in the pre-S2 region coincides with human leukocyte antigen-restricted T- and B-cell epitopes. In vitro, HBsAg was retained in hepatocytes, and the synthesis and secretion of major surface antigen decreased for most of the pre-S mutants.[6,12] Pre-S mutants prevailed with the evolution of chronic HBV, probably under immune pressure.[13] Pre-S mutants usually give rise to a decrease in major S transcripts and an inverse increase in pre-S1 transcripts, leading to the relative overexpression and the subsequent accumulation of large surface proteins in the cytoplasm,[14,15] raising the possibility that the emergence of pre-S mutants may contribute to the inverse relationship between the serum level of HBsAg and the intrahepatic accumulation of HBsAg in chronic infection. Hence, it is possible that the accumulation of non-enveloped viral core particles occurs within the cells and that complete viral particles from pre-S deletion mutants would then decrease in serum.

Multivariate analysis in the present study showed that the absence of BTH was also significantly associated with precore mutation ($P = 0.0085$). This observation is supported by recent reports that in HBV genotype C patients, the emergence rate of YMDD motif mutants in HBeAg-positive patients was statistically significantly higher than that in the HBeAg-negative patients.[16] In the present study, most HBeAg-positive patients had no

mutation in precore nt 1896. By contrast, the frequent emergence of viral resistance with long-term lamivudine monotherapy in HBeAg-negative precore mutant HBV chronic liver disease is followed by increasing viremia levels, culminating in the development of biochemical breakthrough.[17] Thus, the relationship between the presence of the precore mutation and the emergence rate of the YMDD motif during lamivudine therapy remains unclear.

In conclusion, the reversion from pre-S deletion mutants to wild types during lamivudine monotherapy may be associated with BTH. The course of BTH resembles that of acute exacerbations in chronic HBV infection, with increasing levels of active viral replication, which trigger the immune response, culminating in episodes of ALT flares. Such flares may be caused by the immune response to pre-S wild clones with T-cell and B-cell epitopes. As many patients with liver cirrhosis, and genotype C patients, had pre-S deletion mutants, caution should be exercised when lamivudine is used to treat HBV. This study also raises other questions as to the dynamics between the changes in pre-S and BTH. Such dynamics can be studied further using an HBV replication system in vitro.

Acknowledgments. This study was supported by a Grant-in-Aid from the Ministry of Health, Labour, and Welfare of Japan (H13-kanen-2), and a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Science, and Sports of Japan (14770239). We thank Saori Kawakita for technical assistance.

References

1. Lai CL, Chine RW, Leung NWY, Chang TT, Guan R, Tai DI, et al. A 1-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61–8.
2. Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrell DLJ, et al. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Hepatology 1998;27:1670–7.
3. Mukaide M, Tanaka Y, Katayose S, Tano H, Murata M, Hikata M, et al. Development of real-time detection direct test for hepatitis B virus and comparison with two commercial tests using the WHO international standard. J Gastroenterol Hepatol 2003;18: 1264–71.
4. Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, et al. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J Gen Virol 2001;82:883–92.
5. Minami M, Okanoue T, Nakajima E, Yasui K, Kagawa K, Kashima K. Significance of pre-S region-defective hepatitis B virus that emerged during exacerbation of chronic type B hepatitis. Hepatology 1993;17:558–63.
6. Fan YF, Lu CC, Chang YC, Chang TT, Lin PW, Lei HY, et al. Identification of a pre-S2 mutant in hepatocytes expressing a novel marginal pattern of surface antigen in advanced diseases of chronic hepatitis B virus infection. J Gastroenterol Hepatol 2000;15:519–28.
7. Lu CC, Chen M, Ou JH, Yen TS. Key role of a CCAAT element in regulating hepatitis B virus surface protein expression. Virology 1995;206:1155–8.
8. Nakayoshi T, Maeshiro T, Nakayoshi T, Nakasone H, Sakugawa H, Kinjo F, et al. Difference in prognosis between patients infected with hepatitis B virus with genotype B and those with genotype C in the Okinawa Islands: a prospective study. J Med Virol 2003;70:350–4.
9. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. Hepatology 2001;33:218–23.
10. Sugauchi F, Ohno T, Orito E, Sakugawa H, Ichida T, Komatsu M, et al. Influence of hepatitis B virus genotypes on the development of preS-deletions and advanced liver diseases. J Med Virol 2003;70:537–44.
11. Baron JL, Gardiner L, Nishimura S, Shinkai K, Locksley R, Ganem D. Activation of nonclassical NKT cell subset in a transgenic mouse model of hepatitis B virus infection. Immunity 2002;16:583–94.
12. Kajiya Y, Hamasaki K, Nakata K, Nakagawa Y, Miyazoe S, Takeda Y, et al. Full-length sequence and functional analysis of hepatitis B virus genome in a virus carrier: a case report suggesting the impact of pre-S and core promoter mutations on the progression of the disease. J Viral Hepatol 2002;9:149–56.
13. Fan YF, Lu CC, Chen WC, Yao WJ, Wang HC, Chang TT, et al. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection. Hepatology 2001;33:277–86.
14. Melegari M, Scaglioni PP, Wands JR. The small envelope protein is required for secretion of a naturally occurring hepatitis B virus mutant with pre-S1 deleted. J Virol 1997;71:5449–54.
15. Xu Z, Yen TSB. Intracellular retention of surface protein by a hepatitis B virus mutant that release virion particles. J Virol 1996;70:133–40.
16. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315–21.
17. Papatheodoridis GV, Dimou E, Laras A, Papadimitropoulos V, Hadziyannis SJ. Course of virologic breakthroughs under long-term lamivudine in HBeAg-negative precore mutant HBV liver disease. Hepatology 2002;36:219–26.

J Gastroenterol 2004; 39:776–782
DOI 10.1007/s00535-003-1388-0

Journal of Gastroenterology

Interferon monotherapy for patients with chronic hepatitis C and normal serum aminotransferase levels at commencement of treatment

Satoshi Mamori, Fumitaka Suzuki, Tetsuya Hosaka, Norio Akuta, Takashi Someya, Masahiro Kobayashi, Akihito Tsubota, Yoshiyuki Suzuki, Satoshi Saitoh, Yasuji Arase, Kenji Ikeda, and Hiromitsu Kumada

Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105-8470, Japan

Background. Approximately 30% of patients with chronic hepatitis C have normal serum alanine amino transferase (ALT) levels. While interferon (IFN) monotherapy is approved for patients with chronic hepatitis C infection, the effectiveness of such therapy for chronic hepatitis C patients with normal ALT levels at commencement of treatment remains poorly understood. ***Methods.*** Ninety-four individuals (M/F, 54:40; median age, 46 years) with normal ALT levels (< 50 IU/l) at the commencement of treatment who were positive for both anti-hepatitis C virus (HCV) and serum HCV-RNA were studied. Among this group, 18 individuals (M/F, 9:9; median age, 50 years) had had persistently normal ALT levels for at least 3 months prior to treatment. All patients received their first course of IFN therapy in this study. ***Results.*** Forty-three (45.7%) of 94 individuals had lost serum HCV-RNA at 6 months after cessation of therapy (complete response; CR). The proportion of patients with genotype 2a and HCV-RNA level over 1 Meq/ml who showed CR was significantly lower in those with normal ALT levels than in those with elevated ALT levels (23.8% vs 55.6%; $P = 0.0189$). Two patients who had persistently normal ALT levels and HCV-RNA level over 1 Meq/ml were non-responders (NR) and had ALT flare-ups after IFN therapy. Patients with HCV-RNA levels of less than 1 Meq/ml did not show differential responses based on ALT levels. ***Conclusions.*** Our data suggest that IFN therapy is effective for patients with normal ALT levels and less than 1 Meq/ml HCV-RNA. Thus, such patients should be considered for curative IFN therapy.

Key words: interferon, chronic hepatitis C, normal ALT

Introduction

Antiviral treatment for patients with chronic hepatitis C virus (HCV) infection has generally been limited to those with significantly abnormal serum transaminase activity. Results of interferon (IFN) monotherapy in patients with normal serum alanine amino transferase (ALT) levels were the basis for the conclusions of the 1997 Consensus Conference that these patients should not be treated.[1] Thirty of 52 patients treated in seven studies, for whom data were available, had de novo elevations of ALT levels during therapy, and some patients continued to have ALT elevation after IFN was discontinued.[2–8] Approximately 30% of patients with chronic hepatitis C have normal ALT levels.[9] It is true that if ALT levels are persistently normal, the possibility of significant and progressive liver disease tends to be low, but a significant proportion of patients with persistently normal ALT levels show some histological signs of fibrosis—the degree of which is usually mild but is sometimes more marked—and in rare cases, cirrhosis may be present.[1] IFN treatment is effective for patients with chronic hepatitis C, reducing ALT levels, improving histological activity,[10,11] and eliminating HCV-RNA.[12–15] However, the effectiveness of IFN therapy for patients with normal ALT remains poorly understood. Few studies have compared patients with elevated ALT levels and matched patients with normal ALT levels with respect to assessing sustained virological response rates.[1] Moreover, the changes in ALT levels after IFN therapy in patients with normal ALT levels before treatment is not clear.

The aim of this retrospective study was to determine the incidence of sustained virological response after IFN therapy and the changes in ALT levels after therapy in patients with normal ALT levels at commencement of therapy.

Received: April 10, 2003 / Accepted: December 19, 2003
Reprint requests to: F. Suzuki

Journal of Medical Virology 72:26–34 (2004)

Genetic Heterogeneity of the Precore and the Core Promoter Region of Genotype C Hepatitis B Virus During Lamivudine Therapy

Reiichiro Kuwahara,* Ryukichi Kumashiro, Shiro Murashima, Kei Ogata, Kazuo Tanaka, Akiko Hisamochi, Teruko Hino, Tatsuya Ide, Eisuke Tanaka, Yuriko Koga, and Michio Sata

The Second Department of Medicine, Kurume University School of Medicine, Kurume, Japan

It has been reported that spontaneous or interferon (IFN)-induced hepatitis B e (HBe) seroconversion has usually been associated with the development of a stop codon in the precore region. However, the difference between lamivudine-induced seroconversion and spontaneous or IFN-induced seroconversion is not known. The aim of this study was to investigate the correlation between the evolution of the precore and core promoter mutations and lamivudine-induced seroconversion. Forty-five patients with chronic hepatitis B virus (HBV) infection who were treated with lamivudine for more than 1 year were enrolled. The nucleotide sequence of the precore and core promoter region was determined before and after treatment with lamivudine for 1 year. Among 29 patients who were hepatitis B e antigen (HBeAg)-positive before treatment, 12 (41.3%) lost HBeAg during the course of treatment for 1 year. Of these, eight patients (66.7%) still had precore wild type HBV after 1 year. After 1 year, reversion to precore wild type HBV was detected in 11 (64.7%) of 17 patients who had precore mutant HBV before treatment. Twelve (70.6%) of 17 patients who were persistently HBeAg-positive had precore wild type HBV before and after treatment for 1 year. Despite the loss of HBeAg, two thirds of the patients still had precore wild type HBV after the 1-year treatment. It is suggested that lamivudine-induced seroconversion differs from spontaneous or IFN-induced seroconversion in the change of nucleotides in the precore region. The reversion in the precore region may be caused by the difference of drug-susceptibility to lamivudine. The antiviral effect of lamivudine may be more effective in the precore mutant HBV than in the precore wild type HBV. ***J. Med. Virol. 72:26–34, 2004.***

KEY WORDS: chronic hepatitis B; antiviral effect; reversion; lamivudine-resistant HBV strains

INTRODUCTION

Hepatitis B virus (HBV) infection is a serious worldwide problem. The number of people with chronic HBV infection is 350 million globally [Lee, 1997]. The clinical consequences of HBV infection are various, ranging from becoming an asymptomatic carrier to developing fulminant hepatic failure. Chronic HBV infection is one of the major causes of cirrhosis and hepatocellular carcinoma in endemic areas [Lee, 1997].

Until recently, interferon (IFN) was the only effective antiviral agent for chronic HBV infection but its efficacy was limited [Hoofnagle et al., 1988; Lok et al., 1993; Lau et al., 1997]. Lamivudine, a potent inhibitor of HBV replication by suppressing HBV-DNA polymerase, has become the main therapeutic option for the treatment of chronic hepatitis B. Previous studies have shown that lamivudine led to a median 4-log decrease in serum

Abbreviations: HBV, hepatitis B virus; IFN, interferon; ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; anti-HBe, antibody to hepatitis B e antigen; G, guanine; A, adenine; LGE, log genome equivalents; PCR, polymerase chain reaction; nt, nucleotide.

*Correspondence to: Reiichiro Kuwahara, The Second Department of Medicine, Kurume University School of Medicine, 67 Asahi-Machi, Kurume 830-0011, Japan.
E-mail: ray@med.kurume-u.ac.jp

Accepted 26 June 2003
DOI 10.1002/jmv.10558
Published online in Wiley InterScience (www.interscience.wiley.com)

HBV-DNA levels and to an improvement in serum alanine aminotransferase (ALT) levels [Dienstag et al., 1995], and lamivudine treatment increased significantly the rate of e-seroconversion [Dienstag et al., 1999; Lai et al., 1998; Schiff et al., 1998] as well as leading to histological improvement [Lai et al., 1998; Dienstag et al., 2003]. The emergence of lamivudine-resistant strains is a major drawback of lamivudine treatment. Nevertheless, lamivudine is of great benefit for patients with chronic hepatitis B since it prevents progression to cirrhosis [Lai et al., 1998] and acute exacerbation leading to hepatic failure.

The loss of hepatitis B e antigen (HBeAg) or the presence of antibody to hepatitis B e antigen (anti-HBe) was considered to be associated with decreasing levels of viral replication and HBV-DNA, and also as a move towards disease remission or resolution [Hoofnagle et al., 1981]. Spontaneous or IFN-induced seroconversion from HBeAg to anti-HBe has usually been associated with the development of a stop codon in the precore region [Carman et al., 1989; Omata et al., 1991; Karasawa et al., 1995]. It has been reported that the precore mutant HBV, which has a mutation from guanine (G) to adenine (A) at nucleotide 1896 resulting in conversion of tryptophane to a stop codon at 28 in HBeAg, replaces precore wild type HBV during or after e-seroconversion. HBeAg is derived from a cleavage in the translation product in the precore region and core gene [Carman et al., 1989]. The mutation results in a premature translational stop codon and failure in HBeAg production or cessation of its secretion [Okamoto et al., 1990]. HBe-seroconversion induced by lamivudine appeared to be different from spontaneous or IFN-induced e-seroconversion. For example, spontaneous or IFN-induced e-seroconversion follows a transitory exacerbation of hepatitis in many patients. But e-seroconversion during lamivudine therapy is not accompanied by ALT flare in most patients. It is said that the mutation from G to A at nucleotide 1896 is paralleled by e-seroconversion in general and that the nucleotide sequence at nucleotide 1896 reflects the activity of hepatitis.

There have been some reports that the clinical and virological features of HBV infection were affected by the heterogeneity of the precore region of HBV-DNA [Carman et al., 1989; Okamoto et al., 1991; Omata et al., 1991] and HBV genotype [Kao et al., 2000; Orito et al., 2001]. However, few studies have focused on the correlation between the loss of HBeAg and the evolution of the precore mutant HBV during lamivudine therapy in a homogeneous patient group infected with HBV genotype C. In Japan, genotype C is the most common genotype, accounting for 80% or more of the total [Orito et al., 2001], and there may be some differences in seroconversion between different genotypes of HBV. Previously, it was reported that HBV genotype B was associated with earlier e-seroconversion than HBV genotype C [Chu et al., 2002] and that HBe antigen was significantly less frequent in genotype B than genotype C patients [Orito et al., 2001].

Accordingly, in order to investigate the difference between lamivudine-induced HBe-seroconversion and spontaneous or IFN-induced seroconversion, we selected patients infected with HBV genotype C for this study and analyzed the nucleotide sequence in the precore and core promoter region of HBV-DNA.

PATIENTS AND METHODS

Patients, Blood Tests, Clinical Samples, and HBV-DNA Extraction

Forty-five patients who were treated with lamivudine for at least 1 year at Kurume University Hospital between 1995 and 2001 were included in this study. All patients were positive for HBsAg and HBV-DNA, and had elevated ALT levels within 6 months of the initiation of the lamivudine therapy. No patient was treated with antiviral agents such as IFN or other nucleoside analogs throughout this study. The patients were followed up every week for the first month and then every month during the treatment with lamivudine. Routine liver function tests and serum levels of HBV-DNA were determined before and every 2 weeks for the first 4 weeks and then every 4 weeks during the treatment with lamivudine. Of the 45 patients, 29 were HBeAg-positive and 16 were HBeAg-negative before the treatment. Lamivudine was given at a dosage of 100–300 mg per day. Serial samples were taken before and during the treatment and were stored at −70°C until analysis. HBeAg and anti-HBe were tested with commercial radioimmunoassay kits (Abbott Laboratories, North Chicago, IL). HBV-DNA were quantified by transcription-mediated amplification and hybridization protection assay [Kanmisango et al., 1999]. The detection range is 3.7–8.7 log genome equivalents (LGE)/ml ($10^{3.7}$–$10^{8.7}$ copies/ml). HBV-DNA was extracted from each 100 μl serum sample using a nucleic acid extraction kit, the SMITEST EX-R&D kit (Sumitomo Metal Industries, Tokyo, Japan) according to the manufacturer's instructions. The extracted DNA was dissolved in 20 μl of sterile distilled water.

Genotyping of HBV-DNA

To determine the HBV genotype, a commercially available kit, the SMITEST HBV genotype detection kit (Genome Science Laboratories Co., Ltd., Fukushima, Japan) was used. This kit uses ELISA with monoclonal antibodies to type-specific epitopes in the preS2-region product [Usuda et al., 1999].

Amplification of the Core Promoter and the Precore Region of HBV-DNA

The fragments of 263 base pairs corresponding to nucleotides 1679–1941 were amplified using the polymerase chain reaction (PCR) with nested primers. These fragments included the core promoter region (nucleotides 1742–1849) and the precore region (nucleotides 1814–1900). The pair of primers for the first-round PCR was 5′-CATAAGAGGACTCTTGGACT-3′ (sense;

nucleotides 1653–1672) and 5′-AAAGAATTCAGAAGGCAAAAAAGA-3′ (antisense; nucleotides 1949–1972). The pair of primers for the second-round PCR was 5′-AATGTCAACGACCGACCTTG-3′ (sense; nucleotides 1679–1698) and 5′-TCCACAGAAGCTCCGAATTC-3′ (antisense; nucleotides 1922–1941). One microlitre of resuspended DNA was used in a final volume of 50 μl. The first-round PCR solution contained 10 mM Tris-HCl buffer (pH 8.3), 0.2 mM dNTPs (dATP, dTTP, dCTP, dGTP), 0.6 μM sense primer, 0.6 μM antisense primer, 1.5 mM $MgCl_2$, and 25 U/ml Taq DNA polymerase (PE Biosystems Japan, Chiba, Japan). The sample was heated at 95°C for 10 min at the beginning of the first reaction. The reaction of the first-round PCR was carried out for 35 cycles at 94°C for 30 sec, at 50°C for 30 sec, and at 72°C for 30 sec. For the second-round PCR, 2 μl of the first-round PCR product was added to 98 μl of a reaction mixture with a composition of 10 mM Tris-HCl buffer (pH 8.3), 0.2 mM dNTPs (dATP, dTTP, dCTP, dGTP), 0.6 μM sense primer, 0.6 μM antisense primer, 1.2 mM $MgCl_2$, and 25 U/ml Taq polymerase. The sample was heated at 95°C for 10 min at the beginning of the first reaction. The reaction of the second-round PCR was performed in 35 cycles at 94°C for 30 sec, at 55°C for 30 sec, and at 72°C for 30 sec. Five microlitres of the second-round PCR product was analyzed using electrophoresis in 1.5% agarose gel stained with ethidium bromide and these were visualized under ultraviolet light. To purify the DNA fragments, the second-round PCR product was centrifuged in the QIAquick PCR Purification Kit (QIAGEN GmBH, Hilden, Germany). The nucleotide sequence was determined with an automated ABI DNA sequencer (Model 377, PE Applied Biosystems, Foster City, CA) to verify the specificity and was compared with the published HBV sequences. The sequencing condition was specified in the procedure for the Taq BigDye terminator Cycle Sequencing Kit (PE Applied Biosystems).

Detection of Mutations in the Core Promoter and the Precore Region

HBV complete nucleotide sequence for standard genotype C was obtained from international DNA database (DDBJ/EMBL/GenBank) and HPBADRA (database accession no. M12906) was retrieved as a normal nonmuted phenotype. The sequence was aligned by using the CLUSTAL W software and confirmed by visual inspection.

Subcloning of the Core Promoter and the Precore Region of HBV-DNA

In addition to the nucleotide sequence of the 45 patients, the cloned HBV-DNA of four patients was analyzed. Of the four patients treated with lamivudine, three seroconverted to anti-HBe within 6 months. One patient who was treated with lamivudine seroconverted to anti-HBe but was still positive for HBV-DNA after 1 year. The second-round PCR product was purified with the QIAquick PCR Purification Kit (QIAGEN GmBH). The purified PCR product was used for ligation with the pCR4-TOPO vector (Invitrogen Corp., San Diego, CA) and TOP10F′-competent *Escherichia coli* (Invitrogen Corp.) was used for the transformation. The transformed bacteria were incubated at 37°C in 3 ml of the Luria-Bartani medium for 16 hr and an individual recombinant clone was selected. For each pool, 11–15 clones were subjected to investigation in order to determine the nucleotide sequences of the core promoter and the precore region. A colony-direct PCR was performed using a pair of primers, M13 forward primer (5′-GTAAAACGACGGCCAG-3′) and T7 primer (5′-TAATACGACTCACTATAGGG-3′), to amplify the nucleotides including the core promoter and the precore region. The reaction of the PCR was performed in 35 cycles at 94°C for 30 sec, at 55°C for 30 sec, and at 72°C for 30 sec. The nucleotide sequence was determined using the same method as mentioned above.

Detection of Lamivudine-Resistant Mutants

To detect the lamivudine-resistant HBV strains, the SMITEST HBV-YMDD motif ELMA (Sumitomo Metal Industries) was used according to the manufacturer's instructions. The principle of this procedure is a combination of PCR–ELISA and a mini-sequence method [Kobayashi et al., 2000].

Statistical Analysis

Fisher's exact test and the student's *t*-test were used to make comparisons between the groups for certain variables. A probability value of less than 0.05 was considered significant.

RESULTS

Characteristics of Patients at Baseline

The characteristics of the 45 patients at baseline are shown in Table I. All 45 patients had HBV-DNA with an average of 7.1 ± 1.3 LGE/ml, and the average of serum ALT level was 153 ± 116 U/L. Twenty-nine (64.4%) of the 45 patients were HBeAg-positive and 16 (35.6%) were HBeAg-negative. All patients included in this study had HBV genotype C strains. Between those who were HBeAg-positive and those who were HBeAg-negative, there were significant differences in the HBV-DNA level ($P = 0.0015$) and the number of patients with the precore mutant HBV ($P = 0.0015$) (Table II).

HBeAg Seronegative Rate

Of the 29 patients who were HBeAg-positive at baseline, 12 (41.4%) lost HBeAg during the 1-year treatment and 17 (58.6%) were persistently HBeAg-positive. There were no significant differences in age, gender, serum ALT levels, HBeAg titer, HBV-DNA levels, core promoter mutation, or precore mutation between those who lost HBeAg and those who did not (Table III).

TABLE I. Clinical Profile of 45 Patients With CH-B at Baseline and 1 Year

	Baseline										At 1 year					
									Precore	Core promoter			Precore	Core promoter		
Patient	Age (yr)	Gender	ALT (IU/L)	HBeAg	HBeAg (S/N ratio)	Anti-HBe	Anti-HBe (INH %)	HBV-DNA (LGE/ml)	nt 1896	nt 1762 nt 1764	HBeAg		nt 1896	nt 1762	nt 1764	HBV-DNA (LGE/ml)	YMDD motif
1	39	M	20	Positive	19.7	Negative	0	8	G	T A	Negative		G	T	A	<3.7	YMDD + YVDD
2	49	M	73	Positive	13.6	Negative	38.9	5.7	G	T A	Negative		G	T	A	3.7	YMDD
3	46	M	193	Positive	132.6	Negative	0	>8.7	G	T A	Negative		G	T	A	4.1	YMDD
4	59	M	125	Positive	32.6	Negative	0	7.7	G	T A	Negative		G	T	A	<3.7	YMDD
5	45	M	43	Positive	7.2	Negative	68.9	7.3	G	T A	Negative		G	A	G	<3.7	YMDD
6	50	M	236	Positive	72.7	Negative	0	7	A	T A	Negative		G	T	A	<3.7	YMDD
7	45	M	45	Positive	99.8	Negative	0	6.3	A	T A	Negative		G	T	A	<3.7	YMDD
8	40	M	132	Positive	3.8	Negative	60	8.6	A	Deletion	Negative		G	T	A	4.1	YMDD
9	73	F	41	Positive	188.8	Negative	0	7	G	T A	Negative		A	T	A	<3.7	YMDD
10	57	F	87	Positive	282.5	Negative	0	7.8	G	T A	Negative		A	T	A	4.6	YMDD
11	37	M	314	Positive	112.7	Negative	0	5.8	G	A G	Negative		A	T	A	<3.7	YMDD
12	43	M	148	Positive	57.6	Negative	0	8.4	A	A G	Negative		A	T	A	<3.7	YMDD
13	53	F	204	Positive	162.5	Negative	0	8	G	T A	Negative		G	T	A	8.1	YIDD
14	73	F	74	Positive	135.8	Negative	0	6.5	G	T A	Positive		G	T	A	6.4	YVDD
15	73	F	301	Positive	277.2	Negative	0	8.5	G	T A	Positive		G	T	A	<3.7	YMDD
16	39	M	334	Positive	193.7	Negative	0	>8.7	G	T A	Positive		G	T	A	<3.7	YMDD + YIDD
17	42	M	324	Positive	16	Negative	0	7.3	G	T A	Positive		G	T	A	5.2	YMDD + YIDD
18	47	M	106	Positive	8.7	Negative	57.3	5.7	G	T A	Positive		G	T	A	4.7	YMDD
19	45	M	39	Positive	258	Negative	0	>8.7	G	T A	Positive		G	T	A	7.1	YIDD
20	37	M	320	Positive	208.3	Negative	0	7.6	G	A G	Positive		G	T	A	3.9	YMDD
21	36	M	93	Positive	54	Negative	0	>8.7	G	Deletion	Positive		G	A	G	4.8	YMDD + YIDD
22	38	M	53	Positive	212	Negative	0	8.6	G	T A	Positive		G	A	G	<3.7	YMDD
23	59	F	119	Positive	99.4	Negative	0	8.5	G	T A	Positive		G	Deletion		<3.7	YMDD
24	46	M	227	Positive	274	Negative	0	8.2	G	A G	Positive		G	A	G	<3.7	YMDD + YVDD
25	28	M	201	Positive	43	Negative	0	6	A	T A	Positive		G	A	G	<3.7	YMDD
26	38	M	130	Positive	144.1	Negative	0	6.4	G	T A	Positive		A	A	G	<3.7	YMDD
27	40	M	129	Positive	188.4	Negative	0	7.9	G	T A	Positive		A	A	G	<3.7	YMDD
28	44	M	51	Positive	2.3	Positive	85.4	5.7	G	Deletion	Positive		A	A	G	3.9	YMDD
29	40	M	94	Positive	124.3	Negative	0	8.4	A	A G	Positive		A	A	G	7.3	YMDD + YIDD
30	44	F	87	Negative	0.8	Positive	96.7	5.9	G	T A	Negative		G	A	G	<3.7	YMDD
31	41	M	88	Negative	0.5	Positive	97.9	7.1	G	T A	Negative		G	A	G	<3.7	YMDD
32	51	M	27	Negative	0.9	Positive	96.7	<3.7	G	T A	Negative		G	A	G	<3.7	YMDD
33	61	F	96	Negative	1.7	Positive	89.7	4	G	T A	Negative		G	Deletion		<3.7	YMDD
34	53	M	56	Negative	0.6	Positive	96.4	4.9	A	T A	Negative		G	T	A	<3.7	YVDD
35	56	M	313	Negative	0.2	Positive	96.9	7.7	A	T A	Negative		G	T	A	<3.7	YMDD
36	49	M	251	Negative	0.8	Positive	97.8	7.1	A	T A	Negative		G	T	A	<3.7	YMDD
37	41	M	298	Negative	0.5	Positive	96.6	7.1	A	T A	Negative		G	T	A	<3.7	YMDD
38	50	F	538	Negative	1	Positive	97.8	7.1	A	T A	Negative		G	T	A	<3.7	YMDD
39	34	F	56	Negative	0.5	Positive	93.6	5.4	A	A G	Negative		G	T	A	<3.7	YMDD
40	50	M	73	Negative	0.6	Positive	97.5	6.3	A	T A	Negative		G	A	G	<3.7	YMDD
41	56	M	106	Negative	0.4	Positive	96	6.6	G	T A	Negative		A	T	A	<3.7	YMDD
42	41	F	41	Negative	1	Positive	70.9	7.6	A	T A	Negative		A	T	A	5	YIDD
43	45	F	58	Negative	0.7	Positive	96.7	6.2	A	T A	Negative		A	T	A	<3.7	YMDD
44	42	M	211	Negative	0.7	Positive	98.3	7	A	A G	Negative		A	T	A	<3.7	YMDD
45	40	M	336	Negative	0.6	Positive	93.9	7.3	A	T A	Negative		A	A	G	<3.7	YMDD

Abbreviations: M, male; F, female; LGE, log genome equivalent.
The upper limit of the normal range of ALT is 42 IU/L.