TABLE I. Patient Profile and Laboratory Data at Commencement of Interferon Monotherapy

Demography	
Number	364
Sex (M/F)	239/125
Age (years)[a]	51 (17–68)
History of blood transfusion	150 (41.2%)
Familial history of liver disease	65 (17.9%)
Body mass index (kg/m^2)[a]	23.1 (16.3–35.7)
Body surface area (m^2)[a]	1.70 (1.22–2.19)
Laboratory data[a]	
Alanine aminotransferase (IU/l)	83 (8–642)
Albumin (g/dl)	4.0 (2.7–5.1)
Cholinesterase (ΔpH)	1.1 (0.4–1.9)
Hemoglobin (g/dl)	14.6 (9.6–18.3)
Platelet count (×1,000 μ/L)	178 (43–331)
Serum iron (μg/dl)	153 (16–355)
Unsaturated iron-binding capacity (μg/dl)	192 (24–509)
Serum ferritin (μg/l)	128 (<10–2008)
Level of viremia (Meq/ml)	0.8 (<0.5–43.5)
Histological findings	
Stage (F1/F2/F3)[b]	259/81/24
Hepatocyte steatosis (none/mild/moderate/severe)	44/291/29/0

[a]Expressed as median (range).
[b]Stage of chronic hepatitis by Desmet et al. [1994].

(2) patients were cases without steatosis (none) or with the highest grade of steatosis (moderate); and (3) the body mass index was less than 25.0 kg/m^2 (i.e., non-obese patients). Previous studies showed that the body mass index might influence the grade of hepatocyte steatosis and the response to IFN therapy [Akuta et al., 2002; Bressler et al., 2003]. Therefore, to examine the IFN-resistance mechanism specific for hepatocyte steatosis, the relationship between amino acid substitutions of HCV core region/NS5A and the grade of hepatocyte steatosis was assessed only in resistant non-obese cases so as to minimize the influence of obesity.

Laboratory Investigations

Blood samples were frozen at −80°C within 4 hr of collection and were not thawed until used for testing. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region [Chayama et al., 1993]. In all cases, HCV-RNA viremia level was measured by branched DNA assay version 2.0 (Chiron Corp., Emeryville, CA) at commencement of therapy using frozen samples, and the results were expressed as 10^6 genomic equivalents per milliliter (Meq/ml). The lower limit of the assay was 0.5 Meq/ml. Samples were evaluated by HCV-RNA qualitative analysis with PCR (nested polymerase chain reaction or Amplicor™, Roche Diagnostic Systems, CA) during and after therapy, and the results were expressed as positive or negative. The lower limit of the assay was 100 copies/ml. With regard to the IFN resistant cases, samples obtained at the commencement of therapy were also evaluated by quantitative analysis of HCV-RNA with PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics). The lower limit of the assay was 0.5 kIU/ml.

Histopathological Examination of the Liver

Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained six or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al. [1994]. Hepatocyte steatosis was classified into four grades based on the criteria of D'Alessandro et al. [1991]: none (absent), mild (involvement of less than 1/3 of hepatocytes), moderate (involvement of greater than 1/3 but less than 2/3 of hepatocytes), or severe (involvement of greater than 2/3 of hepatocytes).

Nucleotide Sequencing of the Core Region and NS5A Gene

As described in previous reports with some modifications [Chayama et al., 1997; Murakami et al., 1999], the sequences of amino acids 1–191 in the core region [Rubbia-Brandt et al., 2000] and amino acids 2163–2254 in the NS5A [Akuta et al., 2003] were determined by the direct sequencing method using sera of nine patients. The sequences of amino acids were compared with the consensus sequence of genotype 2a, which were determined by comparing the sequences obtained in this study and prototype sequence (HC-J6) [Okamoto et al., 1991]. HCV-RNA was extracted with a SepaGene RV-R kit (Sanko Junyaku, Tokyo, Japan) from serum samples

at the start of treatment and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo, Japan). Nucleic acids were amplified by PCR using the following primers.

Nucleotide sequences of the core region. Nucleic acids of the Core region were amplified by division into two parts of the N- and C-terminal regions. The first-round PCR in the N-terminal region was performed with 2aC1Fo (sense, 5′-TGCTAGCCGAGTAGCGTTGG-3′) and 2aC1Ro (antisense, 5′-TTCACTTCGGCAGCGGAGAC-3′), and the second-round PCR with 2aC1Fi (sense, 5′-CTTGTGGTACTGCCTGATAG-3′) and 2aC1Ri (antisense, 5′-CAGTGGAGCGCCGATCCTTA-3′). The first-round PCR in the C-terminal region was performed with 2aC2Fo (sense, 5′-CCAGATCGTTGGCGGAGTAT-3′) and 2aC2Ro (antisense, 5′-TCCAGCACCGAGATGTATTC-3′), and the second-round PCR with 2aC2Fi (sense, 5′-TATACTTGTTGCCGCGCAGG-3′) and 2aC2Ri (antisense, 5′-AGTCGTTGGTCACCATGTAG-3′).

Nucleotide sequences of the NS5A. The first-round PCR was performed with 5′ outer primer (sense, 5′-CCAGA(AG)TT(CT)TT(CT)TC(CT)TGGGTGGATGG-3′) and 3′ outer primer (antisense, 5′-GGTT(CG)(AG)TA(GA)(CT)C(CT)GGCCTCTTCCA-3′), and the second-round PCR with 5′ inner primer (sense, 5′-TGTAAAACGACGGCCAGTCAGCTCCCTTGCGATCCTGA-3′ with the sequence of the M13 forward primer underlined) and 3′ inner primer (antisense, 5′-CAGGAAACAGCTATGACC(AT)GG(GA)TTGTA(AG)TC(AT)GG(AC)CG(GT)GCCCA-3′ with the sequence of the M13 reverse primer underlined). All samples were initially denatured at 95°C for 15 min. Forty cycles of amplification were set as follows: Denaturation for 1 min at 94°C, annealing of primers for 1 min at 55°C, and extension for 1 min at 72°C with an additional 7 min for extension. Then, 1 μl of the first PCR product was transferred to the second PCR reaction. Other conditions for the second PCR were the same as the first PCR, except that the second PCR primers were used instead of the first PCR primers. PCR-amplified complementary DNA (cDNA) was purified after agarose gel electrophoresis and used for direct sequencing using each of the second-round PCR primer or M13 primer as the sequencing primer with the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). To avoid false-positive results, the procedures recommended by Kwok and Higuchi [1989] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Statistical Analysis

Non-parametric tests were used to examine the background characteristics of patients and amino acids changes, including the chi-squared test or Fisher's exact probability test, and Mann–Whitney U-test. The cumulative HCV-RNA negative rates by qualitative analysis with PCR were calculated using the Kaplan–Meier technique, and differences between the curves were tested using the log-rank test. Univariate and multivariate logistic regression analyses were used to determine those factors associated with hepatocyte steatosis. All P-values of less than 0.05 by the two-tailed test were considered significant. The odds ratios and 95% CI were also calculated. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Potential factors associated with hepatocyte steatosis included the following 16 variables: sex, age, history of blood transfusion, family history of liver disease, body mass index, body surface area, alanine aminotransferase (ALT), albumin, cholinesterase, hemoglobin, platelet count, serum iron, unsaturated iron binding capacity, ferritin, level of viremia, and pathological staging. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, IL).

RESULTS

Factors Associated With Hepatocyte Steatosis in Multivariate Analysis

Data of patients classified with low-grade steatosis (none [n = 44] to mild [n = 291]) and high-grade (moderate [n = 29] to severe [n = 0]) were examined to determine the factors associated with steatosis. Univariate analysis identified three parameters that significantly influenced hepatocyte steatosis. These included age ($P = 0.001$), serum ferritin level ($P = 0.002$), and body mass index ($P = 0.016$). Of these, multivariate analysis identified serum ferritin level ($P = 0.004$) and body mass index ($P = 0.049$) as two parameters that independently influenced hepatocyte steatosis (Table II).

Treatment Efficacy According to Severity of Steatosis and IFN Regimen

As a whole, 249 of 364 (68.4%) patients achieved sustained virological response, while the remaining 115

TABLE II. Factors Associated With Hepatocyte Steatosis in 364 Patients Infected With HCV Genotype 2a, Identified by Multivariate Analysis

Factor	(Category)	Odds ratio (95% CI)	P
Serum ferritin (μg/L)	1: <200	1	
	2: 200	3.48 (1.50–8.07)	0.004
Body mass index (kg/m^2)	1: <25.0	1	
	2: 225.0	2.37 (1.00–5.57)	0.049

Variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.

TABLE III. Sustained Virological Response Rates Estimated by Interferon Treatment Regimens in 364 Patients Infected With HCV Genotype 2a

		Continuous[a] + intermittent[b]	
Grade of steatosis	8 weeks continuous	24 weeks	>24 weeks
Moderate to severe	25.0% (1/4)	44.0% (11/25)	—
None to mild	61.8% (34/55)	72.1% (191/265)	80.0% (12/15)

[a]2- or 8-week continuous.
[b]Three times a week.

patients (31.6%) failed to respond to therapy. Among 335 patients with the low-grade hepatocyte steatosis (grade none to mild), 237 (70.7%) achieved sustained virological response, while of 29 patients with high-grade steatosis (grade moderate to severe), only 12 (41.4%) showed sustained virological response. The rate of sustained virological response in patients with low-grade steatosis was significantly higher than in those with high-grade steatosis ($P = 0.0017$). The clinicopathological characteristics (16 variables, evaluated as potential risk factors of steatosis) of non-sustained virological response patients (115 patients) at the start of treatment of low-grade (98 patients) and high-grade (17 patients) steatosis groups were not significantly different.

With regard to the efficacy of monotherapy according to IFN regimen in 335 patients with low-grade steatosis, sustained virological response rate was 61.8% in patients who received daily IFN therapy for only 8 weeks. In patients on continuous followed by intermittent therapy, 72.1% achieved sustained virological response after 24 weeks of treatment, and 80.0% showed sustained virological response after >24 weeks of treatment (Table III). Table IV[Q1]

Early Viral Kinetics and Severity of Hepatocyte Steatosis

The cumulative HCV-RNA negative rates of the low- and high-grade hepatocyte steatosis were 10.0% and 11.1% at day 1; 10.0% and 13.0% at day 2; 20.0% and 40.7% at week 1; 30.0% and 75.9% at week 2; 60.0% and 94.4% at week 4; and 80.0% and 98.2% at week 8, respectively. Statistical analysis showed that the cumulative HCV-RNA negative rate was significantly higher in the low-grade group than the high-grade steatosis group ($P = 0.0055$; Log-rank test, Fig. 1).

Virological Features of Cases Resistant to IFN According to Severity of Steatosis

Figure 2A,B show the amino acid sequences of HCV core region/NS5A of 9 IFN-resistant patients. In eight patients, the level of viremia was more than 100 kIU/ml, while in the remaining patient (Case 5, who was free of liver steatosis), the level of viremia was 76 kIU/ml. Heterogeneity of two cases or more was found at the following amino acid positions: amino acid 4 (Cases 6 and 7; I/N), amino acid 10 (Cases 5 and 9; Q/K), amino acid 78 (Cases 2 and 4; R/K), amino acid 2240 (Cases 1,

TABLE IV. Clinical and Virological Features of 64 Patients Who Were Evaluated for Early Viral Kinetics, According to the Grade of Hepatocyte Steatosis

	Grade of hepatocyte steatosis		
	None to mild	Moderate to severe	Differences
N	54	10	NS
Age (years)[a]	17–67 (50)	41–66 (52)	NS
Sex (M/F)	31/23	3/7	NS
Body mass index (kg/m^2)	16.8–32.6 (22.2)	19.2–31.1 (25.3)	$P = 0.049$
Level of viremia level (Meq/ml)[b]	<0.5–22.0 (2.2)	<0.5–18.0 (5.8)	NS
Histological staging (F1/F2/F3)[c]	45/6/3	7/1/2	NS
ALT level (IU/l)[a]	15–618 (64)	31–175 (64)	NS
Serum albumin (g/dl)[a]	3.1–4.5 (3.9)	3.3–4.0 (3.6)	$P = 0.024$
Cholinesterase (ΔpH)[a]	0.5–1.8 (1.1)	0.8–1.4 (1.1)	NS
Hemoglobin (g/dl)[a]	9.6–18.3 (14.0)	12.9–15.8 (14.0)	NS
Platelet count (×1,000 μ/L)[a]	89–304 (176)	113–229 (163)	NS
Serum iron (μg/dl)	31–264 (146)	64–283 (196)	NS
Unsaturated iron-binding capacity (μg/dl)	61–484 (198)	24–331 (112)	NS
Serum ferritin (μg/l)	<10–1,417 (102)	<10–698 (243)	NS
IFN dose per day (MU/day)	6–7.5 (6)	6–7.5 (6)	NS

ALT, alanine aminotransferase; IFN, interferon; MU, million units; NS, not significant.
[a]Expressed as range (median).
[b]Viral levels measured by branched DNA assay version 2.0.
[c]Stage of chronic hepatitis by Desmet et al. [1994].

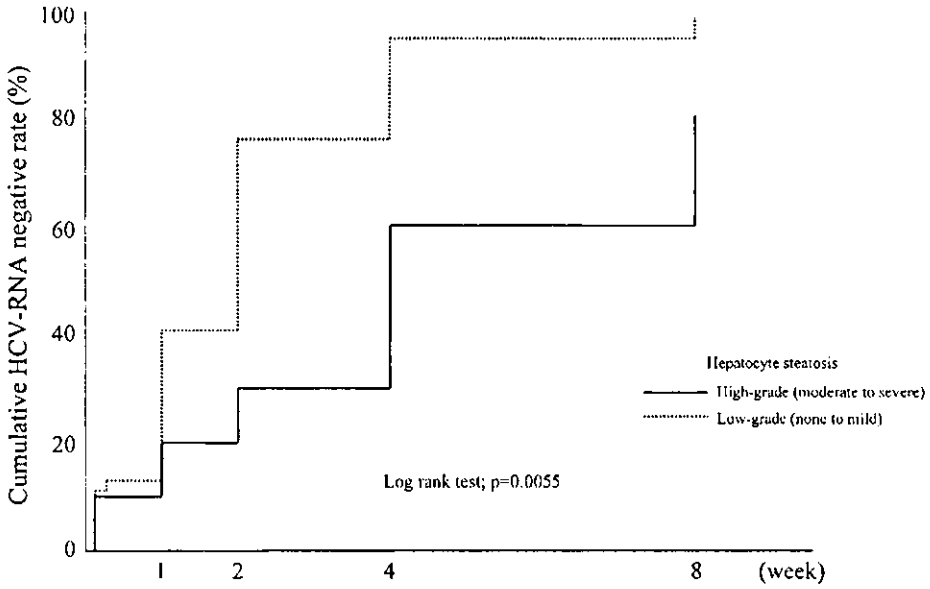

Fig. 1. In early viral kinetic study during IFN monotherapy, the cumulative HCV-RNA negative rate of the low-grade (none to mild) hepatocyte steatosis was significantly higher than that of the high-grade (moderate to severe) steatosis.

2, and 4; V/A, T/A, and V/A, respectively), and amino acid 2243 (Cases 5, 6, and 7; E/R, M/R, and V/R, respectively). These results indicate that the amino acids sequences of four patients resistant to IFN monotherapy with the highest grade of liver steatosis (moderate grade) did not show specific amino acid substitutions, compared with the sequences of five patients also resistant to IFN monotherapy but had no hepatocyte steatosis (none grade).

DISCUSSION

In this study, the factors associated with hepatocyte steatosis were analyzed in HCV genotype 2a. Known factors associated with hepatocyte steatosis include chronic alcohol consumption, high body mass index (obesity), elevated serum concentrations of cholesterol or triglycerides, diabetes mellitus, hepatotoxic drugs, and HCV genotype 3 infection [Wanless and Lentz, 1990; Mihm et al., 1997; Sheth et al., 1997; Czaja et al., 1998; Westin et al., 2002; Castera et al., 2004; Rubbia-Brandt et al., 2004; Sharma et al., 2004]. Recent studies have focused on the mechanism of HCV-related liver damage associated with hepatocyte steatosis. Patton et al. [2004] discussed the possibility of non-inflammatory-mediated mechanism for fibrosis secondary to steatosis. They indicated that one possible mechanism might include lipid peroxidation by HCV, which could result in activation of hepatic stellate cells and subsequent collagen deposition, based on in vitro evidence [Paradis et al., 1997; Okuda et al., 2002; Patton et al., 2004]. Farinati et al. [1995] reported that HCV-related liver damage might be characterized by increased iron storage, which elicits a free-radical-mediated peroxidation, with consequent steatosis and activation of glutathione turnover. In a subsequent study, they also showed that the serum levels 8-hydroxydeoxyguanosine (8-OHdG), a reliable marker of oxidative stress in HCV patients, correlated with serum ferritin levels and the grade of hepatocyte steatosis [1999[Q2]]. In the study of patients infected with HCV genotype 2a, multivariate analysis also identified high serum ferritin level as an independent factor associated with high-grade hepatocyte steatosis. Thus, the results of the present study confirm these early reports that hepatocyte steatosis associated with HCV-related liver damage, including HCV genotype 2 might be characterized by increased iron storage, which elicits a free-radical-mediated peroxidation. Interestingly, steatosis in this study of genotype 2a was not associated with fibrosis. Rubbia-Brandt et al. [2004] reported that liver fibrosis might be associated with steatosis only in genotype 3. Considered together, the results suggest that hepatocyte steatosis might influence progression of liver fibrosis in a viral genotype-specific manner.

Hepatocyte steatosis has been considered recently as an important pretreatment predictor of response to IFN therapy. It is reported to be an important predictive factor of sustained virological response in combination therapy of IFN-ribavirin in patients infected with HCV genotype 1 or 3 [Bjøro et al., 2002; Poynard et al., 2003; Patton et al., 2004; Zeuzem et al., 2004]. However, there is no information on whether hepatocyte steatosis in patients infected with genotype 2 affects the virological response to IFN therapy, a part from a previous report [Akuta et al., 2002]. Akuta et al. [2002] reported previously that hepatocyte steatosis was a negative predictor of sustained virological response to IFN monotherapy in patients infected with genotype 2a, based on

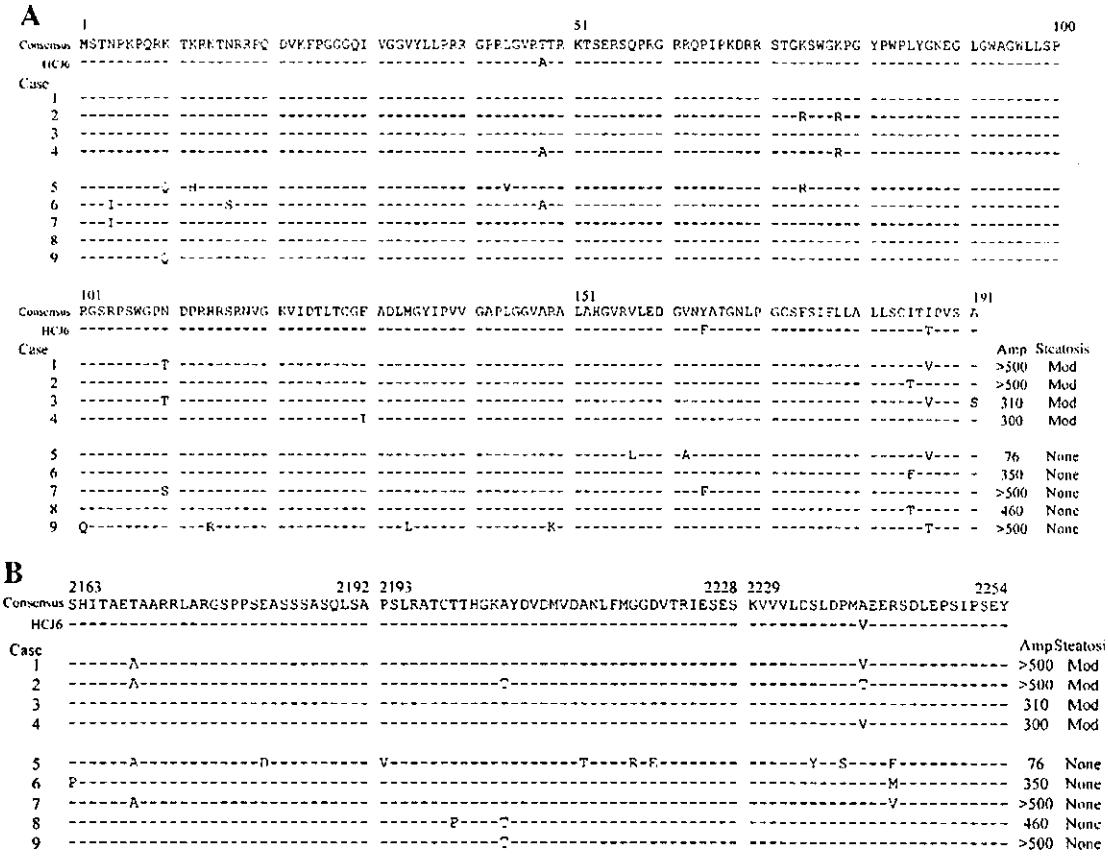

Fig. 2. Sequences of amino acids 1–191 in HCV core region (**A**) and amino acids 2163–2254 in HCV NS5A (**B**) at the start of IFN monotherapy of nine IFN-resistant patients infected with HCV genotype 2a with the highest grade of hepatocyte steatosis (moderate = Mod; Cases 1–4) or without steatosis (none; Cases 5–9). Dashes indicate amino acids identical to the consensus sequence of genotype 2a, and substituted amino acids are shown by standard single-letter codes. Eight IFN-resistant patients had high viremia (HCV more than 100 kIU/ml) while the viral level in the other IFN-resistant case (Case 5), free of steatosis, was 76 kIU/ml. Amp (kIU/ml) = quantitative analysis of HCV-RNA with PCR of Amplicor version 2.0.

multivariate analysis. A recent report [Patton et al., 2004] indicated that patients with genotype 1 who showed an early virologic response (defined as ≥ 2 $\log_{10}$ decline in HCV-RNA at week 12) were more likely to have low-grade steatosis than those who did not show an early response. The results of the present study of early viral kinetics and its relationship to the grade of steatosis in patients infected with genotype 2a were similar to the above study, and is the first report to indicate that the grade of steatosis influences early viral kinetics of HCV-RNA in patients infected with genotype 2a and treated with IFN. Further prospective studies should be performed based on a large number of patients who are matched for factors associated with hepatocyte steatosis (such as alcohol consumption, glucose/lipid metabolism) and pretreatment efficacy predictors (including IFN regimen, viral load, and serum albumin level [Akuta et al., 2002]).

Previous studies that patients infected with genotype 3 who could achieve sustained virological responses by IFN treatment often demonstrated a marked decrease in steatosis as confirmed by repeated posttreatment biopsies, and it was concluded that steatosis of genotype 3 might be considered as a consequence of viral infection [Kumar et al., 2002; Poynard et al., 2003; Castera et al., 2004; Patton et al., 2004]. However, it is not known whether steatosis of genotype 2a is also a consequence of viral infection or other non-viral factors (e.g., metabolic factors). Hence, it is important to investigate the relationship between treatment efficacy and improvement of steatosis grade, based on repeated biopsies in future studies.

IFN-resistance mechanisms specific for hepatocyte steatosis are so far unknown. Steatosis may be associated with reduced liver metabolism probably due to reduced activity of hepatic cytochrome induced by high liver fat content [Leclercq et al., 1998]. Previous studies showed that liver fibrosis correlates with steatosis and obesity [Hourigan et al., 1999; Clouston et al., 2001; Hwang et al., 2001]. Thus, lipid deposits within hepatocytes might cause functional disturbances by increasing the architectural distortion of the hepatic lobule caused by fibrosis and decrease in the contact area between drugs and hepatocytes [Taliani et al., 1995; Giannini

et al., 1999]. Another study reported a close correlation between the level of intrahepatic HCV-RNA and severity of steatosis [Rubbia-Brandt et al., 2000]. In the present study, no significant clinicopathological parameter (including serum HCV-RNA levels, fibrosis, and body mass index) was identified in non-sustained virological response patients with low-grade and high-grade steatosis. Thus, the results failed to establish a link between IFN-resistance and hepatocyte steatosis.

It is also not clear whether the virological characteristics of HCV play any pathogenic role in the derangement of lipid metabolism, and hence contribute to hepatocyte steatosis. Experiments conducted in vitro and in transgenic mice suggested that HCV core protein and NS5A region might be involved in the pathogenesis of lipid accumulation [Barba et al., 1997; Moriya et al., 1997; Shi et al., 2002]. On the other hand, Rubbia-Brandt et al. [2000] reported that steatosis might be a morphological expression of viral cytopathic effect in patients infected with HCV genotype 3, but that analysis of the HCV core protein failed to identify a sequence specially associated with the development of steatosis [Rubbia-Brandt et al., 2000]. No study has investigated the effects of HCV core protein and NS5A region on IFN efficacy in patients with hepatocyte steatosis. In this context, the relationship between amino acid substitutions in HCV core protein/NS5A region and the grade of hepatocyte steatosis was analyzed in the present study in IFN-resistant patients. However, the analysis showed no specific amino acid substitutions in these regions that could establish a role for hepatocyte steatosis in IFN-resistance. It should be noted, however, that the present study was based on a small group of patients and sequence analysis of the defined regions should be investigated in large-scale studies to confirm the present findings.

β IFN is rarely used and is not licensed outside Japan. It was reported previously that the type of IFN (α vs. β) is not a predictor of sustained virological response to IFN monotherapy in 394 patients infected with genotype 2a, based on multivariate analysis [Akuta et al., 2002], and accordingly when the present study was designed, it was thought that the type of IFN should not affect the outcome of patients infected with genotype 2a. Incidentally, based on data from Toranomon Hospital, the frequency of β IFN-related adverse events seems to be lower than those by α IFN, especially in elderly patients (unpublished data). Therefore, the use of β IFN rather than α IFN is recommended at least for elderly patients.

In conclusion, the present study of patients infected with HCV genotype 2a suggested that hepatocyte steatosis is possibly associated with excessive iron storage, and that it might be an important predictor of the efficacy of IFN monotherapy. Further studies should be performed to investigate whether hepatocyte steatosis associated with HCV genotype 2a might be also a predictor of other treatments, including IFN-ribavirin combination therapy and pegylated IFN. In this study, amino acid substitutions associated with IFN-resistance specific for hepatocyte steatosis could not be identified, and large-scale studies should be conducted to confirm the present findings. Further analysis of IFN-resistance mechanisms should be conducted in future studies taking into consideration pharmacokinetic, viral, and host-related factors.

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Virological and Biochemical Relapse after Discontinuation of Lamivudine Monotherapy for Chronic Hepatitis B in Japan: Comparison with Breakthrough Hepatitis during Long-Term Treatment

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Chronic hepatitis B · Lamivudine monotherapy · Biochemical and virological relapse · Basic core promoter · YMDD motif mutant · Breakthrough hepatitis · Retreatment · HBV genotype

Abstract

Objective: Comparison of virological and biochemical relapse in patients with chronic hepatitis B, based on continuation or discontinuation of lamivudine monotherapy. ***Methods:*** In Japanese genotype C-dominant hepatitis B patients, 25 patients who stopped treatment at normal levels of alanine transferase (ALT) were retrospectively compared with 75 patients who continued treatment. Both groups were matched for age, sex, and observation period after start of treatment. We investigated the relapse rates, and evaluated predictive factors for relapse and efficacy of retreatment of the discontinuous group. ***Results:*** Virological and biochemical relapse occurred significantly earlier in the discontinuous than continuous group, and the peak levels and ratios of peak to pretreatment levels of serum bilirubin and ALT after relapse were not significantly different between the two groups. Multivariate analysis identified three independent factors at discontinuation of treatment associated with early biochemical relapse: HBeAg positivity, presence of liver cirrhosis, detection of basic core promoter mutant. Normalization of ALT levels with retreatment occurred in 62.5% of patients, but 2 HBeAg-positive patients retreated after the emergence of YMDD motif mutant developed severe relapse with hyperbilirubinemia. ***Conclusion:*** Our results in Japanese patients with genotype C-dominant hepatitis B suggest that discontinuation of lamivudine monotherapy, and retreatment after the emergence of YMDD mutant should be given attention.

Introduction

Lamivudine, an oral cytosine nucleoside analog clinically used for the treatment of chronic hepatitis B virus (HBV) infection, potently inhibits HBV replication by interfering with HBV reverse transcriptase activity [1–4], and results in a marked decrease of HBV DNA and serum transaminase levels, seroconversion of HBe antigen (HBeAg) to anti-HBe, and histopathological improvement [4–9].

The optimal duration of lamivudine administration for HBV-infected patients is still controversial for two main problems; drug resistance and sustainability of the response to treatment. In particular, there is a need to evaluate short-term treatment with respect to post-treatment safety and the sustainability of responses, and long-term treatment with regard to biochemical relapse (breakthrough hepatitis) associated with the emergence of YMDD motif mutant [10–17]. The American Association for the Study of Liver Diseases practice guidelines suggested that lamivudine could be discontinued in patients who had completed one year of treatment and had persistent HBeAg seroconversion on more than one occasion determined 2–3 months apart [18]. However, this is not completely evaluated in Japanese genotype C-dominant hepatitis B patients.

The present study was designed to deal with the following three issues: (1) To compare the virological and biochemical relapse rates according to the continuation or termination of lamivudine monotherapy, and to compare the risk of biochemical relapse after the termination of the treatment and breakthrough hepatitis during long-term treatment; (2) to determine the independent predictive factors at discontinuation of treatment that contributed to early biochemical relapse in discontinuous patients, and (3) to evaluate the efficacy of retreatment with lamivudine monotherapy.

Patients and Methods

Patients

Lamivudine therapy was provided to 394 consecutive patients with chronic hepatitis B who tested positive for HBs antigen at Toranomon Hospital between September 1995 and December 2002. Among these, 269 patients started lamivudine monotherapy at abnormal alanine transferase (ALT) levels (normal for ALT, 6–50 IU/l) and were able to achieve ALT normalization during treatment, and were enrolled in this retrospective study. The latter group consisted of 25 patients who stopped the lamivudine monotherapy during ALT normalization (discontinuous group) and 244 patients who did not stop the lamivudine monotherapy (continuous group), and the discontinuation or not of lamivudine during ALT normalization was selected at their own request. To compare the cumulative virological and biochemical relapse rates between the discontinuous group and continuous group, all 25 patients of the discontinuous group entered this study along with 75 patients of the continuous group. The latter group was selected from among the 244 because they matched patients of the discontinuous group with respect to sex, age, and observation period after the start of lamivudine monotherapy. They had been confirmed to have hepatitis by liver biopsies, were free of decompensated liver cirrhosis and hepatocellular carcinoma. Coinfection and superinfection with hepatitis A, C, and delta viruses, and human immunodeficiency virus were ruled out serologically or genomically using commercially available kits or conventional polymerase chain reaction (PCR)-based assays. None of the patients had a history of other liver diseases, such as autoimmune hepatitis, alcoholic liver disease, and metabolic disease.

Patients were given a dose of 100 mg of lamivudine once a day. The median period of treatment in the discontinuous group (0.72 years, range; 0.10–5.6 years) was significantly shorter than that of the continuous group (1.8 years, range; 0.71–7.6 years, $p < 0.0001$). The median observation period after the commencement of lamivudine therapy was not significantly different based on the matching of the two groups, and the periods were 2.1 years (range; 0.68–7.9 years) in the discontinuous group and 1.8 years in the continuous group (range; 0.71–7.6 years). In the discontinuous group, the median observation period after discontinuation of lamivudine therapy was 1.4 years (range; 0.15–6.7 years). With regard to the observation period, patients of the discontinuous group who received another course of lamivudine treatment for biochemical relapse and those of the continuous group who received additional interferon treatment for biochemical relapse, were treated as censored data at the time of lamivudine retreatment and additional interferon treatment in the statistical analysis of cumulative relapse rates. The clinical characteristics of enrolled patients are summarized in table 1, and those of discontinuation are shown in table 2.

Methods

Our study compared virological and biochemical relapse in continuous and discontinuous lamivudine monotherapy groups, and determined the independent predictive factors at discontinuation that contributed to early biochemical relapse in the discontinuous group. Furthermore, we also evaluated the efficacy of retreatment with lamivudine monotherapy. Patients in whom ALT levels became abnormal (>50 IU/l) after a period of ALT normalization were defined as biochemical relapsers. Patients in whom levels of HBV DNA re-elevated after the minimum levels, ignoring undetectable HBV DNA levels, were defined as virological relapsers. Especially, virological relapse during lamivudine treatment associated with the emergence of YMDD motif mutant were defined as DNA breakthrough, and biochemical relapse associated with DNA breakthrough were defined as breakthrough hepatitis. Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by a detailed interview and medical examination at least once every month. Patient compliance with treatment was evaluated by a questionnaire.

Blood samples were obtained at least once every month before, during, and after treatment, and were analyzed for various laboratory data including ALT levels, HBV DNA levels, and the presence of YMDD motif mutant. The serum samples were stored in aliquots at −80°C until use. HBs antigen and HBeAg/eAb were determined by radioimmunoassay (Abbott Diagnostics, Chicago, Ill., USA). HBV DNA was measured by transcription-mediated amplification and hybridization protect assay (TMA-HPA) (Chugai Diagnostica, Tokyo, Japan). The lower and upper limits of detection of TMA-HPA are 5×10^3 and 5×10^8 viral genomic equivalents (GE)/ml, respectively. HBV genotype was determined using a previously reported method [19, 20]. Antibody against HCV was detected with a third-generation enzyme-linked immunoassay (Ortho Diagnostic Japan, Tokyo). YMDD motif mutant was detected using the sensitive PCR-restriction fragment length polymorphism [21].

Liver biopsy specimens were obtained percutaneously or at laparoscopy using a modified Vim Silverman needle of 2 mm internal diameter (Tohoku University style, Kakinuma Factory, Tokyo). Each specimen was scored according to the system of Desmet et al. [22].

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from each patient. The study was approved by the Human Ethics Committee of Toranomon Hospital.

Nucleotide Sequencing of HBV Basic Core Promoter (nt 1762/1764) and Precore (nt 1896)

Nucleotide sequences of HBV were compared with the prototype sequences of the HBV genotype C [19]. HBV DNA was extracted with a Smitest EX & R kit (Genome Science, Tokyo). Nucleic acids were amplified by nested PCR using the following primers. Nucleotide sequences of basic core promotor (BCP) nt 1762/1764 and precore (PC) nt 1896: The first-round PCR was performed with BCP-F7 [sense, 5'-TGC ACT TCG CTT CAC CTC TG-3' (nt 1580–1599)] and BCP-R8 [antisense, 5'-TAA GCG GGA GGA GTG CGA AT-3' (nt 2295–2276)] primers, and the second-round PCR with BCP-F5 [sense, 5'-GCA TGG AAA CCA CCG TGA AC-3' (nt 1606–1625)] and BCP-R6 [antisense, 5'-ATA CAG AGC AGA GGC GGT AT-3' (nt 2014–1995)] primers. All samples were initially denatured at 95°C for 4 min. Thirty-five cycles of amplification were set as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C with an additional 7 min for extension. Then 1 μl of the first-round PCR product was transferred to the second-round PCR reaction. Other conditions for the second-round PCR were the same as the first-round PCR, except that the second-round PCR primers were used instead of the first-round PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Perkin-Elmer, Chiba, Japan). To avoid false-positive results, the procedures recommended by Kwok and Higuchi [23] to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

Statistical Analysis

The χ^2 test, Fisher's exact probability test, and Mann-Whitney's U test were used to compare the background characteristics between groups. The cumulative virological and biochemical relapse rates were calculated using the Kaplan-Meier technique, differences between the curves were tested using the log-rank test. Statistical analyses of virological and biochemical relapse periods according to the mode of monotherapy (continuous and discontinuous groups) were calculated using the period from the start of lamivudine monotherapy, and those concerned with the characteristics of the discontinuous group were calculated using the period after discontinuation of the treatment. Stepwise Cox regression analysis was used to determine independent predictive factors at discontinuation of lamivudine monotherapy that contributed to early biochemical relapse after discontinuation of the treatment. We also calculated the odds ratios and 95% confidence intervals. Potential predictive factors associated with early biochemical relapse included the following ten variables at discontinuation of treatment: sex, age, histological stage, HBV genotype, levels of HBV DNA, HBeAg, pattern of BCP and PC, presence of YMDD motif mutant, and duration of lamivudine therapy. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance ($p < 0.05$) or marginal significance ($p < 0.10$) on univariate analysis were tested by multivariate Cox proportional hazard model to identify significant indepen-

dent factors. Statistical comparisons were performed using the SPSS software (SPSS, Chicago, Ill., USA). All p values <0.05 by the two-tailed test were considered significant.

Results

Virological and Biochemical Relapse

Virological relapse occurred in 24.0% (18 of 75 patients) of patients of the continuous group and 84.0% (21 of 25) of the discontinuous group. The cumulative virological relapse rates of the continuous and discontinuous group were 12.3 and 54.1% at the end of one year after the commencement of lamivudine monotherapy; 26.0 and 70.8% at 2 years; and 30.1 and 87.8% at 3 years, respectively. Virological relapse in the discontinuous group emerged significantly earlier than the continuous group ($p < 0.0001$; log-rank test) (fig. 1A).

Biochemical relapse occurred in 22.7% (17 of 75 patients) of patients of the continuous group and 68.0% (17 of 25 patients) of the discontinuous group. The cumulative biochemical relapse rates of the continuous and discontinuous group were 4.27 and 32.2% at the end of one year after commencement of lamivudine monotherapy; 26.5 and 61.9% at 2 years; and 33.9 and 66.7% at 3 years, respectively. Biochemical relapse in the discontinuous group emerged significantly earlier than in the continuous group ($p = 0.0011$; log-rank test) (fig. 1B).

YMDD mutants were not detected in any of the pretreatment serum samples. Emergence of YMDD motif mutant was noted in 29.3% (22 of 75 patients) of patients of the continuous group and 12.0% (3 of 25 patients) of the discontinuous group. In the continuous group, all of 18 virological relapsers showed DNA breakthrough associated with the emergence of YMDD motif mutant, and all of 17 biochemical relapsers showed breakthrough hepatitis associated with DNA breakthrough.

ALT and Bilirubin Levels after Biochemical Relapse or Emergence of YMDD Motif Mutant

The peak levels of serum ALT and bilirubin after biochemical relapse, and the ratios of peak levels to pretreatment were not significantly different between continuation or discontinuation groups (table 3). Likewise, the peak levels of serum ALT and bilirubin after the emergence of YMDD motif mutant, and the ratios of peak levels to pretreatment were also not significantly different between the two groups (table 3).

Factors Associated with Early Biochemical Relapse after Discontinuation of Lamivudine Monotherapy

The cumulative biochemical relapse rates of the discontinuous group were 48.0, 64.8, 69.2, and 69.2% at the end of 0.5, 1, 2, and 3 years after discontinuation of lamivudine monotherapy, respectively. Potential predictive factors associated with early biochemical relapse after discontinuation of treatment were explored in 25 patients of the discontinuation group. In univariate analyses, the following six factors tended to or significantly influenced the early biochemical relapse: HBeAg ($p = 0.0048$), levels of HBV DNA ($p = 0.039$), pattern of BCP ($p = 0.026$), pattern of PC ($p = 0.033$), age ($p = 0.083$), and liver cirrhosis ($p = 0.096$). In multivariate analysis using these factors, HBeAg ($p = 0.0035$), liver cirrhosis ($p = 0.0052$), and pattern of BCP ($p = 0.015$) were independent significant predictors of early biochemical relapse after discontinuation of the treatment (table 4). The odds ratio of liver cirrhosis was 16.1 compared with the absence of cirrhosis. The odds ratio of HBeAg-positive was 5.61 compared with HBeAg-negative. The odds ratio of detectable BCP mutant virus was 3.93 compared with undetectable BCP mutant virus.

Retreatment for Biochemical Relapse after Discontinuation of Lamivudine Monotherapy

Eight of 17 patients, who showed relapse after the termination of the treatment, received another course of lamivudine monotherapy at the same dose after a median stop (no treatment) period of 0.61 years (range, 0.15–1.8 years). The median period of retreatment was 1.1 years (range, 0.14–2.7 years). Six of these patients were HBeAg-positive, and the remaining 2 were HBeAg-negative at the commencement of retreatment. Five of 8 (62.5%) patients successfully showed normalization of ALT level and disappearance of HBV-DNA after retreatment; of whom 2 were HBeAg-negative (100%) and 3 were HBeAg-positive (50%). The other 3 patients, who did not show normalization of ALT, were HBeAg-positive, and especially 2 patients showed HBeAg reversion. Furthermore, in 2 of the latter 3 nonresponders, lamivudine therapy was terminated following the emergence of YMDD motif mutant, and both developed severe biochemical relapse (a rise in ALT level to ≥ 300 IU/l, accompanied by the elevation of total bilirubin level to ≥ 2.0 mg/dl) during retreatment. In particular, one of them developed severe relapse despite HBeAg seronegative conversion and was HBV DNA undetectable for one year (fig. 2). In summary, 5 of 6 patients (83.3%) without YMDD motif mutant at discontinuation could achieve ALT normalization again with retreatment, but 2 of 2 patients with YMDD motif mutant developed severe biochemical relapse during retreatment. Hence, retreatment with lamivudine monotherapy was effective, but tended to be not very effective

for HBeAg-positive patients retreated after the emergence of YMDD motif mutant.

Discussion

Previous studies reported that the estimated half-life of hepatocytes infected with HBV was 10–100 days, suggesting that prolonged administration of lamivudine for a period longer than one year might be needed to clear HBV in the liver by turning over most of cccDNA-containing hepatocytes [24, 25]. However, a recent report by Ryu et al. [26] showed that HBV DNA and HBeAg reappeared in 31 and 16% of their patients, respectively at 2 years after the termination of lamivudine, even when HBV DNA and HBeAg had been persistently negative for 2 years or more. Based on these findings, they suggested that long-term additional administration of lamivudine might enhance the durability of lamivudine-induced HBeAg seroconversion [26]. Our results of the discontinuous group also indicated higher cumulative biochemical relapse rates of 64.8 and 69.2% at 1 and 2 years after discontinuation, similar to the Korean report (relapse rates, 37.5 and 49.2% at 1 and 2 years) [27], although this might be due to the criteria used for the definition of the discontinuous group, regardless of HBeAg seroconversion and inclusion of subjects who were HBeAg-negative at the start of the treatment.

With regard to long-term treatment, while continued disease suppression, or even HBeAg seroconversion, still occurred in some patients, in others, breakthrough hepatitis associated with the appearance of YMDD mutant may occur. Severe breakthrough hepatitis has been reported despite the continuation of lamivudine [28–32], even though previous studies showed that YMDD mutants are less replication-competent compared with the wild-type, and are associated with lower HBV DNA levels compared with pretreatment HBV DNA levels [4, 5, 33–37]. We have recently reported that 3-year lamivudine therapy induced histopathological improvement regardless of the appearance of YMDD mutants, associated with DNA breakthrough and breakthrough hepatitis, and suggested the benefit of long-term treatment [38].

In our study based on patients matched for age, sex, and observation period, the cumulative virological and biochemical relapse rates were compared according to the continuation or not of lamivudine monotherapy. Our results showed that the relapse rates in the discontinuous group emerged significantly earlier than the continuous group. Furthermore, the peak levels of serum ALT and bilirubin and the ratios of peak to pretreatment levels were not significantly different between the continuation and discontinuation groups, regardless of the emergence of YMDD motif mutant. To our knowledge, this is the first report based on matched patients' backgrounds that

mpares the virological and biochemical relapse rates ccording to continuation or discontinuation of lamivudine monotherapy.

One limitation of our study is the small number of patients, the use of various treatment periods, and differences in the discontinuation criteria regardless of HBeAg seroconversion in the discontinuous group. Large-scale prospective studies of each group should be conducted in the future to confirm these findings.

Previous studies showed that HBeAg-positivity, old age, high pretreatment viral loads, and the presence of PC mutant at the start of the treatment might affect the biochemical relapse after treatment [39–41]. Our study based on multivariate analysis-evaluated various aspects of clinicopathological characteristics at the termination of treatment, and identified HBeAg-positivity, liver cirrhosis, and detectable BCP mutant virus as independent significant determinants of early biochemical relapse. Mutations in BCP, increase viral replication and enhance disease activity [42, 43], and are also associated with HBV genotype C and a longer duration of infection (including the higher age, and more advanced liver disease) [44]. These results suggest that the presence of BCP mutant and liver cirrhosis might indicate the more active state of disease, and might be the responsible factors of an early relapse. In our study, the majority of patients of the discontinuous group were Japanese patients infected with HBV genotype C and were positive for a family history of HBV infection (namely, genotype C patients with the longer duration of infection), and thus the presence of BCP mutant together with genotype C and a longer duration of infection might explain the higher viral replication and biochemical relapse after treatment in endemic areas of HBV genotype C infection, such as Japan and Korea, where most HBV infection is considered to be transmitted vertically [27]. To our knowledge, this is the first report of early post-treatment biochemical relapse based on characteristics at discontinuation of lamivudine monotherapy. Previous reports in the United States indicated that viral suppression was maintained after the termination of treatment [45]. The discrepancy between the USA reports and our results are probably due to the differences in HBV genotypes, duration of infection, and follow-up period after the termination of treatment. Further studies of a large group of patients are required to clarify whether the patients' characteristics including HBV genotype and duration of infection affect the early virological and biochemical relapse after the termination of lamivudine monotherapy.

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Reinstitution of lamivudine monotherapy is usually effective in controlling exacerbations in patients who have not experienced breakthrough and may result in subsequent HBeAg seroconversion [39], but the benefits of retreatment are usually transient in patients with breakthrough since YMDD mutant rapidly reappears (often within weeks) when lamivudine is resumed [46, 47] because of possible persistence of YMDD mutant over long periods after the cessation of therapy [48]. In the present study, 83.3% of patients without YMDD motif mutant at discontinuation achieved ALT normalization again with retreatment, but all (100%) patients with YMDD motif mutant developed severe biochemical relapse during retreatment. These results suggest that care should be exercised in the management of patients in whom lamivudine is first discontinued then used again, especially those who show the emergence of YMDD motif mutants.

In conclusion, the present study indicates that the discontinuation of lamivudine monotherapy for Japanese genotype C-dominant hepatitis B should be followed carefully for virological and biochemical relapses. Further prospective studies are necessary to determine the true risk of post-treatment relapse by discontinuation and breakthrough hepatitis by continuation of long-term treatment. However, it should be stated here that it would be difficult to perform such studies based on ethical grounds. Interferon therapy and new nucleotide analogs (for example, adefovir dipivoxil and entecavir) have been recently shown to be effective in patients with YMDD mutants induced by long-term lamivudine administration [49–52]. Thus, new combination therapies of antiviral drugs or alternative drugs are expected to appear in the future.

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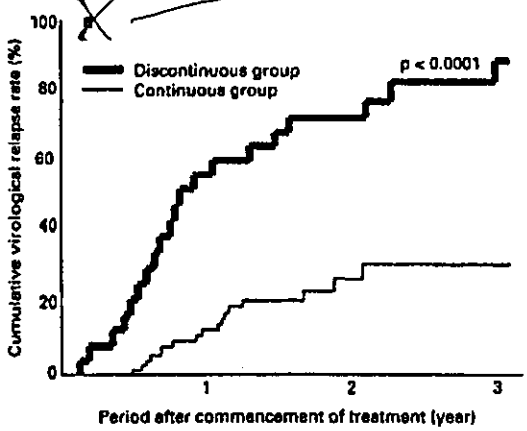

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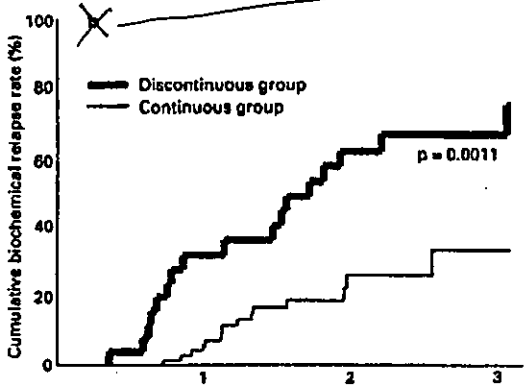

n 915 **Fig. 1.** Virological and biochemical relapse rates according to the
n 916 continuation or discontinuation of lamivudine monotherapy, in
n 917 patients matched for age, sex, and observation period after the start
-918 of treatment. **A** Cumulative virological relapse rates after the com-
n 919 mencement of treatment. **B** Cumulative biochemical relapse rates
n 920 after the commencement of treatment. Virological and biochemical
n 921 relapse in the discontinuous group emerged significantly earlier than
922 in the continuous group.