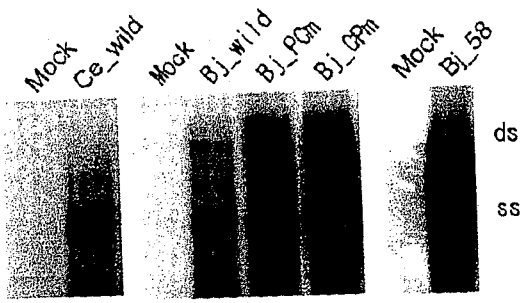

Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

densities of migration patterns of the wild-type, precore, and core-promoter mutants in Southern blotting analysis. The wild-type HBV/Bj displayed a band for single-stranded (ss) HBV DNA and an additional band for double-stranded (ds) HBV DNA. Of note, the densities of these bands were far greater for HBV/Bj mutants incorporated with precore or core-promoter mutation, as well as Bj_58 with the precore mutation, thereby indicating much enhanced replicative activity of precore or core-promoter mutant *in vitro*. Although the intracellular HBV DNA level for the wild-type HBV/Bj was comparable with that for the wild-type Ce (Fig. 3), the extracellular HBV DNA level in culture media was approximately threefold higher for Bj than Ce ($P < .01$) (Sugiyama M et al., manuscript in submission).

Discussion

A nationwide survey of genotypes/subgenotypes in patients with acute HBV infection from Japan during the past 2 decades has examined their influence on fulminant and chronic outcomes. The study was feasible in a country where mass vaccination has not been performed because of an extremely high efficacy of immunoprophylaxis on babies born to carrier mothers; it has decreased the persistent HBV carrier rate from 1.4% to 0.3%.[26] Acute HBV infection keeps increasing, however, predominantly through promiscuous sexual contacts in Japan.

Fulminant hepatitis developed rather frequently in 40 of the 301 (13%) patients. This is likely due to selection bias because the study included only patients who were hospitalized for acute hepatitis B. Exclusion of subclinical cases of acute HBV infection would have overestimated the incidence of fulminant hepatitis. Regardless of such a selection bias, influence of HBV genotypes/subgenotypes was evident in comparison with the 40 patients with fulminant and the 261 with acute self-limited hepatitis. Remarkably, none of the 33 patients infected with HBV/Ae developed fulminant hepatitis. In sharp contrast, 12 of the 22 (55%) patients infected with HBV/Bj developed it. Furthermore, both precore (G1896A) and core-promoter (A1762T/G1764A) mutations were detected significantly more frequently in patients with fulminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of core-promoter mutation was much higher in the patients with fulminant (67%) than that reported in those with chronic hepatitis (16%).[27] Precore and core-promoter mutations are very frequent in patients with fulminant hepatitis from Asia[28-30] and the Middle East.[31] The failure in detecting these mutations in Western countries[32-35] could be attributed to frequent HBV/Ae and rare Bj there. In multivariate analysis, HBeAg-negative, HBV/Bj, and the precore stop-codon mutation for G1896A were independent risk factors for the development of fulminant hepatitis (Table 4). Various mutations at nt 1753 for enhanced HBV replication,[36] as well as those adjacent at nt 1754 prevailing in patients with fulminant hepatitis,[37] occurred more frequently in patients with fulminant than acute self-limited hepatitis. Host factors, such as age and total bilirubin, contributed to the development of fulminant hepatitis as well (Table 4).

In vitro replication analysis demonstrated the intracellular HBV DNA level of the wild-type HBV/Bj comparable with that of the wild-type Ce (Fig. 3). The extracellular HBV DNA level of HBV/Bj-clone, however, was much higher than those of the other genotypes, indicating its strong inclination to be secreted from cells (Sugiyama et al., manuscript in submission). Such a high concentration of HBV/Bj in the circulation of patients would rapidly and extensively promote infection of hepatocytes.

Enhanced replication capacities of precore (G1896A) and core-promoter (A1762T/G1764A) mutants for HBeAg-minus and -reduced phenotypes, respectively, were demonstrated in a replication model *in vitro* (Fig. 3). These observations were concordant with those in previous reports[38,39]; however no data are available on the replication of HBV/Bj *in vitro*, either of the wild-type or variants with these mutations. Extremely high intracellular and extracellular expressions of viral DNA were observed for the HBV/Bj clone with precore stop-codon mutation from a patient with fulminant hepatitis. These results might implicate high replication due to mutations of precore region and core-promoter in the induction of fulminant hepatitis. In support of this view, Bocharov et al.[40] have proposed that enhanced HBV replication would efficiently stimulate immune reactions, represented by the cytotoxic T lymphocyte response, suggesting that enhanced replication by HBV/Bj or precore/

core-promoter mutation might lead to fulminant hepatitis.

That HBV DNA levels were lower in patients with fulminant than acute hepatitis, despite a high replication capacity of HBV/Bj incriminated in the development of fulminant hepatic failure, may seem surprising. Because destruction of hepatocytes proceeds swiftly in patients with fulminant hepatitis, hepatic mass for HBV to thrive would have been extremely reduced in them at presentation. As a consequence, some patients with fulminant hepatitis B are without serum HBsAg; they are diagnosed by high-titered IgM anti-HBc.[41] On the contrary, HBV DNA levels were higher in the patients with HBV/Ae than Bj (Table 1); those with Ae tend to delay reducing HBV DNA, some of whom have chronic outcome. Combined, correlating HBV DNA levels with the clinical outcome in acute HBV infection would be difficult.

A wide variation has been seen in the rate of persistence after acute HBV infection in adulthood. No chronic outcomes of acute hepatitis B were seen in female recipients of red blood cells contaminated with HBV (0/28)[42] or patients in an acupuncture-associated outbreak (0/35).[43] In marked contrast, they ranged from 0.2% (14/715) in Greece[44] through 2.7% (1/37) in university students in Taiwan[45] to 10.4% (5/8) in Alaskan Eskimos[46] and 12.1% (7/58) in Germany.[47] HBV genotypes are implicated in a high rate of persistence in European countries where HBV/A is predominant.[48] In Japan, also, adulthood infection tends to persist longer with HBV/A than B or C (23% 3/13 vs. 13% 1/8 or 12% 3/25).[49] In the current series on 256 patients with acute hepatitis B in Japan who were followed rigorously, HBV infection persisted in only three (1%), representing 2 of the 32 (6%) with HBV/Ae and 1 of the 21 (5%) with Ba. Hence, 99% of patients lost their HBsAg by 6 months. Persistence of HBV observed in the patients with HBV/Ae (6%) is less frequent than that in 4 of the 31 (13%) patients with Ae from a hospital in metropolitan Tokyo.[49] The difference would be ascribable, at least in part, to lamivudine given to some patients in this study (18%). All patients treated with lamivudine recovered from acute hepatitis, whereas none of the three patients with chronic outcome had received antiviral treatment during their acute phase of illness, indicating that lamivudine might be able to prevent the chronic outcome. Likewise, some patients from metropolitan Tokyo, in whom HBV persisted,[49,50] had received immunosuppressants in the acute phase of infection before referral to their hospital.

Using cell culture and chimeric mice models for the replication system of different genotype/subgenotype clones, we have observed that the replication of HBV is the highest for HBV/Bj or C and the lowest for Aa/Ae (Sugiyama M et al., manuscript in submission). It is probable that the propensity of HBV/A infection to chronicity would be due to less intensive immune response against its slow viral dynamics. Taken together, the infection with HBV/A appears to persist longer than those with the other genotypes; this needs to be confirmed by further investigation in patients from various countries.

In conclusion, persistence of HBV after acute infection is rare and occurs more often in patients infected with HBV/Ae than others. Fulminant outcome is frequent in hospitalized patients and associated with HBV/Bj accompanied by the lack of serum HBeAg as well as high replication due to precore stop-codon mutation (G1896A), a finding supported by an *in vitro* replication model.

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Pharmacokinetics and enhanced PKR response in patients with chronic hepatitis C treated with pegylated interferon alpha-2b and ribavirin

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SUMMARY. This study investigated the molecular and pharmacokinetic mechanisms of the enhanced antiviral efficacy associated with pegylated interferon (PEG-IFN) alpha-2b and ribavirin. The study involved comparing the expression of serial double-stranded RNA-activated protein kinase (PKR) before and during treatment in 26 PEG-IFN alpha-2b and 26 conventional IFN alpha-2b recipients matched for age, body weight and dose of ribavirin. The pharmacokinetics of PEG-IFN alpha-2b and ribavirin was analysed in 15 of the 26 PEG-IFN recipients. There was a rapid increase in PKR expression in both treatment groups, although expression from day 2 onwards was maintained at a significantly higher level in the PEG-IFN recipients ($P < 0.05$). C_{max} of PEG-IFN occurred 12–48 h after the initial administration, with $t_{1/2}$ and C_{min} being 49 h and 190 pg/mL, respectively. In contrast to ribavirin, accumulation of PEG-IFN was minimal. There was no association between serum PEG-IFN and ribavirin levels and virological response. Although baseline expression of PKR before treatment was marginally higher in nonresponders (NRs), from day 2 onwards, sequential PKR expression in response to PEG-IFN was higher in sustained viral responders compared with the NRs ($P < 0.05$). Significant correlations were found between kinetics of PKR expression and viral decline rates in each phase of hepatitis C virus dynamics (first phase, $r = 0.67$, $P = 0.0006$; second phase, $r = 0.67$, $P = 0.001$). In conclusion, improvement in pharmacokinetics following pegylation led to higher intracellular PKR expression, which was associated with enhanced virological efficacy of PEG-IFN-based combination therapy. The concentrations of both ribavirin and PEG-IFN alpha-2b were not associated with viral response and PKR expression.

Keywords: hepatitis C virus, hepatitis C virus dynamics, interferon-stimulated gene, treatment.

INTRODUCTION

Combination therapy with pegylated interferon (PEG-IFN) alpha and ribavirin results in a higher sustained virological response (SVR) rate than conventional IFN alpha and ribavirin therapy [1,2] and is now established as the standard treatment for chronic hepatitis C virus (HCV) infection. However, the mechanism responsible for this improved response rate remains to be elucidated.

Interferon induces transcription of IFN-stimulated genes (ISG), including double-stranded RNA-activated protein kinase (PKR) [3]. PKR has many cellular roles, including inhibition of translational responses to viral infection, growth control, differentiation activity and proapoptotic functionality [4,5]. However, the clinical significance of PKR expression during PEG-IFN therapy is not fully understood. Moreover, the pharmacokinetic effects of PEG-IFN on PKR expression and the relationship between the expression of PKR and viral response remain unknown.

In addition, although the serum concentration of ribavirin has been reported to affect the outcome of conventional IFN alpha and ribavirin combination therapy [6], the relationship between serum ribavirin, PEG-IFN concentrations and viral response has not been studied.

Abbreviations: PEG-IFN, pegylated interferon; SVR, sustained virological response; HCV, hepatitis C virus; ISG, IFN-stimulated genes; PKR, double-stranded RNA-activated protein kinase; ALT, alanine aminotransferase; NR, nonresponder; PBMC, peripheral blood mononuclear cells; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction..

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In the present study, we sought to elucidate the underlying mechanism of the enhanced antiviral efficacy seen with PEG-IFN alpha-2b and ribavirin combination therapy by analysing PKR gene expression and pharmacokinetics of PEG-IFN and ribavirin in patients with chronic HCV genotype 1b infections. The relationships between the viral response and PKR expression and pharmacokinetics of PEG-IFN and ribavirin were also studied.

MATERIALS AND METHODS

Patients

Fifty-two patients infected with chronic hepatitis C of genotype 1b and high viral load, admitted between November 2001 and June 2002, were included in the study. Twenty-six patients were treated with PEG-IFN alpha-2b and ribavirin combination therapy, with the remaining 26 patients matched for age, body weight and dose of ribavirin being treated with conventional IFN alpha-2b and ribavirin. The inclusion criteria for the study were as follows: Persistent elevation of serum alanine aminotransferase (ALT) levels above the upper limit of the normal for ≥6 months prior to therapy; the presence of HCV genotype 1b in the serum; the presence of serum HCV-RNA of >100 000 IU/mL detected by the Amplicor-HCV monitor assay (Roche Molecular Diagnostic Co., Tokyo, Japan); no evidence of hepatocellular carcinoma in an ultrasound examination; a haemoglobin level ≥14 g/dL, neutrophil count ≥1500/mm^3, platelet count ≥100 × 10^3/mm^3, creatinine clearance ≥51 mL/min and fasting blood sugar <110 mg/dL. Exclusion criteria included the presence of hepatitis B surface antigen or human immunodeficiency viral antibodies and a history of excess alcohol consumption. Eleven of the 26 PEG-IFN alpha-2b recipients and all 26 conventional IFN alpha-2b recipients had been enrolled previously in a viral dynamics study [7].

Written informed consent was obtained from all the patients and the study protocol was approved by the institutional ethical committee in accordance with the revised version of the Helsinki Declaration of 1983.

Treatment

Twenty-six patients were treated for 48 weeks with subcutaneous injections of PEG-IFN alpha-2b (PegIntron®; Schering-Plough Corporation, Kenilworth, NJ, USA) at a dose of 1.5 μg/kg/week. Ribavirin (Rebetol®, Schering-Plough Corporation) was administered concomitantly over the 48-week period, provided orally twice daily at a total daily dose of 800 mg. At the start of the study, 400 mg of ribavirin was administered, with serum concentrations being measured after 48 h. As the body weight of the patients in the study ranged between 60 and 80 kg, the dose of ribavirin for the remainder of the study period was fixed at 800 mg/day. The dose of PEG-IFN alpha-2b was reduced to 0.75 μg/kg/week when either the neutrophil count was <750/mm^3 or the platelet count was <80 × 10^3/mm^3. The dose of ribavirin was reduced to 600 mg/day when the haemoglobin concentration decreased to <10 g/dL.

The remaining 26 patients were treated for 48 weeks with intramuscular IFN alpha-2b (Intron-A®; Schering-Plough Corporation) in combination with daily oral ribavirin at a dose of 800 mg. For the first 2 weeks of therapy, 6 MU of IFN alpha-2b was administered daily, followed for the next 46 weeks by 6 MU given three times a week.

Measurement of PKR mRNA before and during therapy

Serial measurements of PKR expression before and during treatment were determined in both treatment groups. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood samples collected before, and at 4, 8, 24 h and 2, 4, 7, 14, 21, 28, 56, 84, 112, 140, 168 and 336 days after the initiation of either PEG- or conventional IFN alpha-2b and ribavirin combination therapy. After extraction of total RNA from the PBMCs, the expression of PKR mRNA was quantified at each specified time point using real-time quantitative polymerase chain reaction (PCR) as described previously [8]. The assays were performed in triplicate, and as an internal control, the expression levels of PKR transcript were normalized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression quantified by real-time quantitative PCR. The level of PKR gene expression at each time point during IFN treatment was calculated relative to baseline expression levels measured prior to IFN treatment.

Pharmacokinetics of pegylated interferon alpha-2b and ribavirin

The pharmacokinetics of PEG-IFN and ribavirin was analysed in 15 PEG-IFN alpha-2b recipients who consented to be enrolled in the additional pharmacokinetic study. Of these 15 patients, two were naïve , nine had relapsed and four had not responded to previous conventional IFN monotherapy. Blood samples were collected immediately before, and at 2, 4, 6, 8, 10, 12, 14, 16, 24, 36, 48, 72, 96, 120, 144 and 168 h after the first dose of PEG-IFN alpha-2b and ribavirin. Blood samples were also collected immediately before each administration at weeks 5, 9, 13, 25 and 37 and the trough values measured. At week 48 (final dose), blood was drawn immediately before, and at 2, 4, 6, 8, 10, 12, 14, 16, 36, 48, 72, 96, 120, 144, 168, 366, 504 and 672 h after administration. The sera were harvested immediately after blood collection and stored frozen at −20°C.

Serum PEG-IFN alpha-2b levels were determined using an electrochemiluminescent immunoassay (IGEN International, Inc., Gaithersburg, MD, USA), with the lower limit of detection for this assay being 27 pg/mL. Serum ribavirin levels were measured by high-performance liquid chromatography

in conjunction with tandem mass spectroscopy (MDS Pharma Services Inc., Montreal, QC, Canada) according to a method reported previously [9]. The maximum serum concentration (C_{max}), time to maximum serum concentration (t_{max}) and C_{168h} (trough value of ribavirin) were then determined. Confirmation of the steady state using circadian changes of the trough value, estimation of the time to reach the steady state, the cumulative coefficient (Rods) based on the area under the curve (AUC), the clearance half-life in the terminal excretion phase ($t_{1/2\lambda_z}$) and comparison of AUC_{0-168h} (PEG-IFN alpha-2b) or AUC_{0-12h} for the first and final administrations were also determined. One patient whose IFN concentration exceeded the upper limit of the therapeutic range was excluded from this analysis.

Final virological response and hepatitis C virus dynamics in serum

Patients who were HCV-RNA negative at week 24 following completion of treatment were defined as having achieved an SVR. Patients who did not achieve an SVR were classified as nonresponders (NRs).

To analyse the effect of treatment on HCV dynamics, the amount of HCV-RNA was quantified at the following time points: immediately before initiation of the therapy and 4, 8, 24 h and 2, 4, 7, 14, 21, 28, 56, 84, 112, 140, 168 and 336 days after initiating therapy. The total RNA was extracted from the serum, and the amount of HCV-RNA at each time point was quantified by real-time detection PCR as reported previously [7,10]. The detection sensitivity of this assay was approximately 10 copies/mL, and the dynamic range for the method was from 10 to 1×10^8 copies/mL [11]. The viral decline curve was plotted on a semilogarithmic graph, and the slope of the exponential viral decline was calculated individually by a straight-line fit to the data for each viral decline phase.

Statistical analysis

Categorical data were compared by the chi-square test or Fisher's exact test. Distributions of continuous variables in the two treatment groups were analysed by Student's *t*-test. All tests of the confidence interval were two tailed, with the level of confidence level being set at 95%. *P*-values of <0.05 were considered statistically significant.

In order to analyse the pharmacokinetics of PEG-IFN alpha-2b and ribavirin, descriptive statistics were calculated at each blood collection, and the relationship between the time point of blood collection and the measured levels of the two drugs displayed graphically for each subject. These graphs included the mean value, standard error and the measured concentrations of the drugs at the first and after the final administration. In addition, these analyses were used to confirm the circadian trough values and to estimate the time to reach the steady state, based on AUC (Rods) and clearance half-life ($t_{1/2\lambda_z}$).

RESULTS

The demographics of the patients are shown in Table 1. No significant differences were found in mean age, gender proportionality, activity and stage of liver histology, serum ALT level and initial viral load between the PEG-IFN alpha-2b and non-PEG-IFN alpha-2b treatment groups. SVR rates in the PEG-IFN alpha-2b and non-PEG-IFN alpha-2b treatment groups were 69% (18/26) and 31% (8/26), respectively.

Differences in PKR mRNA expression in response to the different interferon treatment regimens

Sequential transcript analysis demonstrated an approximately 15-fold increase in PKR mRNA expression within 4 h following administration of conventional IFN alpha-2b. At

Table 1 Clinical characteristics of the patients in the two treatment groups of the study

	PEG-IFN alpha-2b plus ribavirin	IFN alpha-2b plus ribavirin	*P*-value (95% CI)
No. of patients	26	26	
Age (years), median (range)	53 (29–67)	53 (29–70)	0.66 (−4.18–6.57)*
Gender (male/female)	14/12	13/13	0.78†
Histology of the liver			
A1/A2/A3	12/11/3	14/11/1	0.56†
F1/F2/F3	14/10/2	13/7/6	0.28†
ALT (IU/L)	93 (72–113)	84 (63–105)	0.54 (−38.2–20.2)*
Haemoglobin (g/dL)	14.6 (14.2–15.0)	14.2 (13.6–14.9)	0.26 (−1.11–0.31)*
Platelet count ($\times 10^3$/mL)	179 (164–195)	171 (151–190)	0.47 (−3.32–1.56)*
Viral load ($\times 10^6$ copies/mL)	14.6 (9.00–20.2)	8.35 (3.77–12.9)	0.11 (−14.1–1.55)*
Ribavirin concentration at 4 W (ng/mL)	2413 (1451–3376)	2266 (1568–2963)	0.79 (−1281–985)*

Values are expressed as mean (95% CI).
*Unpaired *t*-test. †Chi-square test.

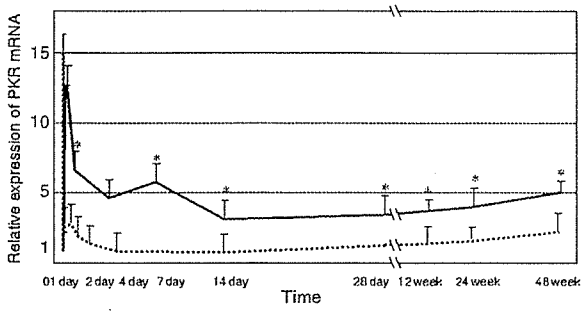

Fig. 1 Sequential expression of PKR mRNA in PBMCs during PEG- (solid line) and conventional (dotted line) IFN alpha-2b and ribavirin combination therapy. Expression of mRNA is shown as the expression level relative to baseline expression. The error bars indicate the standard error. An asterisk indicates a statistically significant difference in relative expression values between the two different IFN regimens ($P < 0.05$).

8 h, the level of PKR mRNA had fallen to a level that was twofold greater than the pre-treatment level (Fig. 1). With PEG-IFN alpha-2b administration, PKR mRNA expression reached a peak at 8 h at a level 12-fold greater than the pre-treatment level. At 24 h post-administration, the level of PKR mRNA had fallen but was still sixfold greater than the pre-treatment level (Fig. 1). This level was maintained until the next dose. No significant difference was observed in peak PKR mRNA expression between conventional IFN alpha-2b and PEG-IFN alpha-2b. However, from the second day of administration onwards, the expression was maintained at a significantly higher level in the PEG-IFN alpha-2b group compared with the conventional IFN alpha-2b group ($P < 0.05$) (Fig. 1).

Pharmacokinetics of serum pegylated interferon alpha-2b

The pharmacokinetic parameters for PEG-IFN alpha-2b at weeks 1 (first administration) and 48 (final administration) are shown in Table 2. Although the trough value of serum PEG-IFN alpha-2b varied between individuals, it almost reached a plateau at week 8. Accumulation of IFN was minimal in the PEG-IFN alpha-2b treatment regimen.

The level of serum PEG-IFN alpha-2b at week 1 increased gradually up to 12–24 h with a $t_{1/2\lambda_z}$ of 40.2 h. These levels were measurable up to 168 h after administration or immediately before the next administration. The trough value following administration showed no significant increase during the 48-week treatment phase (Fig. 2). The blood level after the final administration increased gradually for 12–24 h, remained high for approximately 48 h, and then decreased slowly with a $t_{1/2\lambda_z}$ of 55.3 h. The drug remained measurable up to 2 weeks post-administration. The cumulative coefficients (Rods) of repeated administrations calculated on the basis of C_{max}, C_{168h} and AUC_{0-168h} were 0.917, 2.11 and 1.12, respectively. When a comparison was made between the first and final administrations (weeks 1 and 48), $t_{1/2}$ of serum PEG-IFN alpha-2b levels was slightly prolonged after the final administration, although no changes were observed in C_{max}, AUC and plasma clearance (CL/F) (Table 2; Fig. 3).

Pharmacokinetics of serum ribavirin

The pharmacokinetic parameters for ribavirin at weeks 1 (first administration) and 48 (final administration) are summarized in Table 3. The trough value of serum ribavirin almost reached a plateau 8 weeks after the initial administration. In contrast to PEG-IFN alpha-2b, ribavirin was accumulated significantly during the first 4–8 weeks.

Serum ribavirin levels after the first administration (first day) reached t_{max} by 3.33 h and then decreased rapidly with a $t_{1/2\lambda_z}$ of 27.1 h. In contrast, serum ribavirin levels reached t_{max} by 2.73 h after the final administration and then decreased slowly with a $t_{1/2\lambda_z}$ of 296 h. A comparison of the cumulative coefficient (Rods) in the steady state was made between the first and final administrations and was calculated on the basis of C_{max}, C_{12h} and AUC_{0-12h}. This showed that by the final administration, there was a marked increase in C_{max} and AUC in serum ribavirin levels, an approximately 10-fold prolongation of $t_{1/2\lambda_z}$, a decrease in CL/F of about 1/3, and an approximately threefold increase in Vz/F. There was no change evident in t_{max} (Table 3; Fig. 4).

Clinical and virological response and serum pegylated interferon alpha-2b and ribavirin levels

The dose of PEG-IFN alpha-2b was reduced in two patients after 4 and 25 weeks of treatment because of neutropoenia. Similarly, the dose of ribavirin was reduced in three patients

Table 2 Pharmacokinetic parameters of the patients who received PEG-IFN alpha-2b at weeks 1 (first administration) and 48 (final administration)

	t_{max} (h)	C_{max} (pg/mL)	C_{168h} (pg/mL)	$t_{1/2\lambda_z}$ (h)	AUC (pg h/mL) 0–168 h	CL/F (mL/h/kg)	Vz/F (L/kg)
First	23.1	874	99	40.2	68 926	21.4	1.18
Final	22.2	774	185	55.3	77 039	–	–
Rods	–	0.917	2.11	–	1.12	–	–

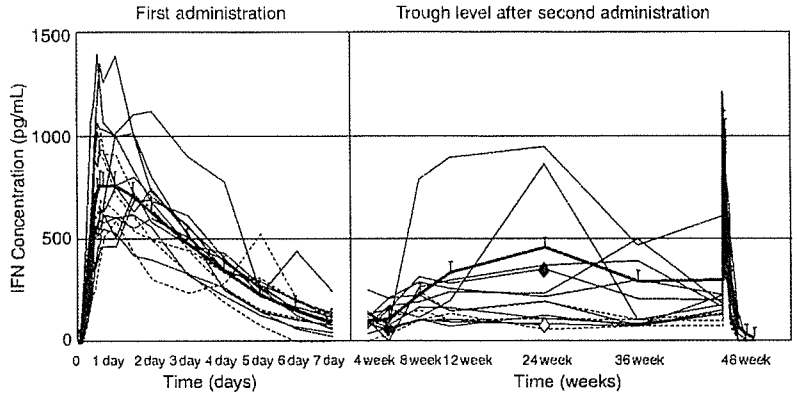

Fig. 2 Changes in serum IFN levels during PEG-IFN alpha-2b and ribavirin combination therapy. No significant increase in the trough value of serum IFN level was found during the 48-week treatment period. The bold lines indicate mean values, while the error bars indicate the standard error. Fine solid lines indicate a sustained virological responder and broken lines a nonresponder. The diamond-shaped symbol indicates a time point and IFN concentration at which either dose reduction (closed diamonds) or discontinuation (open diamonds) occurred.

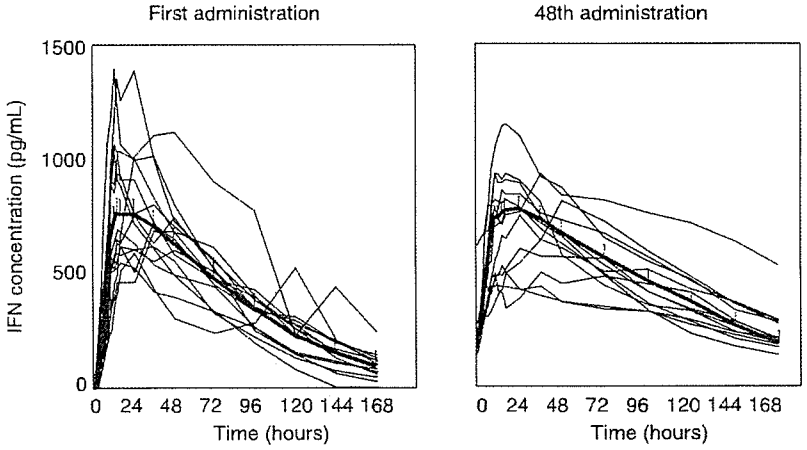

Fig. 3 A comparison of serum IFN levels between the first and 48th doses. Both show very similar values and no accumulation of IFN. It should be noted that PEG-IFN alpha-2b was detectable in all but one patient at 168 h after initial administration. Bold lines indicate mean values and the error bars indicate the standard error.

Table 3 Pharmacokinetic parameters of the patients who received ribavirin at weeks 1 (first administration) and 48 (final administration)

	t_{max} (h)	C_{max} (pg/mL)	C_{168h} (pg/mL)	$t_{1/2\lambda_z}$ (h)	AUC(pg h/mL) 0–168 h	CL/F (mL/h/kg)	Vz/F (L/kg)
First	3.33	604	221	27.1	4019	37.8	1472
Final	2.73	3449	2422	296	33 060	12.7	5374
Rods	–	6.53	12.2	–	9.42	–	–

after 12 and 16 weeks of treatment because of anaemia. In Figs 2 & 4, the individual time points and drug concentration following dose reduction are indicated by closed diamonds. No association could be found between dose reduction and serum concentration for both agents. Treatment was discontinued in 1 of the 15 patients because of depression, as indicated by open diamonds in Figs 2 & 4. Eleven patients including this patient achieved an SVR, with the remaining four patients being classified as NRs.

In order to demonstrate the association between virological response and pharmacokinetics, the final virological response for each individual is indicated in Figs 2 & 4. Serum IFN levels at 2 weeks post-dose tended to be slightly higher in NRs when compared with patients who achieved an SVR. This difference was not statistically significant. There was also no significant difference in serum ribavirin levels between these two groups from the time of the first administration until the completion of the 48-week treatment period.

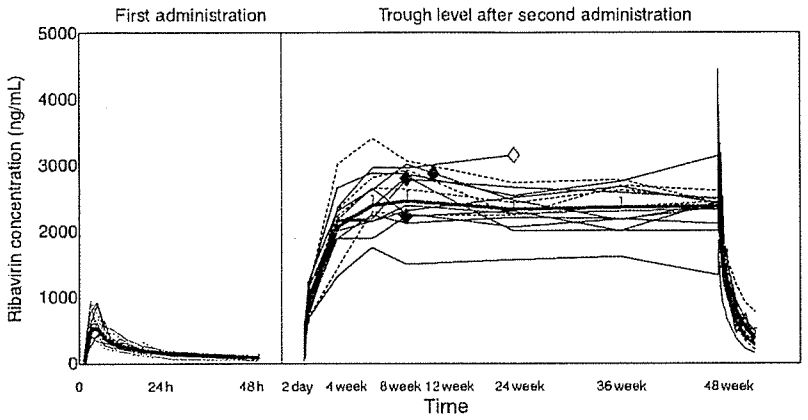

Fig. 4 Changes in serum ribavirin levels during PEG-IFN alpha-2b and ribavirin combination therapy. Serum ribavirin levels reached a peak by the eighth week and then plateaued. Bold lines indicate mean values and the error bars indicate the standard error. Fine solid lines indicate a sustained virological responder and broken lines a nonresponder. The diamond-shaped symbols indicate a time point and ribavirin concentration at which either dose reduction (closed diamonds) or discontinuation (open diamonds) occurred.

Association between PKR mRNA expression and virological response

The absolute expression levels of PKR mRNA at baseline prior to treatment were slightly higher in NRs than in SVR patients (1.8×10^{-2} *vs* 1.3×10^{-2} copies/one copy of G3PDH), although this difference was not statistically significant. Interestingly, in the PEG-IFN alpha-2b group, sequential PKR mRNA expression in response to PEG-IFN administration was significantly higher in patients who achieved an SVR compared with patients classified as NRs ($P < 0.05$) (Fig. 5).

The serum HCV dynamics during PEG-IFN alpha-2b and ribavirin combination therapy showed a biphasic pattern consisting of a rapid decrease within 24 h of initiation of the treatment (first phase), followed by a subsequent slow decrease. The mean viral decay during the first phase was 3.0 $\log_{10}$/day (95% CI: 2.4–3.5) and that calculated from day 2 onwards (the second phase of the response) was 0.075 (95% CI: 0.028–0.12) $\log_{10}$/day. Significant correlation was found between PKR expression at day 1 and viral decline rate calculated from the first phase of HCV dynamics ($r = 0.67$, $P = 0.0006$) (Fig. 6a). Moreover, significant correlation was also found between PKR expression at day 84 and second phase viral decline rate ($r = 0.67$, $P = 0.001$) (Fig. 6b). No significant associations were found between PEG-IFN or ribavirin concentration and kinetics of PKR expression.

DISCUSSION

The data of this study suggests that the higher expression levels of PKR transcripts seen with PEG-IFN alpha-2b from the second day of administration onwards were related, at least in part, to the improved efficacy of PEG-IFN alpha-2b compared with conventional IFN alpha-2b. Our pharmacokinetic study suggests that pegylation may be responsible for the dramatic effect on induction of PKR associated with the PEG-IFN regimen, possibly as a consequence of maintaining blood levels of IFN within the therapeutic range. This concept is supported by our previous work [8], in which we demonstrated that intracellular expression of PKR during the second phase was maintained at a significantly higher level when IFN-beta was administered twice daily.

The expression of PKR transcripts was induced very rapidly following the first administration, and PKR expression at day 1 was significantly correlated with the first phase viral decline rate of HCV dynamics. It is likely this increase in PKR transcripts was associated with the rapid decline of HCV seen in the first phase of serum HCV dynamics, and this change is believed to be a result of the direct effect of IFN on virion production and release from infected target cells [12]. Although we found that there was no significant difference in peak PKR mRNA expression between the PEG-IFN alpha-2b and IFN alpha-2b groups, the expression of PKR transcripts from 24 h onwards was significantly higher with PEG-IFN alpha-2b than conventional IFN alpha-2b administration. The decline in viral numbers and activity seen after the second day (second phase viral decline of HCV dynamics) is believed to reflect the presumed elimination of viral-infected cells in addition to the direct antiviral properties of IFN [12]. It has been suggested recently that apoptosis of HCV-infected cells induced by IFN-stimulated PKR may be an important mechanism for the elimination of viruses [13]. In the present study, expression of PKR transcripts in response to PEG-IFN administration was higher in patients who achieved SVR compared with NR patients, and expression of PKR at day 84 was significantly associated with the viral decline rate calculated from the second phase of HCV dynamics. Therefore, the increased expression of PKR transcripts we observed

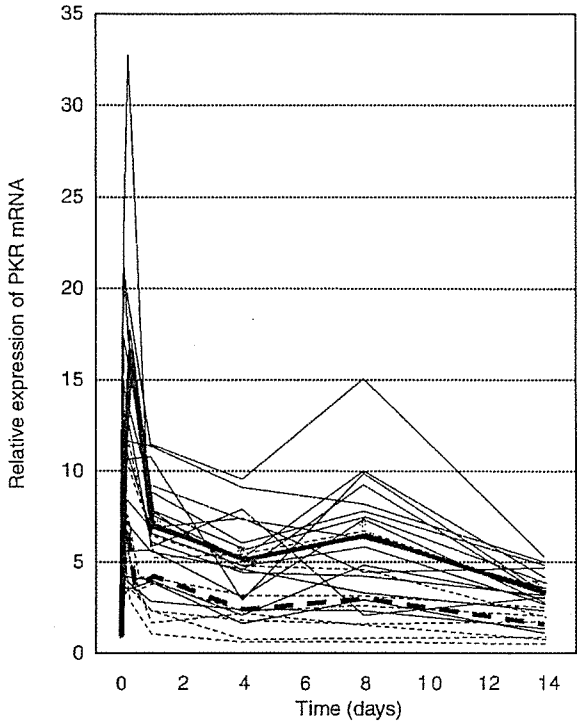

Fig. 5 Sequential expression of PKR mRNA in PBMCs in sustained viral responders (solid line, $n = 18$) and nonresponders (dotted line, $n = 8$). The bold line indicates the mean value for each group. Expression of mRNA is shown as the expression level relative to baseline expression. An asterisk indicates a statistically significant difference in relative expression value between the two different virological responses ($P < 0.05$).

after the second day may be associated with the enhanced efficacy of PEG-IFN alpha-2b. Again, this increased expression may have been due to an improvement in the pharmacokinetics of IFN following pegylation that results in prolonged clearance of IFN from serum.

Gerotto et al. [14] reported previously that higher baseline PKR expression was observed in NR patients compared with patients who achieved an SVR, although no significant difference was found in 'absolute' expression of PKR during treatment between these patients. We observed a similar trend in baseline expression in our study, although the relatively small number of cases meant that this difference did not achieve statistical significance. However, in our study, increased expression of PKR in response to PEG-IFN treatment was found in patients with an SVR. We analysed the changes in PKR expression during treatment relative to baseline expression levels. Because the absolute expression of PKR in response to IFN varies between patients (data not shown), we believe that calculating the level of expression during IFN treatment relative to the level of baseline expression is suitable in comparing PKR responses between patients. While this issue still remains controversial, our results imply that no or low responsiveness of PKR (i.e. less than a twofold increase from baseline) is associated negatively with an SVR, although high responsiveness of PKR during PEG-IFN administration does not always assure an SVR.

Although PBMCs were used as a model to quantify the serial gene expression of PKR, expression of PKR should be studied with hepatocytes, the target cell of HCV. Using liver tissue for sequential analysis is more ideal but ethically impossible. To address this point, we previously demonstrated a significant correlation between basal expression of PKR in liver tissue and the corresponding PBMC [8].

One of the limitation of the present study is that our results specifically concern PKR. Therefore, our present findings cannot be extrapolated to other ISGs such as MxA and 2′,5′-oligoadenylate synthetase. Although expression and response of ISGs to therapy may differ among different ISGs, we previously found significant correlation between sequential expression levels for PKR and MxA during IFN treatment [8].

In the present study, PEG-IFN alpha-2b was detectable in all but one patient at 168 h after initial administration in

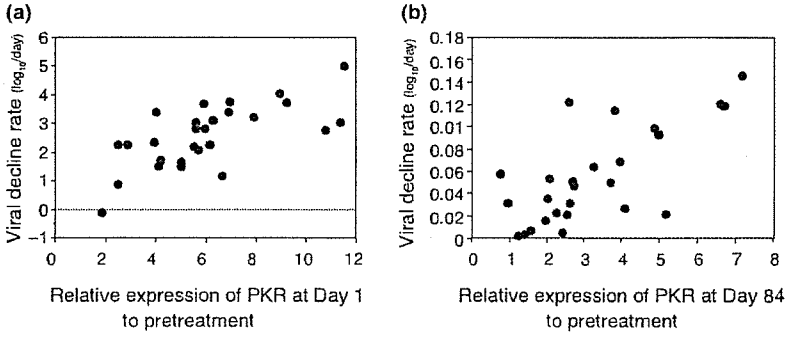

Fig. 6 (a) Significant correlation between expression of PKR mRNA at day 1 and viral decline rate calculated from the first phase of HCV dynamics ($r = 0.67$, $P = 0.0006$). (b) Significant correlation between expression of PKR mRNA at day 84 and viral decline rate calculated from the second phase of HCV dynamics ($r = 0.67$, $P = 0.001$).

contrast to a study reported by Bruno et al. [15]. However, as in that study, no significant accumulation of PEG-IFN alpha-2b was found during therapy, which is marked contrast to the data from PEG-IFN alpha-2a (40 kD) plus ribavirin therapy [15]. In our study, the viral response was not associated with serum PEG-IFN concentration, but it was associated with cellular responses to IFN such as PKR expression. Although both PEG-IFNs appear to show different profiles in absorption, distribution and clearance, it remains unknown how these differences relate to differences in cellular responses *in vivo* such as PKR and the primary clinical endpoint, SVR.

The serum level of ribavirin has been reported previously to be associated with the observed clinical effects [6]. With ribavirin combination therapy, the antiviral effect was more potent after 3 weeks, at which time serum ribavirin levels were shown to have increased [7]. Therefore, accumulation of ribavirin from the third week of administration onwards, during which viral suppression is important for SVR, may be associated with the viral response seen with combination therapy. However, in our study, we found no significant difference in serum ribavirin levels between patients who achieved an SVR and NR patients. There was also no significant difference in serum IFN levels between the SVR and NR patients. As there are only a small number of studies that have reported serum ribavirin levels and associated virological effects in detail, further more comprehensive investigations are therefore required.

In conclusion, the pharmacokinetic improvement provided by pegylation of IFN leads to dramatic changes in PKR transcript expression patterns. In contrast, serum ribavirin concentrations appear not to be associated with the viral response and PKR expression. Our data suggest that the higher intracellular expression of PKR transcripts from the second day onwards is associated with the enhanced virological efficacy of PEG-IFN alpha-2b and ribavirin combination therapy.

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Editorial

Optimal timing of interferon treatment for acute hepatitis C

The incidence of acute hepatitis C is declining owing to a near elimination of transfusion associated hepatitis after the initiation of the screening of blood for hepatitis C virus (HCV). However, acute hepatitis C is not totally eliminated. There is still a risk for HCV infection through medical procedures or by accidental needle-stick injury. Since acute hepatitis C is often followed by chronic hepatitis which may eventually progress to liver cirrhosis and hepatocellular carcinoma, establishment of the effective treatment of this disease is still a serious matter.

An appropriate treatment strategy of acute hepatitis C has not been established to date. Several studies have clearly demonstrated the beneficial effect of the interferon (IFN) treatment in the eradication of HCV during acute infection and preventing the progression to chronic hepatitis [1–5]. However, the controversies remain on the following issues: (1) which patients should be treated, (2) when should therapy be started (immediately at the onset of hepatitis or after a period of waiting for spontaneous remission), and (3) what regimen of therapy should be used (whether to use ribavirin combination therapy rather than interferon mono-therapy).

Theoretically, suppression of HCV replication by IFN therapy during the early phase of acute hepatitis may favor the patient's immune systems to clear the virus and prevent the development of chronic infection. In contrast, if HCV replication is not controlled during the early phase due to the delay of the treatment, the immune responses towards HCV during acute hepatitis, which is usually more vigorous compared to chronic hepatitis, may be weakened which lead to the failure of HCV clearance [6–8]. According to this logic, immediate initiation of therapy for acute hepatitis C is desirable before immunologic mechanisms for persistent infection are established. The major disadvantage of the immediate treatment strategy is that exposing patients who may spontaneously clear the virus to unnecessary treatment. In fact, 20–50% of patients clear the virus spontaneously [9–11]. Thus, optimal timing for the IFN treatment remains unresolved.

In this issue of the journal, Ogata [12] found that delay of IFN therapy later than 24 weeks after the onset is associated with a significant decrease in therapeutic efficacy. The rate of sustained clearance of HCV was significantly high when IFN therapy was initiated within 24 weeks compared to later than 24 weeks. On the other hand, as long as the therapy was initiated within 24 weeks, the earlier timing of therapy was not associated with the improved rate of HCV clearance. In other words, the immediate therapy was not associated with improvement in the efficacy. Their results suggest that immediate therapy at the onset of acute hepatitis is not necessary and the initiation of therapy could be delayed after a period of careful waiting for spontaneous clearance of HCV. The critical time point may be 24 weeks. Recent randomized controlled study by Nomura et al. [13] has demonstrated that delaying the initiation of IFN therapy for a period of 12 months lowered the response rates substantially (87–100% in the early treatment (at 8 weeks after the onset) group and 40–53% in delayed-treatment group). Meanwhile, a recent meta-analysis showed that delaying therapy by 8–12 weeks after the onset of acute hepatitis does not compromise the rate of HCV clearance [14]. It is also reported that the spontaneous clearance of HCV is likely to occur within 4–12 weeks of infection [10,11]. These results imply that immediate therapy is too early and waiting for more than 24 weeks is too late. Optimal timing for the IFN treatment may end up within a period of 8–24 weeks after the onset of acute hepatitis.

Besides when to start therapy, controversy also remains on which patients should be treated, since there is no reliable predictors to identify which patients are unlikely to clear the virus spontaneously. If the likelihood of chronicity in individual patients could be predicted, therapy could be started with no delay in high risk patients. It is reported that symptomatic patients [15] or those with jaundice [11] may have more chance of spontaneous clearance of the virus compared to asymptomatic patients. In addition, Ogata [12] depicted that patients with the fluctuation of ALT levels are unlikely to clear the virus spontaneously. From these observations, asymptomatic, non-icteric patients with the fluctuation of ALT levels may be one of the high risk groups for the development of chronic infection and thus therapy should be initiated without delay.

Another important issue is what regimen of therapy should be used. Higher dose of IFN may be preferable [2] but the optimal dose and duration of therapy has not reached a consensus. Recent reports indicate that PEG-IFN monotherapy is equally effective to conventional IFN mono-therapy

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[16,17]. Combination therapy of ribavirin and IFN or PEG-IFN, which is now the standard regimen for chronic hepatitis, may not have additive value over mono-therapy in acute hepatitis since the rate of sustained clearance of HCV is already high with mono-therapy.

Conclusive recommendations on the treatment of acute hepatitis C could not be made due to a lack of a large scale, prospective and randomized study. However, available evidences suggest that IFN therapy should be recommended as a standard therapy in patients with acute hepatitis C. Immediate therapy is not always necessary and a wait and see may be a reasonable strategy since the later therapy with 8–24 weeks of delay does not compromise the rate of sustained clearance of HCV.

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Emergence of a Novel Lamivudine-Resistant Hepatitis B Virus Variant with a Substitution Outside the YMDD Motif[▽]

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Lamivudine is a major drug approved for treatment of chronic hepatitis B virus (HBV) infection. Emergence of drug-resistant mutants with amino acid substitutions in the YMDD motif is a well-documented problem during long-term lamivudine therapy. Here we report a novel lamivudine-resistant strain of HBV with an intact YMDD motif, which included an amino acid substitution, rtA181T, in the reverse transcriptase (RT) domain of HBV polymerase. The substitution also induced a unique amino acid substitution (W172L) in the overlapping hepatitis B surface (HBs) protein. The YMDD mutant strains were not detected even by using the sensitive peptide nucleic acid-mediated PCR clamping method. The detected nucleotide substitution was accompanied by the emergence of an additional nucleotide substitution that induced amino acid change (S331C) in the spacer domain. The rtA181T mutant strain displayed a threefold decrease in susceptibility to lamivudine in in vitro experiments in comparison with the wild type. In vivo analysis using human hepatocyte-chimeric mice confirmed the resistance of this mutant strain to lamivudine. We developed a method to detect this novel rtA181T mutation and a previously reported rtA181T mutation with the HBs stop codon using restriction fragment length polymorphism PCR and identified one patient with the latter pattern among 40 patients with lamivudine resistance. In conclusion, although the incidence is not high, we have to be careful regarding the emergence of lamivudine-resistant mutant strains with intact YMDD motif.

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma (4, 12, 33). To date, interferon and three nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, and entecavir) have been approved by the United States Food and Drug Administration for the treatment of chronic HBV infection. Lamivudine, an oral cytosine nucleoside analogue, potently inhibits HBV replication by interfering with RNA-dependent DNA polymerase (10, 16, 22). Lamivudine therapy suppresses HBV replication in most patients and improves transaminase levels and liver histology (16, 22, 25, 30). However, prolonged therapy results in the emergence of drug-resistant mutants in 24% and 70% of patients after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels (18).

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Most lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed (1, 9). In vitro analyses have confirmed that the rtL180M mutation augments the level of lamivudine resistance and enhances viral replication, while the rtV173L mutation enhances only viral replication (9, 23). On the other hand, only a few uncommon mutations associated with lamivudine resistance have been reported so far (3, 7, 24, 34). In the C domain of HBV polymerase, rtM204S and rtD205N were detected in patients with lamivudine resistance (3, 7). In the B domain, rtL180C and rtA181T were associated with lamivudine resistance (7, 24, 34). Yeh et al. (34) reported the emergence of rtA181T mutants in 4 of 23 patients who received long-term lamivudine therapy. The mutant appeared concomitantly with or after emergence of YMDD motif mutants and persisted thereafter. The nucleotide substitution in the FLLA motif resulted in early termination of the overlapping HBs gene transcription by creating a stop codon (TGG to TGA). Yeh et al. (34) demonstrated that the mutation reduced the

susceptibility to lamivudine in vitro. They also detected such mutations in virus from a patient with leukemia and speculated that truncated HBs gene might be related to the development of leukemia (7).

Analyzing nucleotide and amino acid sequences of HBV in patients who developed a breakthrough, we identified a novel mutant that showed nucleotide substitutions in the B domain of the reverse transcriptase. The G residues of nucleotides 669 and 670 were mutated to T and A, respectively, and associated with the amino acid substitution rtA181T. The substitutions also induced the amino acid substitution W172L in the overlapping HBs protein. Since the nucleotide substitution was associated with nucleotide and amino acid substitutions in the putative spacer region of the polymerase, we checked the importance of these substitutions for resistance to lamivudine in vitro. We also analyzed the resistance of this new strain in vivo using a human hepatocyte-chimeric mouse (27, 31). Furthermore, we analyzed the susceptibility of the mutant strain to adefovir and entecavir. When used alone or in combination with lamivudine, these drugs are known to be effective against wild-type as well as lamivudine-resistant HBV (2, 5, 14, 17, 32). Infrequent emergence of resistance compared with lamivudine resistance has been reported for both of these two drugs (2, 5). We also developed a detection system to identify the novel and previously reported (7, 34) nucleotide substitutions to study the incidence of such mutations.

MATERIALS AND METHODS

Antiviral compounds. Lamivudine [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, United Kingdom). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and entecavir {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT).

Analysis of virological markers. Hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), and antibody against HBeAg (anti-HBe) were quantified by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using a Light Cycler (Roche, Mannheim, Germany). The primers used for amplification were 5′-TTTGGGCATGGACA TTGAC-3′ and 5′-GGTGAACAATGTTCCGGAGAC-3′. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Cloning of HBV DNA and plasmid construction. HBV DNA was extracted from 100 μl of each serum sample by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H_2O. Full-length HBV DNA was amplified using the above HBV DNA samples by the method of Gunther et al. (13). Nucleotide sequence positions were numbered from the unique EcoRI site. The 1.4-genome-length HBV DNA amplified from the serum of a patient who showed lamivudine resistance was cloned into plasmid vector pTRE (Takara Bio, Tokyo, Japan) (patient strain). In brief, the PCR product amplified using serum from the patient was cleaved with BamHI and ApaI (HBV positions 1400 to 2600) and cloned into pcDNA3 (Invitrogen, San Diego, CA), and the resulting construct was named pcDNA3-1. Similarly, the PCR product was cleaved with ApaI and BamHI (HBV positions 2600 to 3215 and 1 to 1400) and cloned into pBlueScript SK+ (Stratagene, La Jolla, CA), and the resulting construct was named pB-1. The KpnI-BamHI fragment from pB-1 and the KpnI-ApaI fragment from pcDNA3-1 were cloned into pcDNA3-1. Finally, the plasmids were cleaved with HindIII and NotI within the multicloning site and cloned into plasmid vector pTRE. As a laboratory strain, we employed a plasmid containing a 1.4-genome-length wild-type genotype C HBV (wild-type strain; GenBank accession number AB206816) (31). To introduce the nucleotide substitutions into the S331C/rtA181T patient and wild-type strains, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene).

TABLE 1. In vitro susceptibility of the S331/rtA181 mutant to lamivudine[a]

Strain		S331/rtA181 mutation	Lamivudine IC_{50} (μM)	Resistance (fold)
Source	Type			
Patient	WT	−/−	0.19 ± 0.01	1
	S331C	C/−	0.23 ± 0.01	1.2*
	rtA181T	−/T	0.58 ± 0.08	3**
	S331C/rtA181T	C/T	0.57 ± 0.06	3**
Laboratory	WT	−/−	0.23 ± 0.04	1
	S331C	C/−	0.3 ± 0.05	1.3*
	rtA181T	−/T	0.88 ± 0.2	3.9**
	S331C/rtA181T	C/T	0.98 ± 0.12	4.3**

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

The eight plasmids with and without amino acid substitutions in the spacer and reverse transcriptase domain are listed in Table 1.

Cell culture, transfection, and determination of IC_{50}. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in 5% CO_2. Cells were seeded to semiconfluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 cells was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC_{50}s) for each antiviral drug, various concentrations of lamivudine, adefovir, and entecavir were added after 24 h to the culture plate containing the cells, and cells were harvested after 5 days. The medium containing the drugs was changed on days 1, 3, and 4. A plasmid encoding β-galactosidase (β-Gal) was cotransfected to adjust the transfection efficiency. The β-Gal enzyme assay was performed with a β-Gal enzyme assay system (Promega, Madison, WI). All experiments were performed in triplicate. GraphPad Prism software (GraphPad Software, Inc.) was used to determine the best-fit values for individual dose-response equations.

Analysis of replicative intermediate of HBV by Southern blot hybridization and quantitation. The cells were harvested at 3 or 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, and 0.5% [vol/vol] NP-40) followed by centrifugation for 2 min at 15,000 × *g*. The core-associated HBV genome was immunoprecipitated by mouse anticore monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after sodium dodecyl sulfate–proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer. Quantitative analysis was performed by real-time PCR with SYBR green using a Light Cycler. The HBV-specific primers used for amplification were 5′-TTTGGGCATGGACATTGAC-3′ and 5′-GGT GAACAATGTTCCGGAGAC-3′. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Evaluation of effects of antiviral drugs on mutant strains using human hepatocyte-chimeric mice. Human hepatocyte-chimeric mice were generated and used in the drug evaluation studies as described previously (27, 31). Briefly, human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice, which are immunodeficient and develop liver failure. The transplanted cells were characterized in terms of in vivo growth potential and function. The human hepatocytes progressively repopulated the murine host liver and were susceptible to cultured-cell-line-produced HBV. All animal protocols were performed in accordance with the guidelines of the local committee for animal experimentation. The mice were inoculated with 50 μl of serum samples containing wild-type and newly identified drug-resistant strains. Serum samples obtained from mice were stored at −80°C before further analyses. After stable high-level HBV viremia was confirmed, the mice were administered food containing 30 mg of lamivudine/kg of body weight/day. The nucleotide sequences of wild-type and mutant strains were confirmed by sequencing analysis.

Detection of rtA181T mutants by PCR with restriction fragment length polymorphism (RFLP). HBV DNA extracted from serum samples were amplified by

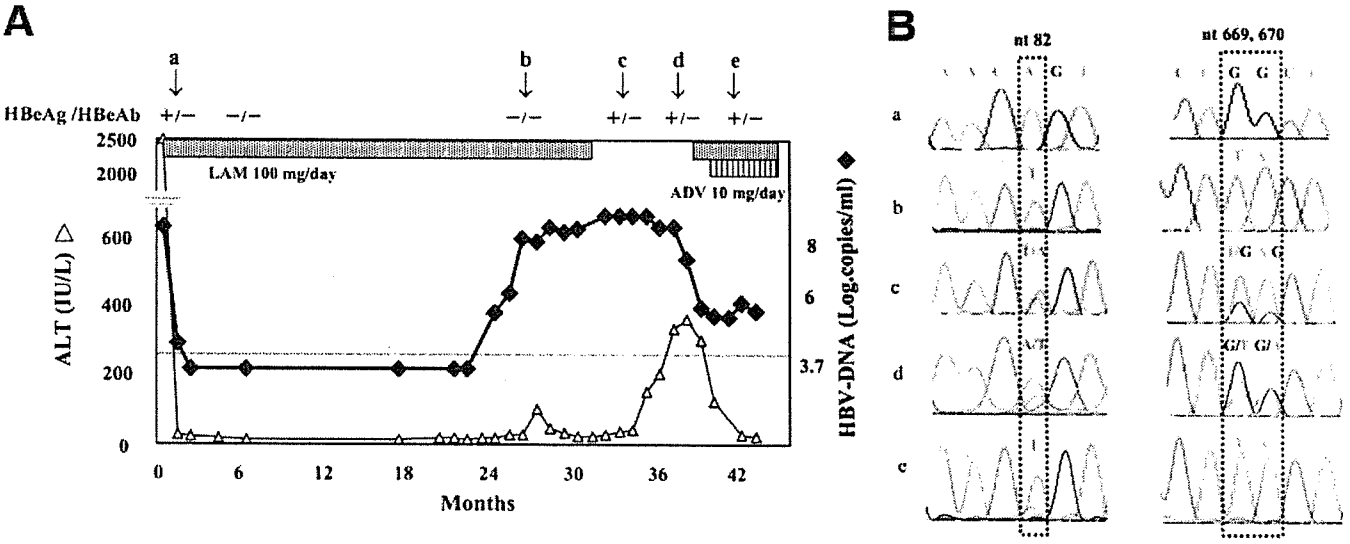

FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis B virus by direct sequencing. Time points of serum sampling (see panel A) were as follows: (a) just before lamivudine treatment, (b) after breakthrough, (c) after cessation of lamivudine treatment, (d) just before readministration of lamivudine, and (e) during adefovir and lamivudine therapy. Note that the wild type reappeared during the cessation of therapy (c and d), but it disappeared after readministration of the drug (e).

PCR using the primers 5′-GCCCGTTTGTCCTCTACTTCCA-3′ and 5′-ACCA CTGAACAAATGGCACTAGTAAGCTGA-3′. The reverse primer was designed to introduce an EspI site (GCTCAGC) into only wild-type sequences. The PCR was performed in a total volume of 25 μl, consisting of a reaction buffer (100 mmol/liter Tris-HCl [pH 8.3], 50 mmol/liter KCl, and 15 mmol/liter $MgCl_2$), 0.2 mmol/liter of each deoxynucleoside triphosphate, 1 μl of the DNA solution, 10 pmol of each primer and 1 U of *Taq* DNA polymerase (Gene Taq; Wako Pure Chemicals, Tokyo, Japan) with 0.2 μg of anti-*Taq* high (Toyobo Co., Osaka, Japan). The amplification conditions included an initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 1 min, annealing of primer at 58°C for 1 min, extension at 72°C for 2 min), and final extension at 72°C for 7 min. Two μl of PCR products was digested with 5 U of EspI and subjected to electrophoresis in a 3.5% agarose gel.

Statistical analysis. Data are expressed as means ± standard deviations (SD). Group comparisons were performed using the Student *t* test. A *P* value of less than 0.05 was considered statistically significant.

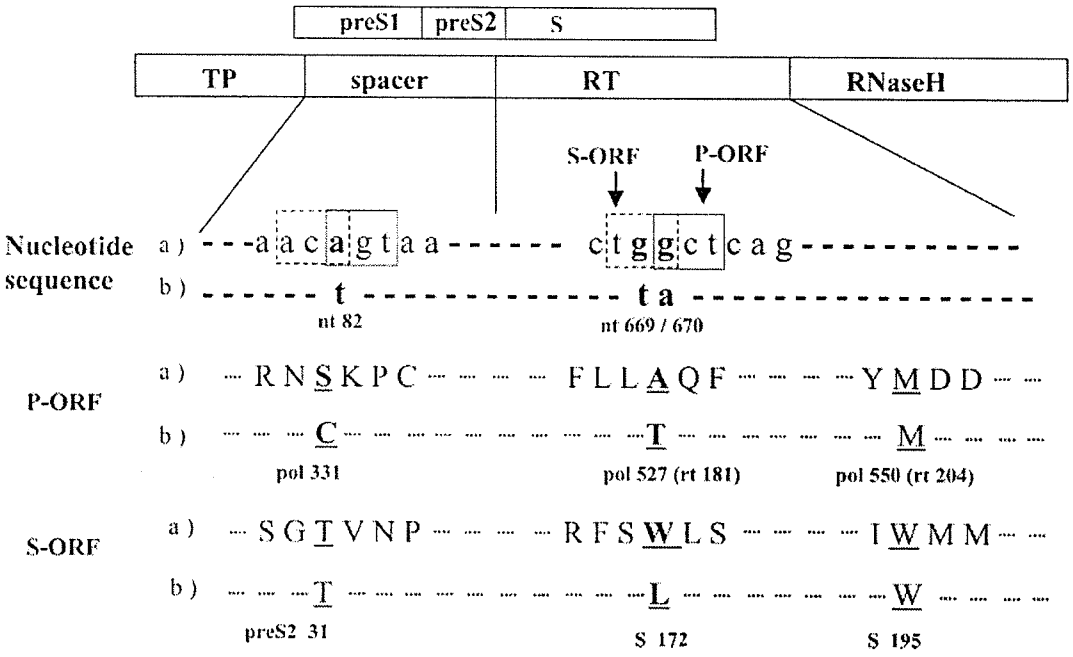

FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were compared. See Fig. 1A for time points of serum sampling. Nucleotide sequence numbers are those of typical HBV (e.g., accession no. AB206816 [31]), which starts from a unique EcoRI site.

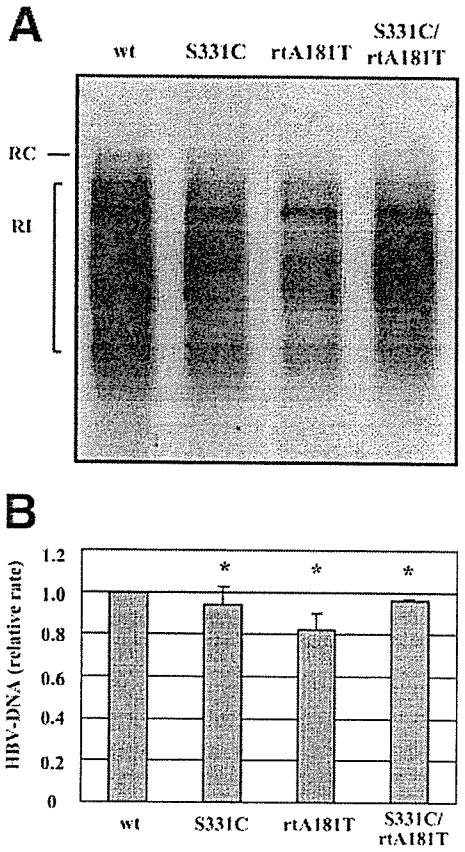

FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates of HBV DNA were isolated from HepG2 cells at 3 days after transfection. The positions of relaxed circular DNA (RC) and replication intermediates (RI) are indicated. (B) Quantitative analyses of core-associated intermediates of HBV. Experiments were performed in triplicate. Values are relative to those of the wild type and are expressed as means ± SD. *, not significant compared to the wild type.

RESULTS

Isolation of a novel lamivudine-resistant strain with an intact YMDD motif. The novel lamivudine-resistant strain of HBV was isolated from a 44-year-old Japanese man with chronic HBV infection (Fig. 1A). In this patient, lamivudine successfully reduced the HBV level at the initial stage of treatment, but viral breakthrough was observed at 24 months after the beginning of therapy. The patient was very punctual and confirmed that he took lamivudine with perfect compliance. The HBV viral load reached up to 8.5 log copies/ml, but nucleotide sequence analysis showed no YMDD mutation. The YIDD and YVDD mutants were not detected even with a peptide nucleic acid-mediated PCR clamping method sensitive for detection of YMDD mutants (6). The analysis also showed that this isolate belonged to genotype C of HBV. Comparison by the direct sequence method of nucleotide sequences obtained before and after the viral breakthrough showed three nucleotide substitutions that induced two amino acid substitutions in both spacer (polS331C) and reverse transcriptase (polA527T or rtA181T) domains of the polymerase (Fig. 1B and 2). The latter nucleotide substitutions induced an amino acid change in the overlapping HBs protein (W172L) (Fig. 2). Twelve HBV genomes were cloned from the serum of this patient after viral breakthrough, and eleven of them showed the above amino acid substitutions. Only one clone showed the wild-type sequence. The new strain of HBV became undetectable when lamivudine therapy was discontinued, and this strain outcompeted the wild-type strain upon administration of the drug (Fig. 1B). These results prompted us to study the significance of each of these mutations.

Effect of substitutions on HBV replication. To assess the effect of nucleotide substitutions on HBV replication, four plasmids containing 1.4-genome-length patient-specific HBV genome (Table 1) were generated and transfected into HepG2 cells. In comparison with the patient's wild-type strain, the replication capacities of the S331C, rtA181T, and S331C/rtA181T mutants were not different (94%, 82%, and 96%, respectively), suggesting that these mutants can replicate at almost the same rate as the wild-type strain (Fig. 3).

Susceptibility of mutants to lamivudine in vitro. To analyze the role of the polS331C and rtA181T mutations in lamivudine resistance, four patient-specific strains and four laboratory strains were transfected into HepG2 cells (Fig. 4; Table 1). A single amino acid substitution in the spacer region did not contribute to resistance in either patient or laboratory strains. In contrast, an amino acid substitution in the polymerase (rtA181T) induced resistance that was 3.0 and 3.9 times greater than that of patient and laboratory strains ($P < 0.001$), respectively. The presence of both of these amino acid changes induced 3.0 and 4.3 times greater resistance in each of the above strains. Thus, the spacer mutation had little effect on the susceptibility to lamivudine (Table 1).

We also compared the rtA181T mutant identified in this study with the rtA181T mutant reported previously, which had premature termination in the HBs protein (7, 34), for replication ability and susceptibility to lamivudine. Although the HBs antigen produced to culture supernatant was different between the two strains (52.5 ± 8.2 and 4.4 ± 0.6 IU/ml, respectively), there was no noticeable difference in replication ability and lamivudine sensitivity between the two mutants (data not shown).

Assessment of drug resistance of novel mutations in vivo using human hepatocyte-chimeric mice. To confirm the lamivudine resistance of the novel mutant strain, two human hepatocyte-chimeric mice were each inoculated with a serum sample obtained from the patient who developed breakthrough without mutations in the YMDD motif (Fig. 1A). The serum was obtained during breakthrough while the patient was still taking the drug. Twelve weeks after the inoculation of the serum samples, both mice developed high-level viremia (7.8 and 6.6 log copies/ml, respectively). Direct sequence analysis showed that the nucleotide sequence of the virus that replicated in the chimeric mice was in accordance with the mutant strain. Cloning and sequencing analysis showed that only 1 of 12 clones obtained from the inoculum was wild type, while the remaining 11 clones were rtA181T mutants with an intact YMDD motif. We also analyzed the serum of the two infected mice before and after lamivudine therapy. All 11 and 15 clones before and all 11 and 12 clones during therapy had the

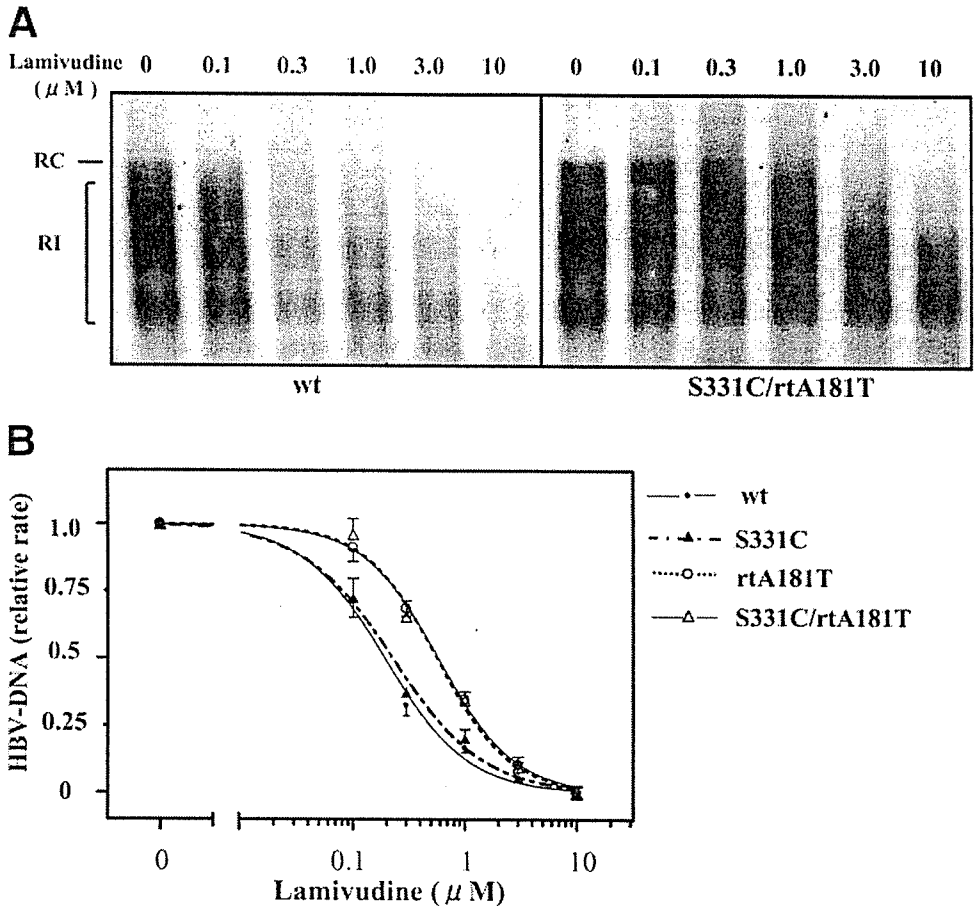

FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (A) Southern blot analysis of replicative intermediate. Representative results for the wild type (wt) and the S331C/rtA181T mutant are shown. The positions of relaxed circular (RC) and replication intermediate (RI) forms of HBV DNA are indicated. (B) Dose-response curves of the four HBV strains against lamivudine. The curves were used to estimate the lamivudine IC_{50}s for each HBV strains. Values are relative to no-lamivudine controls for each strain. Experiments were performed in triplicate. Values are expressed as means ± SD.

rtA181T mutation (data not shown). Two other mice were inoculated with wild-type HBV obtained from a patient not treated with lamivudine as a control, and both mice also developed high-level viremia (8.3 and 9.3 log copies/ml, respectively). Thirteen weeks later, the viremia reached plateau and the mice were fed food containing lamivudine. After 6 weeks of treatment, the mean viral load decreased by 2.8 log copies/ml in the wild type, whereas it decreased by only 0.39 log copy/ml in the mutant ($P < 0.001$) (Fig. 5).

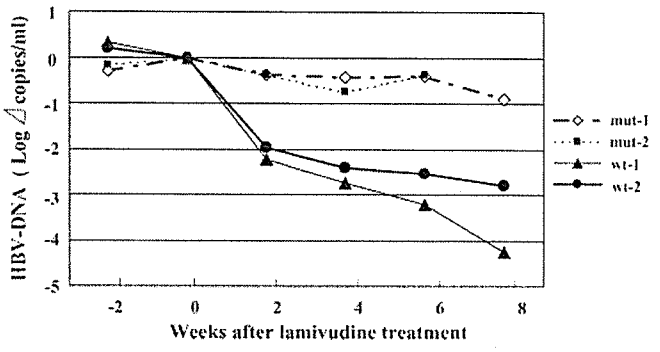

FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

Susceptibility of mutants to adefovir and entecavir in vitro. We also analyzed the effects of adefovir and entecavir against the S331C/rtA181T mutant using a transient-transfection assay with HepG2 cells. The IC_{50}s of these drugs for the mutant strain and wild type were almost the same (Table 2).

Detection of rtA181T mutant in patients treated with lamivudine. In this study, we developed a RFLP PCR method to detect the rtA181T mutants, by which we were able to detect mutant strains even when they were mixed with the wild type (Fig. 6). The system also detected the rtA181T (HBs stop) mutant reported by Chien et al. (7) and Yeh et al. (34). Using this method, we analyzed 40 patients who showed viral breakthrough (increase in viral load equal to or more than 1 log) during lamivudine therapy. We found that only one of these patients was positive (Fig. 6A). Nucleotide sequence analysis of serum samples obtained from this patient showed that the

TABLE 2. In vitro susceptibility of the S331/rtA181 mutant to lamivudine, adefovir, and entecavir[a]

Patient strain	S331/rtA181	Lamivudine		Adefovir		Entecavir	
		IC_{50} (μM)	Resistance (fold)	IC_{50} (μM)	Resistance (fold)	IC_{50} (nM)	Resistance (fold)
WT	−/−	0.19 ± 0.01	1	0.37 ± 0.1	1	0.19 ± 0.02	1
S331C/rtA181T	C/T	0.57 ± 0.06	3**	0.36 ± 0.08	0.98*	0.23 ± 0.05	1.2*

[a] Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

mutant strain had the rtA181T mutation with a truncated HBs antigen, as reported previously (7, 34). The YMDD motif of HBV detected in this patient was of the wild type. All 39 remaining patients with viral breakthrough were positive for YIDD and/or YVDD mutants. The RFLP PCR analysis of these 39 samples showed that four contained a small amount of rtA181T mutants (Fig. 6B). Nucleotide sequence analyses of these samples showed that they contained only a small amount of rtA181T mutants with a truncated HBs antigen (Fig. 6C).

Finally, we examined the presence of YMDD or rtA181T mutants in eight patients who showed a poor response with lamivudine treatment (HBV viral load above 6.0 log copies/ml after 6 months of treatment). None of these patients tested positive for both of these mutations (data not shown).

DISCUSSION

In this study, we identified a novel lamivudine-resistant strain of HBV with an intact YMDD motif in a patient who received long-term lamivudine therapy. YMDD mutants were not detected even by a sensitivity-enhanced detection method, which was reported previously by our group (6). The double nucleotide substitutions (GG to TA) induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L). One might assume that the compliance of the patient was poor. However, the patient was very punctual and confirmed that he took lamivudine with perfect compliance.

Our study demonstrated that the rtA181T mutation reduced the susceptibility to lamivudine 3.0- to 3.9-fold in vitro (Table 1). Furthermore, we also confirmed lamivudine resistance of this mutant strain in vivo using human hepatocyte-chimeric mice. The amino acid substitution in the reverse transcriptase (RT) domain is similar to that reported previously (7, 34). However, in contrast to our results, the mutant strains in the latter reports emerged with or after those with the mutation in the YMDD motif (YIDD or YVDD) and took over them (34). There are two additional differences between the substitutions we identified and those described by Yeh et al. (34), as detailed below.

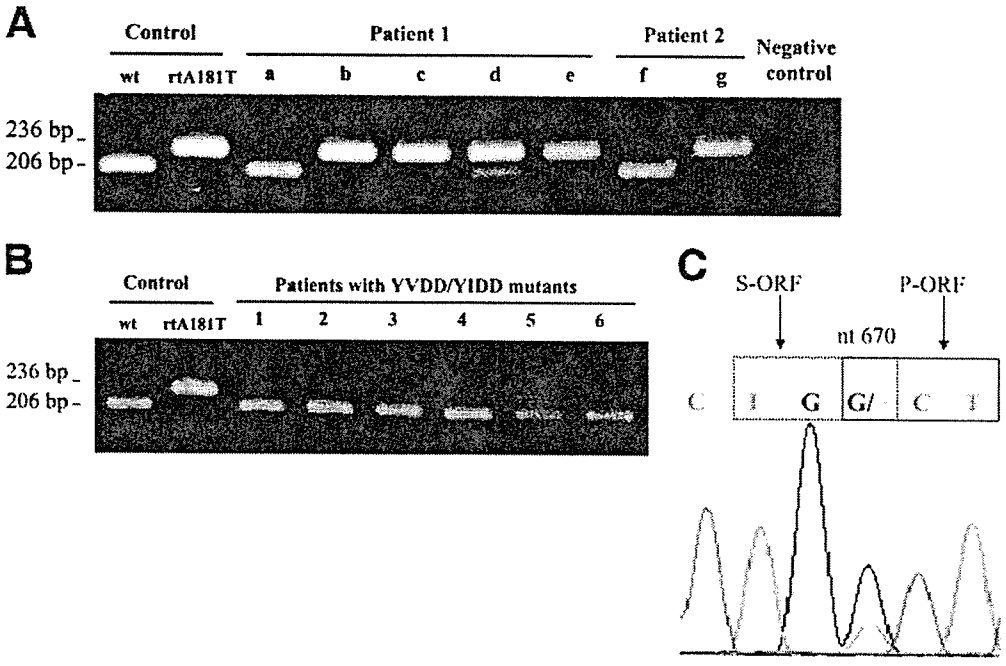

FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with EspI, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. See Fig. 1A for the time points of serum sampling (a to e) for patient 1 and see Fig. 1B for a comparison with nucleotide sequence analyses. f and g indicate the time points before and after viral breakthrough for patient 2. (B) Agarose gel electrophoresis of RFLP PCR products using HBV DNA samples obtained from 39 patients who showed lamivudine breakthrough. Of the 39 samples, 35 were wild type (lanes 1 and 2). The remaining four samples (lanes 3 to 7) showed partial digestion, suggesting a mixture of wild-type and mutant strains. (C) Nucleotide sequence analysis of a sample by RFLP PCR suggested the presence of a wild-type–mutant mixture (lane 5 of panel B).

Firstly, the HBs antigen was prematurely terminated in the mutant strain reported by Yeh et al. (34). In this regard, a similar amino acid substitution of the B domain of the polymerase FLLA motif in woodchuck hepatitis virus (WHV) treated with lamivudine was reported (15, 28). The HBs antigen in these WHV mutant strains also had premature stop codons. These findings suggest that the mutant strains of HBV and WHV cannot replicate and spread by themselves because of the lack of HBs antigen. Such strains are thought to replicate by using in vivo-supplied HBs antigen from wild-type strains as helper antigens. In contrast, the novel strain identified in this study had no premature termination of the HBs gene. The in vitro study suggested that the strain had a replication ability similar to that of the wild type. Furthermore, we also showed that the strain infected and reached a high viral load in human hepatocyte-chimeric mice. Although the inoculum contained only a small amount of wild-type strain (one of 12 clones), all clones obtained from mouse serum were mutant strains (rtA181T). Considering these results and the fact that the index patient showed high viral titers after breakthrough (more than 7.6 log copies/ml), this mutant strain can spread and replicate by itself and has strong replicative ability.

Secondly, the substitutions identified in this study appeared with nucleotide and amino acid substitutions in the spacer region of the polymerase (S331C). There are only a few studies that reported the function of the spacer domain (19–21, 28), leaving the biological significance of this region unknown. The substitution in the spacer region reappeared with the A181T mutation in the RT domain in the index patient after the patient restarted lamivudine therapy. Although our study showed no significant contribution of this mutation to drug resistance (Fig. 3 and 4; Table 1), the significance of the mutation in this region (fingers in the HBV polymerase homology model [8]) should further be investigated.

Recently, the amino acid substitutions rtA181T and rtA181V were reported to emerge with resistance against adefovir (11, 32). Tillmann et al. (29) reported one case in which the virus developed the rtA181T mutation during famciclovir breakthrough. The A556T mutation of WHV, analogous to the rtA181T mutation of HBV, has been reported to be associated with lamivudine resistance (15, 28). These results indicate that the amino acid substitutions at position 181 may associate with resistance against many nucleoside analogues, including lamivudine, famciclovir, and adefovir. Although our in vitro study indicated that the rtA181T mutant had no resistance against adefovir and the animal study showed that combination therapy with lamivudine and adefovir effectively reduced the virus load in woodchucks (15), such combination therapy did not produce sufficient suppression of HBV in the index patient (Fig. 1A). The amino acid substitution at position 181 has to be further analyzed with regard to resistance to anti-HBV drugs.

The rtA181T mutation detection system using RFLP PCR developed in this study is a useful tool, as we were able to distinguish the wild type from all mutants with nucleotide substitutions in a given region. The system also enabled us to monitor the fluctuation of the wild-type/mutant ratio during therapy against HBV (Fig. 1 and 6). The incidence of rtA181T mutants with an intact YMDD motif is rare in Japanese patients with chronic HBV infection treated with lamivudine. Interestingly, 4 of the 39 (10%) patients who developed lamivudine breakthrough and were positive for YMDD mutants were found to have small amounts of rtA181T mutant strains. Different from the previous report (34), the mutants did not take over another strain and were not preceded by exacerbation. We have to monitor these patients carefully for further population change of mutants and for exacerbation of hepatitis.

A recent study reported that the prevalence of genotype A HBV infection is increasing in Japan and that the incidence of disease chronicity is higher than for other genotypes (26). It is thus expected that an increasing number of the sexually active population will receive nucleoside analogue therapy against HBV and multiple mutant strains can potentially emerge and spread along with long-term treatment. There is an increasing possibility of emergence of novel mutants resistant to multiple anti-HBV drugs. The importance and significance of the rtA181 mutations, including the novel mutant strain identified in this study, should be investigated further to develop more useful treatment strategies.

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