East Asia, including Japan, Korea, and Northern China, were determined by the PCR-RFLP method described previously.[12]

Quantification of HBV DNA and Sequencing. HBV DNA sequences spanning the S gene were determined by real-time detection PCR according to the method of Abe et al.,[25] with the detection limit of 100 copies/mL. HBV DNA sequences bearing core promoter, precore region, and the core gene were amplified by PCR with hemi-nested primers by the method described previously.[10] Negative samples were tested by another more sensitive second-round PCR with HB7F and HBV1917R (5′-CTC CAC AGT AGC TCC AAA TTC TTT A-3′). Thereafter, PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer.

Construction of Plasmid and Site-Directed Mutagenesis of HBV DNA. Serum samples were obtained from two patients infected with HBV/Bj and a patient with Ce. HBV DNA was extracted from 100 μL serum using QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Four primer sets were designed to amplify two fragments covering the entire HBV genome. Amplified fragments were inserted into pGEM-T Easy Vector (Promega, Madison, WI) and cloned in DH5a competent cells (TOYOBO, Osaka, Japan). At least five clones of each fragment were sequenced and the consensus sequence determined. Among them, those containing the consensus sequence were identified and adopted as templates for further construction. Finally, 1.24-fold the HBV genome (nt 1413-3215/1-2185), just enough to transcribe oversized pregenome and precore mRNA, was constructed into pUC19 vector (Invitrogen Corp., Carlsbad, CA). For site-directed mutagenesis, the wild-type HBV was digested by *HindIII* and *EcoO65I* and ligated with the fragment carrying T1762/A1764 to produce 1.24-fold the genome carrying the core-promoter double mutation. Similarly, 1.24-fold the HBV genome with the precore stop-codon mutation (1896A) was generated. Further details are available online at: http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html.

Cell Culture and DNA Transfection. For the standard replication assay, 10-cm-diameter dishes were seeded with 1×10^6 Huh7 cells each. After 16 hours of culture, cells were transfected with 5 μg DNA construct using the FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) and harvested 3 days later. Transfection efficiency was measured by cotransfection with 1 μg reporter plasmid expressing secreted alkaline phosphatase and estimating its enzymatic activity in the culture supernatant.

Southern Blot Hybridization. HBV DNA samples from cells at day 3 in culture were separated on 1.2% (wt/vol) agarose gel, transferred to a positive-charged nylon membrane (Roche Diagnostics), and hybridized with full-length HBV DNA labeled with alkaline phosphatase. Detection was performed with CDP-star (Amersham Biosciences, Piscataway, NJ), and signals were analyzed in the LAS-1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Statistical Analysis. Categorical variables were compared between groups by the chi-squared test and non-categorical variables by the Mann-Whitney *U*-test. A *P* value less than .05 was considered significant. Multivariate analyses with logistic regression were used to determine independent factors for fulminant hepatitis. STATA Software (StataCorp LP, College Station, TX) version 8.0 was employed for analyses.

Results

Demographic and Clinical Differences in Patients Infected With Various HBV Genotypes/Subgenotypes. Genotypes of HBV were not classifiable in 28 (8%), and sufficient clinical data were not available in 7 (2%) of the 336 patients with acute hepatitis B. Exclusive of these 35 patients, 301 (90%) were left for evaluation of HBV genotypes in reference to clinical outcome.

HBV genotypes/subgenotypes were Aa in 10 (3%), Ae in 33 (11%), Ba in 22 (7%), Bj in 22 (7%), Cs in 11 (4%), Ce in 192 (64%), D in 5 (2%), and G in 6 (2%); none of them were infected with F or H (Table 1). All six patients with HBV/G were co-infected with another genotype; Ae in two, Ba in two, and Ce in the remaining two. The mean age was lower in the patients with HBV/Ae than Ba ($P = .0001$), Aa ($P < .01$), Bj or Cs ($P < .05$ for each) and Ce than Ba ($P < .05$). Men predominated in HBV infections with foreign (Ae and Ba) compared with domestic genotypes (Bj and Ce) ($P < .05$).

HBeAg was detected in 79% of patients with HBV/Ae at a frequency much higher than that with Bj ($P < .005$), Ce ($P < .001$) or Ba ($P < .05$). HBeAg in four of the six (67%) patients with HBV/G was coded for by HBV of the other genotypes co-infecting them, because it has two stop codons and an insertion in the core gene that prohibit encoding HBeAg.[21] HBV DNA levels as well as HBeAg-positive rates at the presentation were higher in HBV/Ae than Ce ($P < .005$) or Bj ($P < .05$) infection.

The peak alanine aminotransferase (ALT) level was higher in HBV/Bj than Ae infection ($P < .05$). Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (55%) than the other genotypes ($P < .05$); it occurred in two of the five (40%) patients with HBV/D, also. In reflection of severe clinical course,

Table 1. Clinical Characteristics of Patients Acutely Infected With HBV of Distinct Genotypes/Subgenotypes

Features	Genotypes/Subgenotypes							
	Aa (n = 10)	Ae (n = 33)	Ba (n = 22)	Bj (n = 22)	Cs (n = 11)	Ce (n = 192)	D[a] (n = 5)	G[a,b] (n = 6)
Age (years)	42.2 ± 13.1	31.2 ± 10.3[d]	41.5 ± 10.7[e]	43.5 ± 19.1	38.5 ± 11.1	36.3 ± 15.0	38.6 ± 20.8	42.7 ± 17.5
Men	8 (80%)	30 (91%)[f]	19 (86%)[g]	9 (41%)	7 (64%)	122 (64%)	2 (40%)	6 (100%)
HBeAg positive	7 (70%)	26 (79%)[h]	11 (50%)	8 (36%)	8 (73%)	101 (53%)	1 (20%)	4 (67%)
ALT (IU/L)	1875 ± 759	2070 ± 1113[i]	2523 ± 1185	3472 ± 2720	2269 ± 995	2610 ± 1719	2559 ± 1672	2142 ± 722
Duration of elevated ALT (weeks)[c]	7.9 ± 5.8	9.5 ± 6.2	8.8 ± 3.7[j]	6.0 ± 2.5	10.1 ± 7.5	7.7 ± 5.1	5.7 ± 2.1	9.8 ± 1.5
Total bilirubin (mg/dL)	14.1 ± 10.3	9.0 ± 7.2	9.3 ± 5.9	10.9 ± 9.0	11.0 ± 13.8	9.8 ± 10.7	8.2 ± 2.2	13.0 ± 7.8
HBV DNA (log copies/mL)								
Median	4.76	6.08[k]	5.15	4.93	5.61	4.94	5.91	5.97
(range)	(2.90-8.08)	(2.00-8.46)	(2.00-8.19)	(2.00-8.44)	(2.00-8.50)	(2.00-9.06)	(2.00-8.37)	(3.35-7.11)
<2.00 (undetectable)	0 (0%)	1 (3%)	2 (9%)	3 (14%)	2 (18%)	28 (15%)	1 (20%)	0 (0%)
Medication with								
Lamivudine	1 (10%)	9 (27%)	2 (9%)	5 (23%)	2 (18%)	28 (15%)	4 (80%)	2 (33%)
Steroid	0	3 (9%)	0	5 (23%)	1 (9%)	16 (8%)	0	0

[a]Patients with HBV genotype D or G were not included in the analysis.
[b]All patients with HBV genotype G were co-infected with HBV of another genotype; Ae in two, Ba in two, and Ce in two.
[c]Exclusive of the 16 patients who died of fulminant hepatitis, 3 receiving liver transplantation and 10 without clinical data available.
[d]$P = .0001$, Ae vs. Ba. $P < .01$, Ae vs. Aa. $P < .05$, Ae vs. Bj or Cs.
[e]$P < .05$, Ba vs. Ce.
[f]$P = .0001$, Ae vs. Bj. $P < .005$, Ae vs. Ce.
[g]$P < .005$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[h]$P < .005$, Ae vs. Bj. $P < .01$, Ae vs. Ce. $P < .05$, Ae vs. Ba.
[i]$P < .05$, Ae vs. Bj.
[j]$P < .01$, Ba vs. Bj. $P < .05$, Ba vs. Ce.
[k]$P < .005$, Ae vs. Ce. $P < .05$, Ae vs. Bj.

the peak ALT level tended to be high in patients with HBV/Bj.

Presumed infection routes of 301 patients were sexual transmission in 172 (57%), blood transfusion in 4 (1%), medical accidents in 17 (6%), and unknown in the remaining 108 (36%).

Clinical Outcome of Patients With Acute Hepatitis B. Fulminant hepatitis developed in 40 (13%) patients. To cope with severe acute liver disease, lamivudine and steroid were administered to 53 (18%) and 25 (8%) patients, respectively. Fulminant hepatitis led to death in 16 (5%) patients, and three (1%) received liver transplantation. Exclusive of the 40 patients with fulminant hepatitis who received various treatments and five without clinical data, 256 (85%) were followed for the chronic outcome (Fig. 1). Serum ALT levels stayed elevated for longer than 24 weeks for the diagnosis of chronic hepatitis in eight (3%) of them. Among them, five had cleared HBsAg from serum until then, and therefore, their liver function abnormality was not attributed to persistent HBV infection. Table 2 summarizes persistence of HBV infection in the 256 patients with acute hepatitis; 253 (99%) lost serum HBsAg by 6 months. Hence, HBV infection evolved into chronicity in only 3 of the 256 (1%) patients, representing 2 of the 32 (6%) infected with HBV/Ae and 1 of the 21 (5%) with Ba. All of the three with chronic outcome had low-titered IgG anti-HBc at the presentation, and two of them had been negative for HBsAg before the presentation. None of them had received lamivudine or steroid treatment during their acute phase of illness. Of the patients without antiviral therapy, chronic outcome was significantly more frequent in those infected with HBV/Ae than non-Ae genotypes (9% 2/23 vs. 0.5% 1/187, $P = .032$).

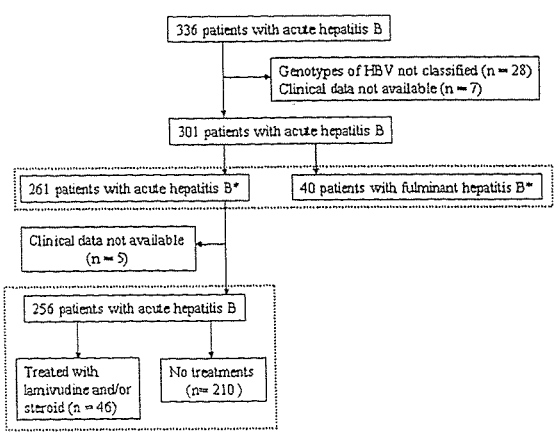

Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including 27 with acute and 10 with fulminant hepatitis.

Table 2. Persistence of HBV Infection in the Patients With Acute Hepatitis Who Did or Did Not Receive Lamivudine or Steroid

Treatment	Total	Genotypes/Subgenotypes							
		Aa (n = 8)[a]	Ae (n = 32)[a]	Ba (n = 21)[a]	Bj (n = 10)[a]	Cs (n = 10)[a]	Ce (n = 167)[a]	D (n = 3)[a]	G (n = 5)[a]
Total (n = 256)	3/256 (1.2%)	0	2/32 (6%)c	1/21 (5%)	0	0	0	0	0
Lamivudine (n = 36)[b]	0/36 (0%)	0/1 (0%)	0/9 (0%)	0/2 (0%)	0	0/1 (0%)	0/19 (0%)	0/2 (0%)	0/2 (0%)
Steroid (n = 16)[b]	0/16 (0%)	0	0/3 (0%)	0	0	0/1 (0%)	0/12 (0%)	0	0
Neither	3/210 (1.4%)	0/7 (0%)	2/23 (9%)c	1/19 (5%)	0/10 (0%)	0/8 (0%)	0/139 (0%)	0/1 (0%)	0/3 (0%)

[a]Exclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.
[b]Six patients received steroid along with lamivudine.
[c]$P < .05$, Ae vs. non-Ae.

Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis. Table 3 compares demographic, clinical, and virological characteristics between the 40 patients with fulminant and the 261 with acute self-limited hepatitis for whom analysis was feasible. Patients with fulminant hepatitis were significantly older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P = .0017$), less predominantly male (43% vs. 71%, $P = .0005$) and less often positive for HBeAg (23% vs. 60%, $P < .0001$) than those with acute hepatitis. Peak ALT and total bilirubin levels were higher for fulminant than acute hepatitis ($P < .0001$), reflecting severe hepatic lesions. Notably, the median HBV DNA level was lower in patients with fulminant than acute hepatitis (4.89 vs. 5.19 log copies/mL, $P = .0178$); the frequency of undetectable HBV DNA at the presentation was higher in fulminant hepatitis (25% vs. 10%, $P = .0086$). Lamivudine or steroid was given significantly more often to patients with fulminant hepatitis.

There were marked differences in the distribution of genotypes between patients with fulminant and acute hepatitis. HBV/Ae was less frequent (0% vs. 13%, $P = .0121$), whereas Bj was more often (30% vs. 4%, $P < .0001$) in patients with fulminant than acute hepatitis. Although HBV/Ce tended to be less frequent in patients with fulminant than acute hepatitis (55% vs. 65%), the difference fell short of being significant.

Precore stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were more

Table 3. Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis Who Were Infected With HBV

Features	Fulminant (n = 40)	Acute (n = 261)	*P* Value
Age (years)	44.7 ± 16.3	36.0 ± 14.3	.0017
Men	17 (43%)	186 (71%)	.0005
HBeAg positive	9 (23%)	157 (60%)	<.0001
ALT (IU/L)	4315 ± 2889	2284 ± 1221	<.0001
Total bilirubin (mg/dL)	20.5 ± 16.4	8.3 ± 7.3	<.0001
HBV DNA (log copies/mL)			
Median	4.89	5.19	.0178
(range)	(2.00-8.44)	(2.00-9.06)	
<2.00 (undetectable)	10 (25%)	27 (10%)	.0086
Treatment			
Lamivudine	16 (40%)	37 (14%)	.0003
Steroid	9 (23%)	16 (6%)	.0022
Genotypes/subgenotypes			
Aa	1 (2.5%)	9 (3%)	NS
Ae	0 (0%)	33 (13%)	.0121
Ba	1 (2.5%)	21 (8%)	NS
Bj	12 (30%)	10 (4%)	<.0001
Cs	1 (2.5%)	10 (4%)	NS
Ce	22 (55%)	170 (65%)	NS
D	2 (5%)	3 (1%)	NS
G	1 (2.5%)	5 (2%)	NS
Mutations[a]			
nt 1753 and/or nt1754[b]	11/30 (37%)	28/234 (12%)	.0003
A1762T/G1764A	15/30 (50%)	39/234 (17%)	<.0001
G1896A	16/30 (53%)	21/234 (9%)	<.0001
G1899A	7/30 (23%)	8/234 (3%)	<.0001

[a]Exclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.
[b]T1753C/A/G and/or T1754C/A/G.

Table 4. Multivariate Analysis for Factors Independently Associated With Fulminant Hepatitis

Factors	Odds Ratio	95% Confidence Interval	*P* Value
Age (yr)			
<34[a]	1		
≥34	3.472	1.094-11.023	.0347
Sex			
Male	1		
Female	2.272	0.780-6.613	.1323
HBeAg			
Positive	1		
Negative	3.344	1.065-10.506	.0387
ALT (IU/L)			
<2200[a]	1		
≥2200	2.094	0.683-6.414	.1957
Total bilirubin (mg/dL)			
<10.0[a]	1		
≥10.0	18.818	4.320-81.980	<.0001
HBVDNA (log copies/mL)			
<5.00[a]	1		
≥5.00	1.042	0.367-2.961	.9383
Treatment			
Lamivudine (−)	1		
Lamivudine (+)	2.650	0.814-8.625	.1056
Steroid (−)	1		
Steroid (+)	2.515	0.668-9.472	.1728
Genotypes/Subgenotypes			
Non-Bj	1		
Bj	7.001	1.737-28.228	.0062
Mutations			
nt 1753 and/or 1754[b]			
Absent	1		
Present	2.316	0.698-7.683	.1700
A1762T/G1764A			
Absent	1		
Present	1..013	0.295-3.478	.9841
G1896A			
Absent	1		
Present	4.157	1.265-13.657	.0189
G1899A			
Absent	1		
Present	2.525	0.534-11.949	.2427

[a]Median values.
[b]T1753C/A/G or T1754C/A/G.

frequent in patients with fulminant than acute hepatitis (53% vs. 9% and 50% vs. 17%, respectively, $P < .0001$ for each). Likewise, mutations in core-promoter at nt 1753 or nt 1754, and G1899A mutation were more frequent in patients with fulminant than acute hepatitis ($P = .0003$ and $P < .0001$, respectively).

Factors Independently Associated With the Development of Fulminant Hepatitis. Various factors found in association with fulminant hepatitis were evaluated for the independence in multivariate analysis (Table 4). Age 34 years or older (odds ratio 3.47 [95% confidence interval 1.09-11.02], $P = .035$), HBV/Bj (7.00 [1.74-28.23], $P = .006$), HBeAg-negative (3.34 [1.07-10.51], $P = .039$), total bilirubin ≥10.0 mg/dL (18.82 [4.32-81.98], $P < .0001$) and G1896A (4.16 [1.27-13.66], $P = .019$) were independent risk factors for the development of fulminant hepatitis.

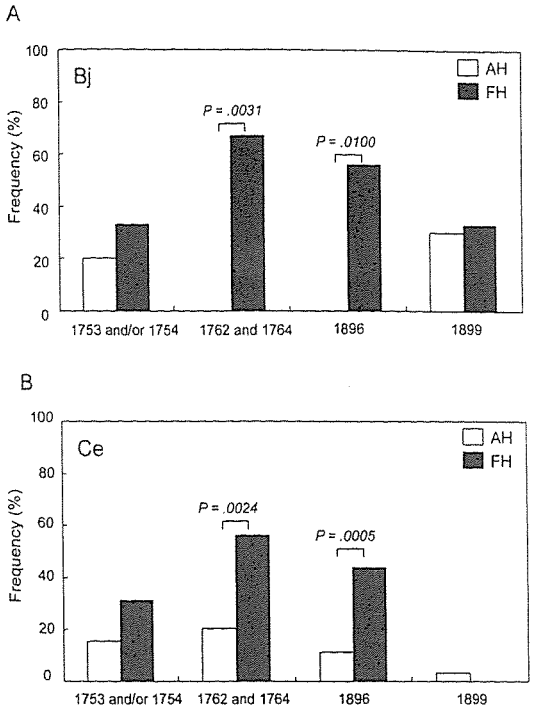

Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

In view of the majority of Japanese patients who were infected with Bj or Ce, mutations in the precore region and core-promoter were compared between those with fulminant and acute self-limited hepatitis for each subgenotype (Fig. 2). G1896A and A1762T/G1764A were significantly more frequent in patients with fulminant than acute hepatitis infected with either HBV/Bj or Ce (56% vs. 0% and 67% vs. 0% for Bj or 44% vs. 11% and 56% vs. 22% for Ce, respectively, $P \leq .01$ for all). For the patients infected with HBV/Bj, in particular, precore and core-promoter mutations were highly frequent in those with fulminant hepatitis (56% and 67%, respectively), whereas they occurred in none of those with acute hepatitis. G1899A was equally frequent in both patients with fulminant and acute hepatitis infected with HBV/Bj; it was rarely seen in those with Ce. Mutations involving nt 1753 or nt 1754 tended to be more frequent in patients with fulminant than acute hepatitis.

***Replication of the Wild-Type HBV as Well as Precore and Core-Promoter Mutants* In Vitro.** Full-length HBV DNA of the wild-type HBV/Bj from a patient with chronic hepatitis B was incorporated with G1896A or A1762T/G1764A mutation *in vitro*. Another plasmid was constructed with HBV/Bj_58 carrying G1896A from a fulminant patient. Figure 3 compares

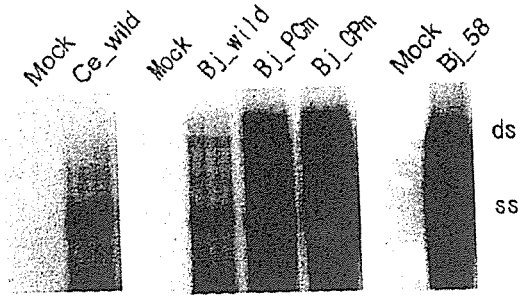

Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

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.

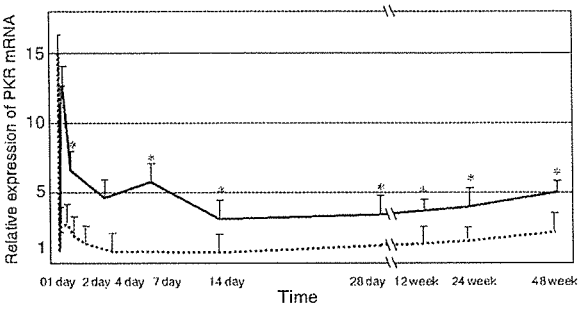

Fig. 1 Sequential expression of PKR mRNA in PBMCs during PEG- (solid line) and conventional (dotted line) IFN alpha-2b and ribavirin combination therapy. Expression of mRNA is shown as the expression level relative to baseline expression. The error bars indicate the standard error. An asterisk indicates 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	–	–

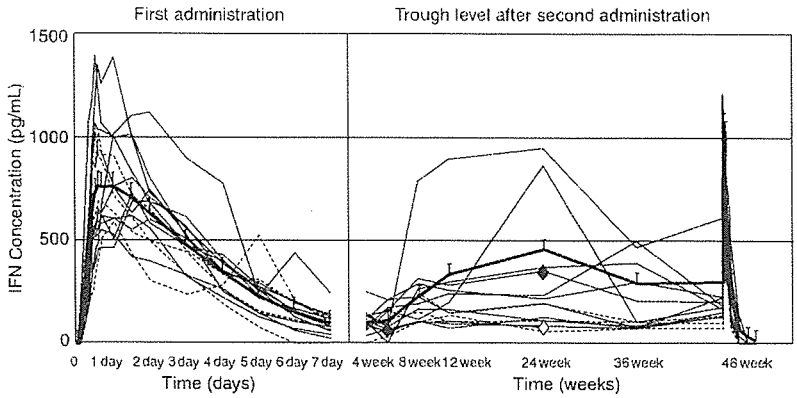

Fig. 2 Changes in serum IFN levels during PEG-IFN alpha-2b and ribavirin combination therapy. No significant increase in the trough value of serum IFN level was found during the 48-week treatment period. The bold lines indicate mean values, while the error bars indicate the standard error. Fine solid 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.

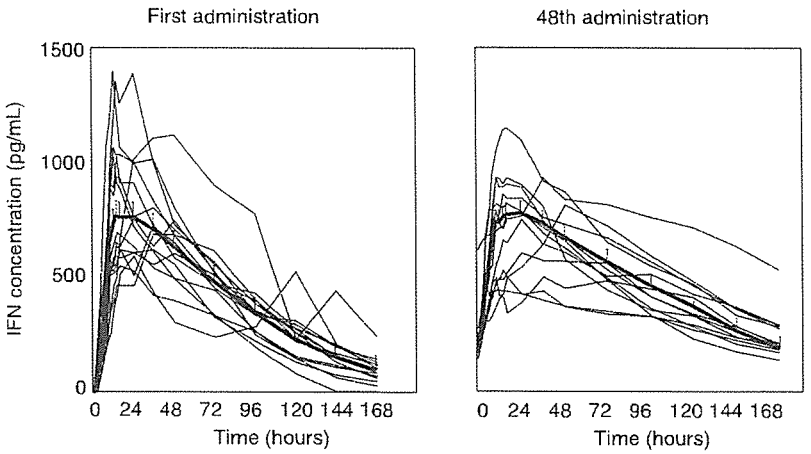

Fig. 3 A comparison of serum IFN levels between the first and 48th doses. Both show very similar values and no accumulation of IFN. It should be noted that PEG-IFN alpha-2b was detectable in all but one patient at 168 h after initial administration. Bold lines indicate mean values and the error bars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

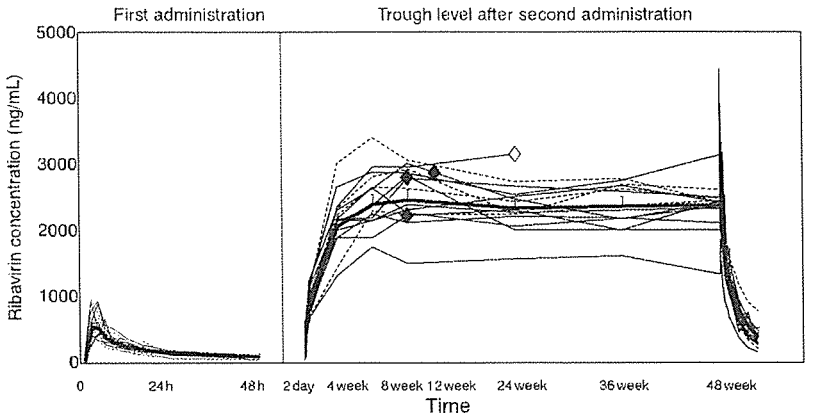

Fig. 4 Changes in serum ribavirin levels during PEG-IFN alpha-2b and ribavirin combination therapy. Serum ribavirin levels reached a peak by the eighth week and then plateaued. Bold lines indicate mean values and the error bars indicate the standard error. Fine solid lines indicate a sustained virological 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

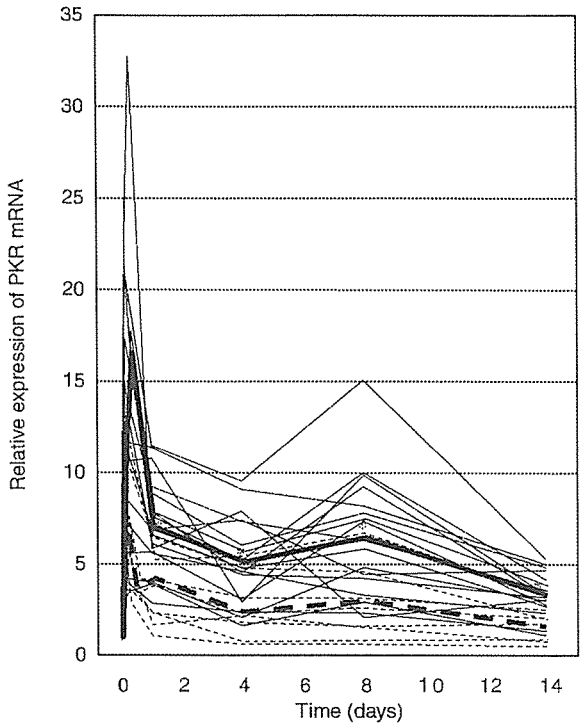

Fig. 5 Sequential expression of PKR mRNA in PBMCs in sustained viral responders (solid line, $n = 18$) and nonresponders (dotted line, $n = 8$). The bold line indicates the mean value for each group. Expression of mRNA is shown as the expression level relative to baseline expression. An asterisk indicates 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

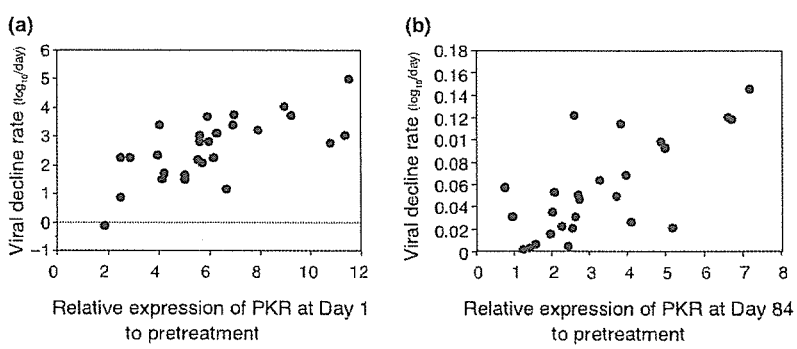

Fig. 6 (a) Significant correlation between expression of PKR mRNA at day 1 and viral decline rate calculated from the first phase of HCV dynamics ($r = 0.67$, $P = 0.0006$). (b) Significant correlation between expression of PKR mRNA at day 84 and viral decline rate calculated from the second phase of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

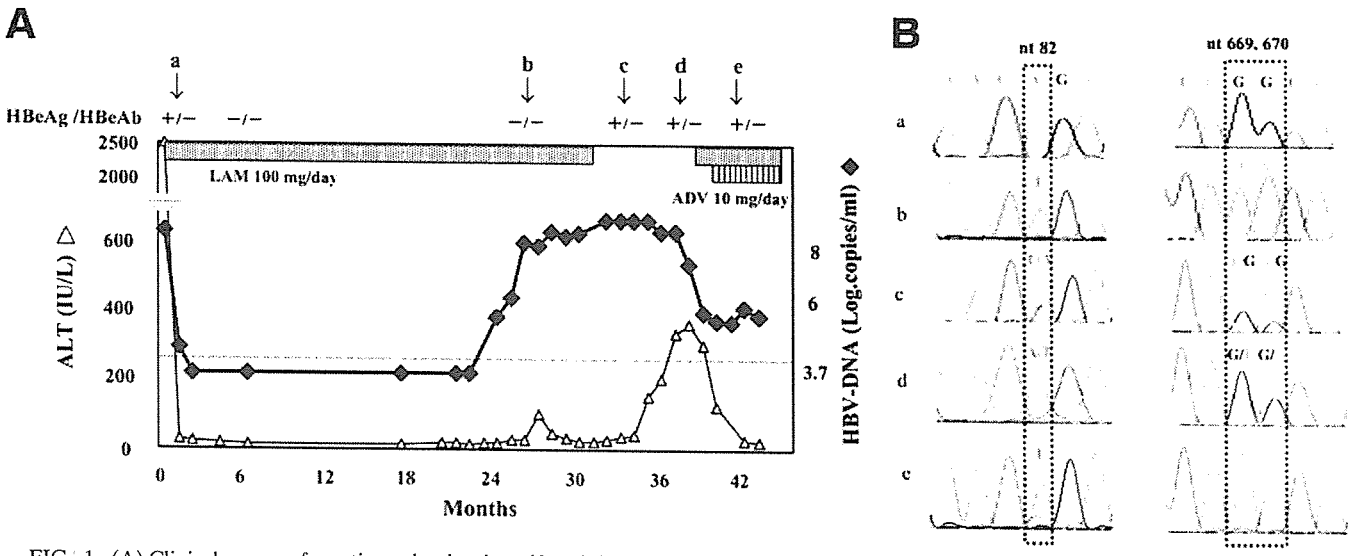

FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis 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.

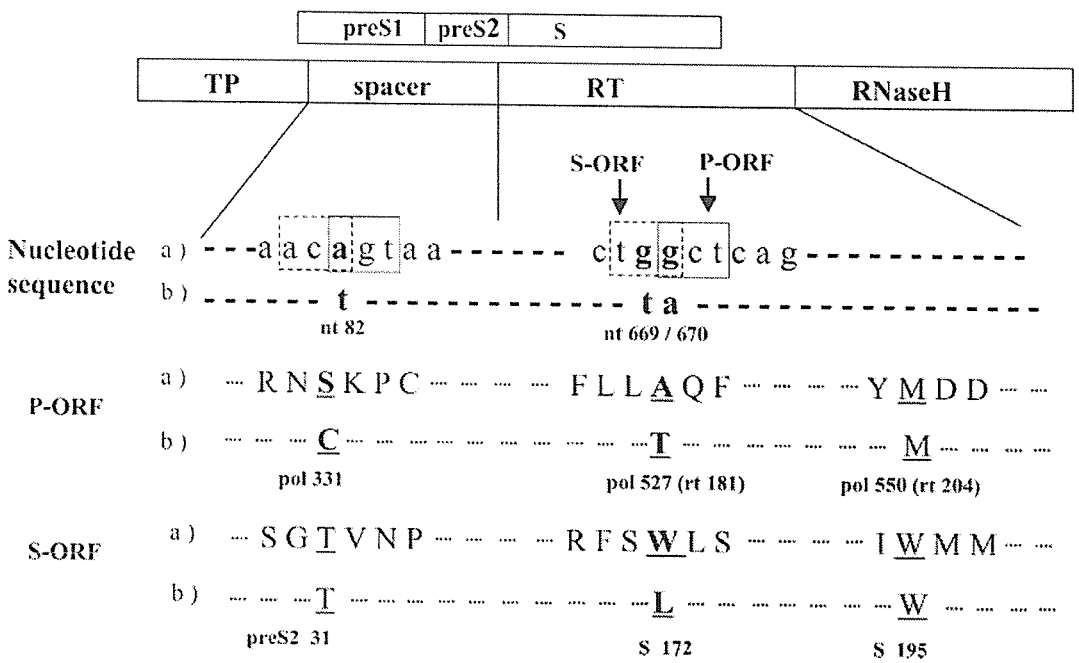

FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were 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.