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 noncategorical 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, $P = .032$).

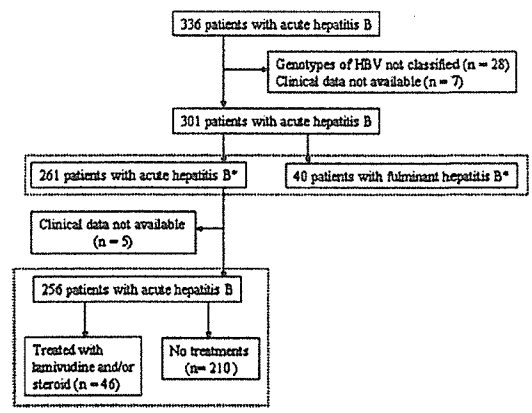

Fig. 1. A flow diagram of 336 patients studied. Comparison was made between patients with fulminant and acute self-limited hepatitis (upper dotted area), and the chronicity was compared between patients with and without treatments (lower dotted area). *Of 301 patients, 37 were negative for HBV DNA, including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

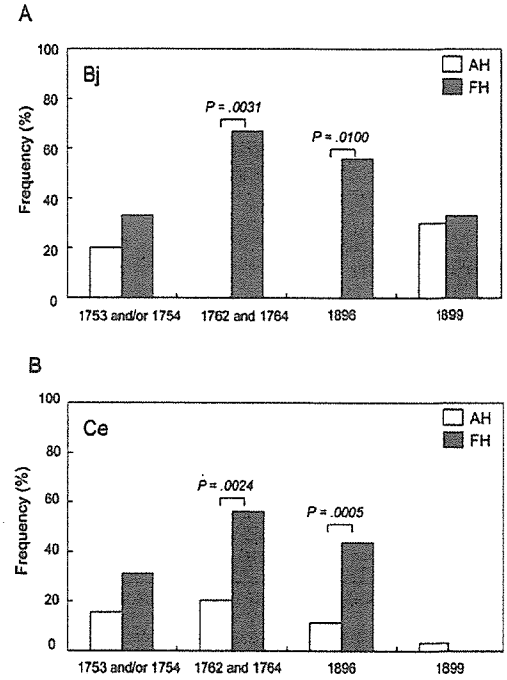

Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

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.

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

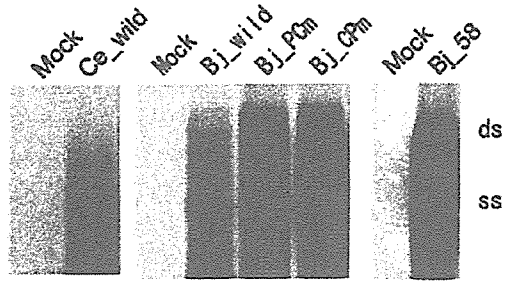

Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

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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Journal of Clinical Virology 35 (2006) 250–256

JOURNAL OF CLINICAL VIROLOGY

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Association of virus infected-T cell in severe hepatitis caused by primary Epstein-Barr virus infection

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Received 13 April 2005; received in revised form 7 July 2005; accepted 12 July 2005

Abstract

Background: Infectious mononucleosis owing to primary Epstein-Barr virus (EBV) infection sometimes causes hepatitis, which is usually self-limiting with mildly elevated transaminases, but can rarely develop into severe hepatitis with jaundice.
Objective: To clarify the pathogenesis of severe hepatitis by primary EBV infection.
Methods: We experienced four cases of severe hepatitis with jaundice caused by primary EBV infection. These cases were analyzed virologically and histologically, and compared with infectious mononucleosis patients without jaundice.
Results and discussion: Using real-time PCR, more EBV-DNA was detected in peripheral blood from patients with severe hepatitis, as compared to those without jaundice. Furthermore, $CD3^+$, $CD4^+$ or $CD8^+$ cells contained more EBV DNA than did other cell populations, indicating that in severe hepatitis, T cells harbor most of the EBV. By contrast, mainly B cells were infected in infectious mononucleosis patients without jaundice. The liver was biopsied in three of the four cases. An in situ hybridization study showed that EBV infected lymphocytes, not hepatocytes. In addition, in one patient, it was confirmed that the infected lymphocytes were $CD8^+$ T cells. These results suggest that a large EBV burden and T cell infection may play major roles in the mechanism of severe hepatitis caused by primary EB virus infection.

Keywords: EBV; Primary infection; Hepatitis; T cells

1. Introduction

Epstein-Barr virus (EBV) is ubiquitous in humans and is the causative agent of infectious mononucleosis. Although primary EBV infection in children is usually asymptomatic, some children or young adults manifest infectious mononucleosis with typical symptoms of fever, pharyngitis, lymphadenopathy, hepatosplenomegaly and atypical lymphocytosis (Sumaya and Ench, 1985). Infectious mononucleosis is caused by an intense cytotoxic T lymphocyte response to eliminate EBV-infected B cells (Rickinson and Kieff, 2001). This disease is usually benign, and self-limiting. In the acute phase of infectious mononucleosis, elevated transaminases are found in 80% of patients, while jaundice is noted in only 5.0–6.6% (Markin, 1994; White and Juel-Jensen, 1984).

Abbreviations: ALT, alanine aminotransferase; EBER1, Epstein-Barr encoded small RNA 1; EBNA, Epstein-Barr nuclear antigen; EBV, Epstein-Barr virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein; PBMCs, peripheral blood mononuclear cells; VCA, viral capsid antigen; WBC, white blood cells

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doi:10.1016/j.jcv.2005.07.009

Hepatitis owing to primary EBV infection is usually mild and self-limited, although the mechanism is unclear. Rarely, it results in hepatic failure with severe jaundice in fatal infectious mononucleosis (Markin et al., 1987; Tazawa et al., 1993).

We previously reported on one severe hepatitis patient with primary EBV infection. In that case, EBV did not infect hepatocytes, but infected T cells (Kimura et al., 2001b). In this study, we report three additional cases of severe hepatitis with jaundice caused by primary EBV infection. We performed virological and histological studies of the four cases, and compared them with results for infectious mononucleosis patients without jaundice.

2. Patients and methods

2.1. Patients

Four female patients, aged 2–22 years old (mean age: 15.5 years) with severe hepatitis caused by EBV, were enrolled in this study (Table 1). Severe hepatitis was defined on the basis of clinical jaundice. All four were previously healthy and had no prior serious infections, and none had contributory family histories. Two were hospitalized in the University Hospital of Nagoya University Graduate School of Medicine, one in the University Hospital of Okayama University Graduate School of Medicine, and one University Hospital of Iwate Medical University. They all had several clinical symptoms typical of patients with infectious mononucleosis, including fever, lymphadenopathy, pharyngitis and hepatosplenomegaly. The percentage of atypical lymphocytes exceeded 20% in three patients and that of normal and atypical lymphocytes exceeded 60% of the white blood cells (WBC) in all of them (Table 1). The Paul-Bunnell test was performed on serum samples from two patients, but was negative. In the EBV-specific serology, viral capsid antigen (VCA)-IgG antibody was positive and EB nuclear antigen (EBNA) antibody was negative in all cases. VCA-IgM was positive in three cases, indicating the presence of primary EBV infection. Antibodies against hepatitis viruses A, B and C, and human immunodeficiency virus were negative. Cytomegalovirus DNA was not detected in their blood.

Table 1
Characteristics of the patients with severe hepatitis

	Case 1	Case 2	Case 3	Case 4
Age	2	17	22	21
Sex	F	F	F	F
Symptoms				
Fever	+	+	+	+
Lymphadenopathy	–	+	+	+
Pharyngitis	+	–	+	+
Hepatosplenomegaly	+	+	+	+
Rash	–	–	–	–
Laboratory data				
WBC count (μl^{-1})	14700	2400	16000	12700
Lymphocytes (%)	64	43	27	53
Atypical lymphocytes (%)	0	27	44	21
Hemoglobin (g/dl)	11	13	12	14
Platelet count ($\times 10^4$ μl^{-1})	13.9	6.5	7.4	18.6
EBV-related antibody				
Anti-VCA-IgG	2560	160	1280	1280
Anti-VCA-IgM	<10	10	20	40
Anti-EBNA	<10	<10	<10	<10
Viral load at peak				
PBMCs (copies/μg DNA)	13000	470[a]	3700	1920[a]
Plasma (copies/ml)	530	290000	32000	43

Note: WBC, white blood cells; VCA, viral capsid antigen; EBNA, Epstein-Barr nuclear antigen; PBMCs, peripheral blood mononuclear cells.
[a] PBMCs were not available in their acute phase.

Each patient received different treatments. In case 1, acyclovir was administered intravenously without clinical improvement, then vidarabine was administered, which led to improvement. Administration of methylprednisolone pulse therapy combined with acyclovir led to clinical improvement in case 3. Case 4 was treated with γ-globulin and acyclovir without clinical improvement; subsequent administration of methylprednisolone pulse therapy led to a remarkable clinical improvement. Glycyrrhizic acid was administered to all of the patients. Cases 1 and 3 were hospitalized for about 1 month, while cases 2 and 4 were hospitalized for about 2 months. They were all discharged without sequelae.

2.2. Controls

As controls, 21 infectious mononucleosis patients without clinical jaundice were enrolled. They aged 1–21 years old (mean age: 6.8 years). All fulfilled at least three of the following criteria for infectious mononucleosis: (1) fever, (2) tonsillopharyngitis, (3) cervical adenopathy, (4) hepatomegaly or splenomegaly, and a peripheral WBC count of (a) at least 50% or 5000 lymphocytes per microliter and (b) at least 10% or $\geq$1000 atypical lymphocytes per microliter. Moreover, they met one or more of the following serologic criteria: (1) early detection of VCA-IgM, (2) four-fold or greater VCA-IgG titer rise during the course of the disease and (3) early presence of VCA-IgG with early absence and later emergence of EBNA (Sumaya and Ench, 1985).

2.3. Samples

Blood samples were collected after obtaining informed consent from the patients or their parents. Plasma was separated from whole blood by centrifugation. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient with Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). Alanine aminotransferase (ALT), total bilirubin and EBV-DNA in plasma were examined sequentially. ALT was measured at no less than three points and EBV-DNA was quantified at no less than two points in all patients and controls.

2.4. Real-time quantitative PCR assay using a fluorogenic probe

Both PBMCs and plasma were used for the real-time PCR assay. DNA was extracted from either 1×10^6 cells or 200 μl of plasma using a QIAamp Blood Kit (Qiagen, Hilden, Germany) and eluted in 50 μl distilled water; 10 μl solution were used for the real-time PCR assay, which was performed with a TaqMan PCR kit and a Model 7700 Sequence Detector (Applied Biosystems, Foster, USA) as previously described (Kimura et al., 1999). The copy number of virus DNA was expressed as copies/μg DNA in PBMCs and copies/ml in plasma samples.

2.5. Determination of EBV-infected cells

The PBMCs from three severe hepatitis patients were fractionated into $CD3^+$, $CD16^+$ and $CD19^+$ cells using an immunobeads method (DynaBeads; Dynal AS, Oslo, Norway), as reported previously (Ito et al., 2001; Kimura et al., 2005, 2001a). In two cases, the PBMCs were further fractioned into $CD4^+$ and $CD8^+$ cells. As controls, the PBMCs obtained from six patients with infectious mononucleosis were fractionated into $CD3^+$, $CD16^+$ and $CD19^+$ cells. The purity of each subpopulation was confirmed by flow cytometry analysis (Ito et al., 2001). The immunomagnetic beads were detached from isolated cells by Detachabeads (Dynal AS). Aliquots of 1×10^5 isolated cells were incubated with Opticlone CD4-fluorescein isothiocyanate/CD8-phycoerythrin or CD3-fluorescein isothiocyanate/CD19-phycoerythrin or CD16-fluorescein isothiocyanate/CD56-phycoerythrin (Immunotech, Marseilles, France). Cell samples were analyzed on FACScan (Becton Dickinson, Mountain View, USA) using CellQuest software (Becton Dickinson). Live cell gating was performed and 5000 events were acquired for each analysis. All purity results were greater than 92% (range: 92–97%). EBV-DNA was quantified in all of the fractionated cells using real-time quantitative PCR. As an internal reference, human β-actin gene was also quantified in the fractionated cells using TaqMan β-actin Control Reagent (Applied Biosystems). The copy number of β-actin DNA was expressed as copies/μg DNA in the fractionated cells.

2.6. Histology

Liver tissue was fixed in 10% formalin, dehydrated in alcohol and embedded in paraffin. Samples were sectioned at 4-μm thickness. Routine hematoxylin- and eosine-stained sections were made for histologic examination. The in situ hybridization assay was performed using the Epstein-Barr encoded small RNA 1 (EBER 1; Dako A/S, Glostrup, Denmark), as previously described (Kimura et al., 2001b). 5-Bromo-4-chloro-3-indoyl phosphate (BCIP) was used for visualization. Immunostaining was performed using the streptavidin–biotin peroxidase complex method using monoclonal antibodies against CD45RO, CD4, CD8, CD20 and CD56 (Kimura et al., 2001b). Diaminobenzydine was used for visualization.

In one case, for double labeling with immunostaining and in situ hybridization, the immunohistochemical studies were performed before in situ hybridization using a previously published technique with monoclonal antibodies against CD4, CD8 and CD56 (Sheibani and Tubbs, 1984).

2.7. Statistical analysis

EBV DNA copy number was compared between the severe hepatitis group and infectious mononucleosis without jaundice group by using analysis of co-variance. Days after onset were used as covariate. Since viral load tends to change in a logarithmic fashion, statistical analysis was completed after logarithmic (base 10) transformation. Statistical analyses were performed by using software StatView 5.0.1 (SAS Institute Inc., Cary, USA).

3. Results

ALT was measured sequentially and compared between patients with severe hepatitis and those without jaundice (Fig. 1). In 4 of 21 patients without jaundice, ALT levels were normal throughout the clinical course. Peak ALT levels in patients with severe hepatitis ranged from 239 to 1426 (average: 886) IU/l, and were much higher than levels in patients without jaundice (range: 21–443, average: 117 IU/l). ALT levels peaked 5–11 (average: 7.3) days after the onset of the disease in patients with severe hepatitis as compared to 2–17 (average: 6.9) days after onset in control patients. In patients without jaundice, ALT levels decreased to normal within 1 month, whereas ALT levels remained elevated for more than 1 month in patients with severe hepatitis (Fig. 1). As Fig. 2 shows, total bilirubin was markedly elevated in the patients with severe hepatitis, peaking at 8.7–19.8 mg/dl 17–29 days after the onset of the disease. Jaundice lasted about 2 months.

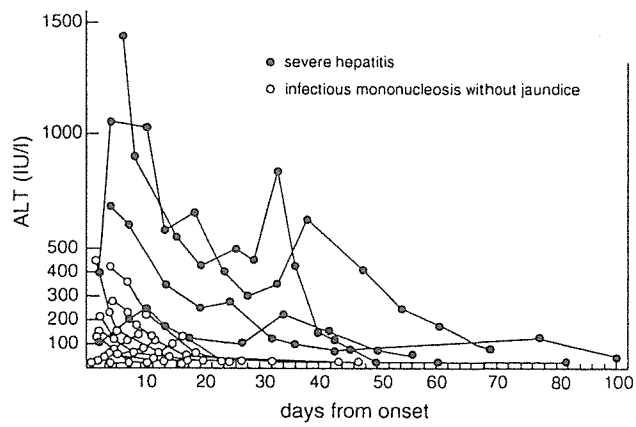

Fig. 1.

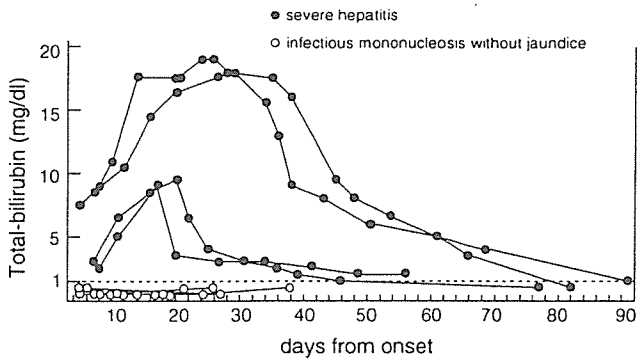

Fig. 2.

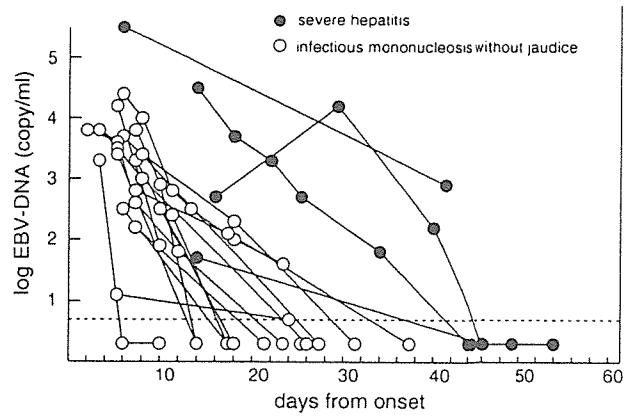

Fig. 3.

Fig. 3 shows sequential data on EBV-DNA in plasma. In infectious mononucleosis patients without jaundice, EBV-DNA copy numbers decreased and became undetectable within 1 month after onset. The mean copy number of EBV-DNA at the onset was $10^{3.3}$ (range: $10^{1.1-4.4}$) copies/ml. On the other hand, the mean copy number at the onset was $10^{4.0}$ (range: $10^{1.7-5.5}$) copies/ml in patients with severe hepatitis. Copy numbers were higher in patients with severe hepatitis and decreased more slowly than in patients without jaundice ($P = 0.02$ by analysis co-variance).

Infected cells were determined using fractionated PBMCs followed by quantitative PCR (Table 2). $CD3^+$, $CD4^+$ or $CD8^+$ cells contained more EBV-DNA than did other cell populations, indicating that EBV infected mainly T cells in patients with severe hepatitis. By contrast, $CD19^+$ cells harbored most of the EBV in infectious mononucleosis patients without jaundice, indicating that mainly B cells were infected. In parallel, human β-actin gene was quantified in the fractionated cells to investigate the quality and quantity of DNA (Table 2). The number of β-actin gene was similar among fractionated cell populations in each patient.

The liver was biopsied in three of the four patients with severe hepatitis. Table 3 summarizes the histological findings.

Table 3
Histological findings of the liver biopsy in patients with severe hepatitis

	Case 1	Case 2	Case 4
Histology			
Spotty necrosis	++	+	++
Lymphoid infiltration of the portal tract	++	+	+
Destruction of the limiting plate	+	+	–
Sinusoidal lymphoid infiltration	++	–	++
Fatty degeneration of liver cells	+	+	+
Cholestasis	+	+	–
Vasculitis	+	–	–
Fibrosis	–	–	–
EBER-1 in situ hybridization			
Lymphocytes	+	+	n.d.
Hepatocytes	–	–	n.d.

Note: EBER-1: Epstein-Barr-encoded small RNAs, n.d.: not done.

In all cases, mononuclear lymphocytes infiltrated the portal and lobular areas. Spotty necrosis of the liver parenchyma and fatty degeneration of liver cells were also seen in all. In the two cases, destruction of the limiting plate was noted. EBER-1 RNA was positive in infiltrating lymphocytes, but negative in hepatocytes in two patients in whom the in situ

Table 2
Determination of Epstein-Barr virus (EBV)-infected cells using peripheral blood mononuclear cells

	EBV-DNA copy number (copies/μg DNA)						β-Actin DNA copy number (log_{10} copies/μg DNA)					
	Unfractionated cells	Fractionated cells					Unfractionated cells	Fractionated cells				
		$CD3^+$	$CD4^+$	$CD8^+$	$CD16^+$	$CD19^+$		$CD3^+$	$CD4^+$	$CD8^+$	$CD16^+$	$CD19^+$
Severe hepatitis												
Case 1	8600	**12000**	7800	**14000**	n.d.	5400	5.9	5.4	5.0	5.3	n.d.	5.1
Case 2	470	**580**	n.d.	n.d.	250	360	5.6	5.8	n.d.	n.d.	6.0	5.7
Case 3	1700	60	**2800**	470	1400	710	5.4	5.8	5.0	5.9	5.7	5.5
Infectious mononucleosis without jaundice												
Case A	580	260	n.d.	n.d.	190	**1600**	5.6	5.7	n.d.	n.d.	5.8	5.9
Case B	1100	120	n.d.	n.d.	40	**2300**	6.0	6.2	n.d.	n.d.	6.3	6.9
Case C	280	310	n.d.	n.d.	450	**4400**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Case D	2800	760	n.d.	n.d.	1800	**3100**	5.5	5.7	n.d.	n.d.	5.8	5.9
Case E	270	130	n.d.	n.d.	270	**1300**	6.0	5.9	n.d.	n.d.	6.0	6.0
Case F	2900	1300	n.d.	n.d.	**3100**	**4300**	5.6	5.8	n.d.	n.d.	5.9	5.9

Note: n.d., not done. Fractions in bold letters indicate that EBV-DNA was concentrated after fractionation.

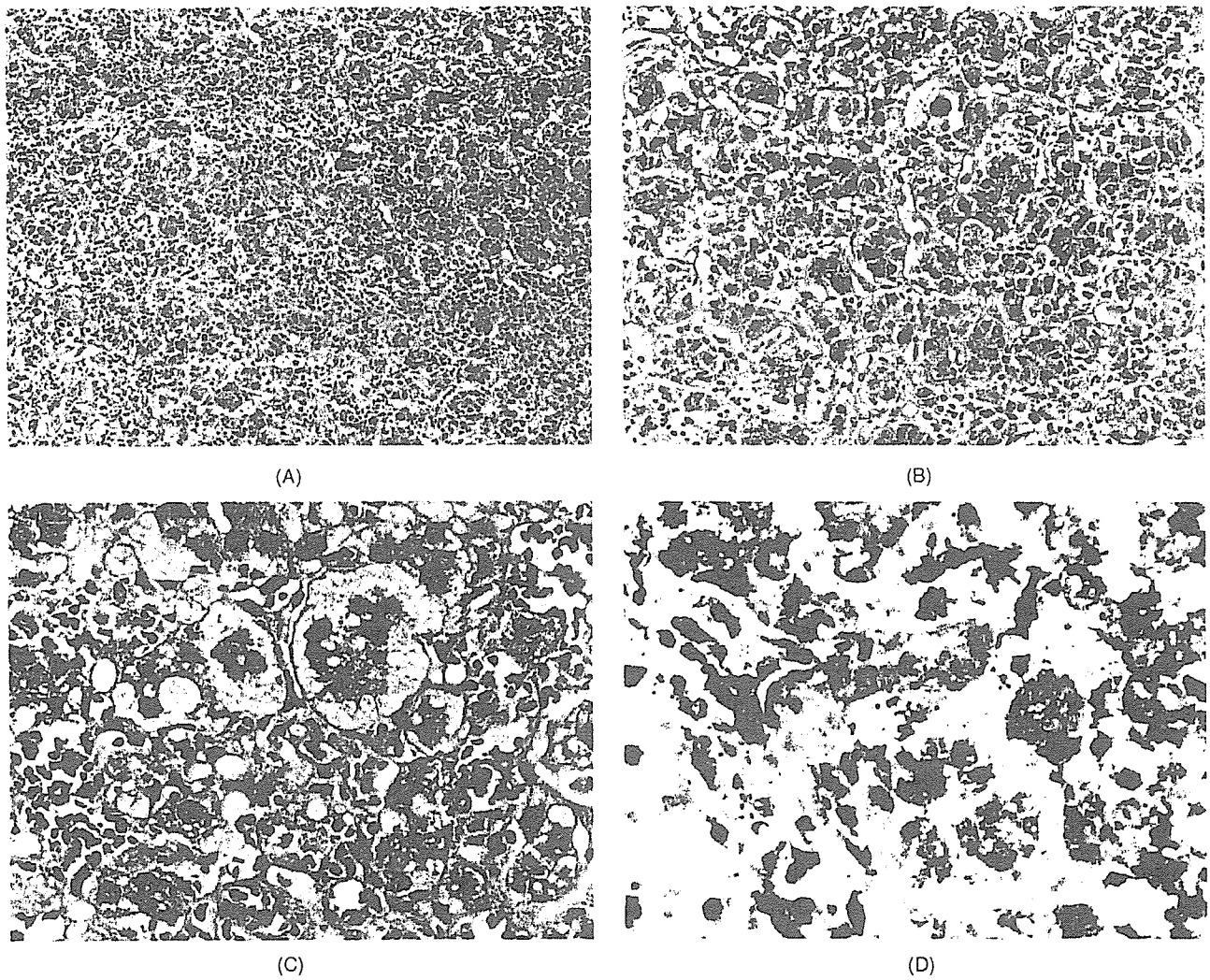

Fig. 4.

hybridization assay was performed. In case 1, double labeling with EBER-1 and surface marker analysis showed that most of the EBER-1-positive cells were $CD8^+$ T cells, as we previously reported (Kimura et al., 2001b). Histology of the case (Case 1) is shown in Fig. 4 as a representative case.

4. Discussion

We present four cases of severe hepatitis with primary EBV infection. Relative to infectious mononucleosis patients without jaundice, patients with severe hepatitis had higher, more prolonged elevation of ALT and total bilirubin levels and larger viral loads in their blood. It is unlikely that they had congenital or acquired immunodeficiency, as they were previously healthy and are now well after recovering from their hepatitis.

In infectious mononucleosis, B cells harbor EBV (Rickinson and Kieff, 2001). We found that in severe hepatitis patients, EBV infected mainly T cells, whereas in patients without jaundice EBV infected B cells. Peripheral blood or bone marrow T cells harbor EBV in life-threatening infectious mononucleosis (Baumgarten et al., 1994; Iijima et al., 1992), EBV-associated hemophagocytic syndrome (Kawaguchi et al., 1993; Su et al., 1994) and chronic active EBV infection (Kimura et al., 2001a; Quintanilla-Martinez et al., 2000; Yuge et al., 2004), all of which have high mortality rates, with multiple organ failure, including hepatic failure. Many papers concerning these EBV-associated severe diseases have emanated from the East Asia. The genetic backgrounds of East Asia people may be associated with the functions of virus-specific or non-specific lymphocytes that allow the expansion of EBV-infected T cells. On the one hand, EBV-infected T cells may escape the host's immunity and thereby proliferate, because they express fewer and less antigenic viral proteins than do B cells (Rickinson and Kieff, 2001).

In this study, we used immunomagnetic cell isolation to fractionate PBMCs, followed by quantitative PCR. Although this method is very convenient and rapid, its disadvantage is the relatively low purity of the selected cells. EBV-infected

cells can contaminate uninfected cell fractions, and the real-time PCR assay is sufficiently sensitive to detect the contaminating genome. Therefore, this method can be used only to determine the main infected cell population. The low levels of EBV-DNA seen in other populations do not always indicate that they are infected with EBV (Kimura et al., 2005). For example, 120 copies of EBV-DNA/μg DNA was detected in the $CD3^+$ fraction from case B (Table 2). The number nearly equals to that of 5% of the $CD19^+$ fraction (2300 copies/μg DNA). If 5% of B cells contaminated the $CD3^+$ fraction, 120 copies of EBV DNA/μg DNA would be detected in T cells. Thus, EBV DNA in the $CD3^+$ or $CD16^+$ fraction does not mean that T or NK cells are infected with EBV in infectious mononucleosis. Another drawback in this method is that the immunomagnetic sorting can be incomplete if surface expression of targeted antigens was decreased. It is possible that some surface marker molecules might be down-regulated in EBV-infected cells. In fact, there was a discrepancy between the $CD3^+$ cell fraction and the $CD4^+$ cell fraction in case 3.

The histological features of acute infectious mononucleosis are characterized by a mild increase in portal inflammation that consists primarily of lymphocytes (Markin, 1994). The bile ducts, hepatic arterioles and portal venules are usually unaffected. The hepatic parenchyma may show mild hepatocellular ballooning (Markin, 1994). In our cases of severe hepatitis caused by primary EBV infection, spotty necrosis of the liver parenchyma was seen in all patients and destruction of the limiting plate was seen in two patients. These findings indicate that the liver was more inflamed in severe hepatitis than in infectious mononucleosis without jaundice. Interestingly, the in situ hybridization assay showed that lymphocytes, not hepatocytes, were infected with EBV. Furthermore, the cells were $CD8^+$ T cells, which is in accord with the results for peripheral blood.

The mechanism of EBV-induced hepatitis remains unclear. T cell infection might be associated with the pathogenesis of severe hepatitis, although this might be a bystander event associated with more severe infection or genetic predisposition. A recent animal model showed that activated $CD8^+$ T cells are trapped in the liver selectively, primarily by intracellular adhesion molecule 1, which is expressed constitutively on sinusoidal endothelial cells and Kupffer cells (Mehal et al., 1999). In EBV-associated hepatitis, EBV-infected $CD8^+$ T cells, presumably activated T cells, may accumulate in the liver. A series of experiments showed that certain soluble products of the immune response, especially interferon γ, tumor necrosis factor α and Fas ligand, induce hepatitis (Bradham et al., 1998; Kondo et al., 1997; Kusters et al., 1996). In EBV-associated hepatitis, these products, which are produced by either EBV-infected $CD8^+$ T cells or infiltrating cytotoxic T lymphocytes, may induce hepatocyte injury. Although further studies are necessary to clarify the precise mechanisms, our observations suggest that a large EBV burden and T cell infection play major roles in severe hepatitis following primary EBV infection.

Acknowledgment

This work was supported by a grant (16591022) from the Ministry of Education, Culture, Sport, Science and Technology of Japan.

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Journal of Medical Virology 78:476-484 (2006)

Analysis of the Full-Length Genome of Genotype 4 Hepatitis E Virus Isolates From Patients With Fulminant or Acute Self-Limited Hepatitis E

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It was suggested that hepatitis E virus (HEV) genotype 4 is associated more closely with the severity of hepatitis E than genotype 3, although the virological basis remains unknown. The aim of this study was to examine whether genomic differences among genotype 4 HEVs are responsible for the development of fulminant hepatitis. Full-length sequences of genotype 4 HEVs from three patients with fulminant hepatitis and six patients with acute self-limited hepatitis were determined. The sequences were analyzed with those of 13 genotype 4 HEV isolates whose entire nucleotide sequence is known. Analysis of 22 full-length sequences (fulminant hepatitis, 5; acute hepatitis, 17) revealed that C at nt 1816 and U at nt 3148 (U3148), both of which do not change the amino acid sequences, were significantly associated with fulminant hepatitis ($P=0.0489$, respectively). When partial nucleotide sequences containing nt 1816 or nt 3148 were determined in 16 additional HEV isolates of genotype 4, a closer association between U3148 and fulminant hepatitis ($P=0.0018$) was observed. The comparison of 86 HEV isolates of all four genotypes showed that U3148 had a stronger association with fulminant hepatitis than other nucleotides at nt 3148 ($P=0.0006$). Patients infected with HEV with U3148 had a significantly lower value of the lowest prothrombin activity ($P=0.0293$). Nt 3148 is located within the RNA helicase domain, and 22-nt sequence including nt 3148 was well conserved among all genotypes. A silent substitution of U3148 in HEV may be associated with the development of fulminant hepatitis. Further studies are needed to clarify the underlying mechanism. ***J. Med. Virol. 78:476-484, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: hepatitis E virus; fulminant hepatitis; full-length genome; genotype; silent mutation

INTRODUCTION

Hepatitis E virus (HEV) is a major cause of epidemic and sporadic hepatitis in many developing countries [Purcell and Emerson, 2001]. Recently, sporadic cases of HEV infections have been reported in industrialized countries [Harrison, 1999; Schlauder and Mushahwar, 2001; Smith, 2001; Okamoto et al., 2003], where zoonotic transmission of HEV has been suggested; animals such as swine serve as reservoirs for HEV [Meng, 2003; Tei et al., 2003; Yazaki et al., 2003]. HEV is currently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* [Emerson et al., 2004a; http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_hepev.htm]. The genome of HEV is a single-stranded, positive-sense RNA of approximately 7.2 kb and consists of a short 5′-untranslated region, three partially overlapping open reading frames (ORF1, ORF2, and ORF3), and a short

The nucleotide sequence data reported in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB220971–AB220979 (full-length sequences) and AB221706–AB221758 (partial ORF1 sequences).

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant sponsor: Ministry of Health, Labor, and Welfare of Japan.

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Accepted 15 December 2005

DOI 10.1002/jmv.20565

Published online in Wiley InterScience (www.interscience.wiley.com)

3′-untranslated region terminated by a poly(A) tract [Tam et al., 1991; Wang et al., 2000]. Based on sequence analysis, HEV sequences have been classified into four major genotypes (1–4). Genotype 1 is the main cause of hepatitis E in developing countries in Asia and Africa, and genotype 2 has been documented in Mexico and Nigeria. Genotype 3 or 4 has been described in the United States, European countries, China, Taiwan, and Japan [Schlauder and Mushahwar, 2001; Mizuo et al., 2002].

Infection of HEV induces self-resolving hepatitis or a subclinical state in most cases, but it can cause fulminant hepatitis. It was reported that HEV infection is a major cause of fulminant hepatitis in endemic areas for HEV [Nanda et al., 1994; Coursaget et al., 1998; Sheikh et al., 2002]. Patients with fulminant hepatitis have been reported in an industrialized country [Suzuki et al., 2002]. Recent observations suggest that the HEV genotype influences the severity of hepatitis E, and that genotype 4 is associated more strongly with the severe form of hepatitis E than genotype 3 [Mizuo et al., 2005]. To date, nine HEV-associated fulminant hepatitis cases have been reported in Japan, and seven of them were infected with genotype 4 HEV [Suzuki et al., 2002; Ohnishi et al., 2003; Takahashi et al., 2003; Yazaki et al., 2003; Kato et al., 2004; Takahashi et al., 2005], although a larger number of patients are infected with genotype 3 than genotype 4 in Japan [Okamoto et al., 2003]. These observations suggest that there is a close relationship between infection with genotype 4 HEV and progression to fulminant hepatitis, which prompted us to investigate whether genomic differences among HEVs are responsible for the development of fulminant hepatitis. The full-length genome of genotype 4 HEV in patients with fulminant hepatitis and in those with acute self-resolving hepatitis was determined.

MATERIALS AND METHODS

Serum Samples

Sera collected between 1998 and 2004 in Japan from three patients with type E fulminant hepatitis and six patients with the mild form of type E acute hepatitis, who had a lowest prothrombin activity of ≥80%, were used for full-length sequencing of HEV. Clinical characteristics and laboratory data of the three patients with fulminant hepatitis (isolate names: HE-JF3, HE-JF4, and HE-JF5) [Suzuki et al., 2002; Yazaki et al., 2003] and six acute hepatitis patients (isolate names: HE-JA2, HE-JA19, HE-JA28, HE-JA36, HE-JA37, and HE-JA41) [Mizuo et al., 2002, 2005; Yazaki et al., 2003] were reported previously.

Serum samples collected between 1993 and 2004 from 20 patients with genotype 3 HEV and 16 patients with genotype 4 HEV were used for partial sequencing of ORF1 of HEV. One each of the patients with genotype 3 or 4 was diagnosed with type E fulminant hepatitis whose isolate was designated as HE-JF2 and HE-JF1, respectively [Suzuki et al., 2002]. The remaining 34 patients were diagnosed as having type E acute hepatitis [Mizuo et al., 2002, 2005; Kuno et al., 2003; Yajima et al., 2003; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Hijioka et al., 2005].

Amplification of Full-Length HEV Genome

Total RNA was extracted from 120 to 300 μl of serum, and subjected to cDNA synthesis and nested polymerase chain reaction (PCR) for six overlapping regions excluding the extreme 5′- and 3′-terminal regions; the amplified regions were nt 37–1199 (1163 nt) (primer sequences excluded), nt 991–3148 (2158 nt), nt 3029–4603 (1575 nt), nt 4401–5325 (925 nt), nt 5240–5998 (759 nt), and nt 5985–7142 (1158 nt).

The 5′-end sequence (nt 1–60) was determined by a modified rapid amplification of cDNA ends (RACE) technique called RNA ligase-mediated RACE (RLM-RACE) with the First Choice RLM-RACE kit (Ambion, Austin, TX), as described previously [Okamoto et al., 2001]. Amplification of the 3′-end sequence (nt 7071–7240: excluding poly [A] tail) was attempted by the RACE method as described previously [Okamoto et al., 2001].

Amplification of Partial Nucleotide Sequences of ORF1 Including Nt 1816 and 3148

Total RNA extracted from 50 μl of serum was reverse-transcribed and subjected to PCR with genotype-specific primers. To amplify the nt 1543–2086 sequence (primer sequences excluded) of genotype 4 HEV, first round PCR was performed with primers HE296 and HE269, and second round PCR was performed with primers HE297 and HE298 (Table I). To amplify the nt 2827–3286 sequence of genotype 4 HEV, HE299 and HE302 were used for the first round PCR and HE300 and HE301 for the second round PCR. Genotype 3-specific PCR was performed to amplify the nt 1546–2074 sequence with HE303 and HE306 in the first round and HE304 and HE282 in the second round. To amplify the nt 2995–3469 sequence of genotype 3 HEV, HE307 and HE310 were used in the first round and HE308 and HE309 in the second round.

Semi-Quantitation of HEV RNA

Semi-quantitation of HEV RNA was performed by the end-point dilution method, with primers targeting the ORF2 region as described previously [Mizuo et al., 2002]. The highest dilution (10^N) of extracted RNA that was found to be positive was estimated and it was converted to the relative titer per 1 ml of serum.

Sequence Analysis

The amplification products were sequenced on both strands either directly or after cloning into pT7Blue T-Vector (Novagen, Inc., Madison, WI), using the BigDye Terminator Cycle Sequencing Ready Reaction Kit. Sequence analysis was performed using Genetyx-Mac (version 12.6.6; Genetyx Corp., Tokyo, Japan) and

TABLE I. Positions and Nucleotide Sequences of Primers Used for Polymerase Chain Reaction (PCR) Amplification of Partial Nucleotide Sequences of Hepatitis E Virus (HEV) ORF1

Primer	Polarity	Nucleotide position[a]	Specificity	Nucleotide sequence[b]
HE269	Antisense	2135–2154	Genotype 4	5′ ARSCCYGAMACCGACCAGGT 3′
HE282	Antisense	2075–2094	Genotype 3	5′ GACTCCCARVYRTGSCCRGG 3′
HE296	Sense	1509–1528	Genotype 4	5′ AGGGTTAYGAYAAYGAGGCA 3′
HE297	Sense	1523–1542	Genotype 4	5′ GAGGCATTTGARGGGTCGGA 3′
HE298	Antisense	2087–2106	Genotype 4	5′ AARGGRTTRGYWGACTCCCA 3′
HE299	Sense	2765–2784	Genotype 4	5′ GAYGCYTGGGARCGYAACCA 3′
HE300	Sense	2807–2826	Genotype 4	5′ CTKACYGAGCCRGCBATAGC 3′
HE301	Antisense	3287–3306	Genotype 4	5′ TTTGTWGGDACMAGCTCAGG 3′
HE302	Antisense	3303–3322	Genotype 4	5′ RGTAASRTGCCACCACTTTG 3′
HE303	Sense	1448–1467	Genotype 3	5′ CRGTGGYTVGGSCAGGAGTG 3′
HE304	Sense	1526–1545	Genotype 3	5′ GCYTAYGAGGRBTCYGAGGT 3′
HE306	Antisense	2087–2106	Genotype 3	5′ AADGGRTTVGCAGRCTCCCA 3′
HE307	Sense	2933–2952	Genotype 3	5′ CGTGCYTGYGCYGGYTGCAC 3′
HE308	Sense	2975–2994	Genotype 3	5′ TAYCAGTTYACYGCYGGGGT 3′
HE309	Antisense	3470–3489	Genotype 3	5′ TGRACHGTRATYGCACCAGG 3′
HE310	Antisense	3518–3537	Genotype 3	5′ GCYGTRGCTATRATTGTGGT 3′

[a]The nucleotide numbers are in accordance with HE-JA1 (AB097812).
[b]R = A or G; S = C or G; Y = C or T; M = A or C; V = A, C or G; W = A or T; K = G or T; B = C, G or T; D = A, G or T; H = A, C or T.

ODEN (version 1.1.1) from the DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan) [Ina, 1994]. Sequences were aligned by CLUSTAL W (version 1.8) [Thompson et al., 1994]. A phylogenetic tree was constructed by the neighbor-joining method [Saitou and Nei, 1987]. Bootstrap values were determined with 1,000 resamplings of the data sets [Felsenstein, 1985]. The final tree was obtained using the TreeView program (version 1.6.6) [Page, 1996].

Statistical Analysis

Statistical analyses were performed using Fisher's exact probability test for comparison of proportions between two groups and the Mann–Whitney U test for comparison of continuous variables between two groups. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Analysis of Full-Length Genome of Genotype 4 HEV Isolates

Seven of the nine HEV isolates, that is, HE-JF4, HE-JF5, HE-JA19, HE-JA28, HE-JA36, HE-JA37, and HE-JA41, had the same genomic length of 7239 nt, excluding the poly(A) tract at the 3′-terminus. HE-JF3 and HE-JA2 had a genomic length of 7240 nt and 7243 nt, respectively; the differences in genomic length were attributed to an insertion of 1 nt or 4 nt in HE-JF3 and HE-JA2, respectively, in the 3′-untranslated region. When the full genome sequences were compared with those of reported HEV isolates, HE-JF3 was closely related to HE-JA1 [Nishizawa et al., 2003] with a nucleotide identity of 99.6%. HE-JF4 shared an identity of 99.7% with HE-JF5, and had the highest identity of 99.9% with both JYW-Sap02 and JTS-Sap02 [Takahashi et al., 2004]. HE-JA19 and HE-JA37, which shared an identity of 99.3%, were 99.2% and 98.9% similar to JKK-Sap00 [Takahashi et al., 2003], respectively. HE-JA28, HE-JA36, and HE-JA41 were 99.5–99.6% identical to each other and had an identity of 98.4–98.7% with JSM-Sap95 [Takahashi et al., 2004]. HE-JA2 was only 84.3–86.8% similar to the other genotype 4 isolates. A phylogenetic tree was constructed based on the full genome sequence of genotype 1–4 HEV (Fig. 1). HE-JF3 segregated into a cluster consisting of JSN-Sap-FH02 [Takahashi et al., 2003] and JSF-Tot03 [Takahashi et al., 2005], both of which had been obtained from fulminant hepatitis patients. HE-JF4, HE-JF5, HE-JA19, and HE-JA37 were grouped into another cluster consisting of JKK-Sap00, JYW-Sap02, and JTS-Sap02. HE-JA28, HE-JA36, and HE-JA41 formed a mini-cluster, separate from the latter cluster.

To investigate nucleotide differences that may be related to fulminant hepatitis, the nine full-length sequences of HEV determined in this study were examined along with 13 previously reported, entire or nearly entire HEV sequences [Wang et al., 2000; Kuno et al., 2003; Liu et al., 2003; Nishizawa et al., 2003; Takahashi et al., 2003, 2004, 2005]. No nucleotide substitutions within the 5′-and 3′-untranslated regions were specific for the HEV isolates from patients with fulminant hepatitis, although some of the reported isolates lacked the extreme 5′-terminal part of the 5′-untranslated region. A total of 7,145 nucleotides within the coding region of the 5 genomes (HE-JF3, HE-JF4, HE-JF5, JSN-Sap-FH02, and JSF-Tot03) recovered from patients with fulminant hepatitis were compared with those of the 17 genomes recovered from patients with acute hepatitis. At each nucleotide position, we examined whether a particular nucleotide was significantly more prevalent among the HEV genomes from patients with fulminant hepatitis than among those from patients with acute hepatitis. A *P*-value was determined for each nucleotide position.

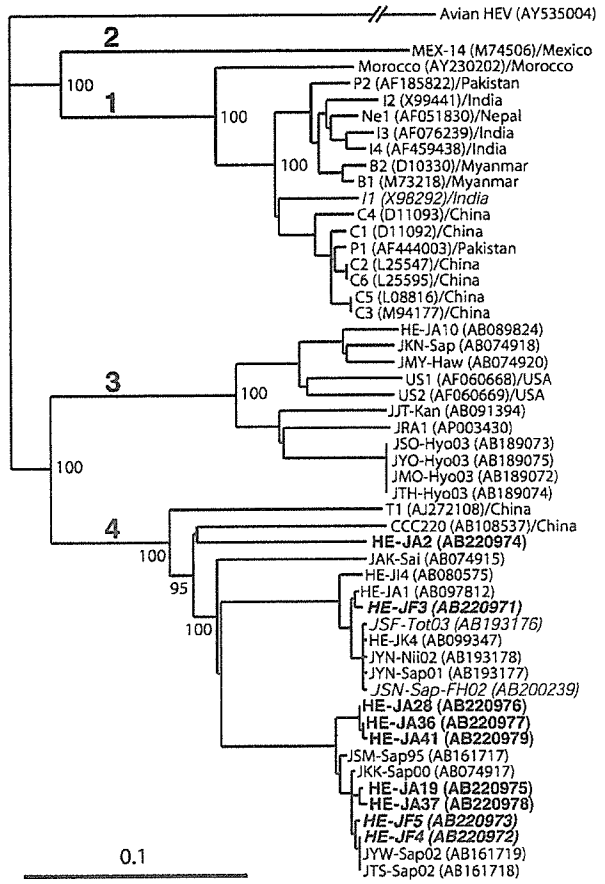

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on the full-length nucleotide sequences of 51 human hepatitis E virus (HEV) isolates, using a chicken HEV isolate (AY535004) as an outgroup. The nine HEV isolates whose full-length sequence was determined in the present study are indicated in bold type for visual clarity. Forty-two isolates whose entire or nearly entire sequence has been reported were included for comparison, with the accession number in parentheses. After the slash, the name of the country other than Japan where the HEV isolate was isolated is shown. The six isolates that were obtained from patients with fulminant hepatitis are indicated in italic type. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings.

All 21 nucleotide substitutions with $P < 0.2$ did not change the amino acids (Table II). Among them, C at nt 1816 (C1816) and U at nt 3148 (U3148) were seen significantly more frequently among the HEV isolates from patients with fulminant hepatitis than among those from patients with acute hepatitis (100% vs. 47%, $P = 0.0489$; 100% vs. 47%, $P = 0.0489$).

Comparison of Nucleotides at Nt 1816 and 3148 in Genotype 4 HEV Isolates

To determine the nucleotides at nt 1816 and 3148 in an additional 16 HEV isolates from patients with genotype 4 HEV, including a patient with fulminant hepatitis (HE-JF1), the partial nucleotide sequences of two different regions within ORF1 (nt 1543–2086 and nt 2827–3286) were determined. The nucleotides at nt 1816 and 3148 in the obtained sequences and those in the 22 full-length genomes were compared between the fulminant and acute hepatitis patients (Table III). C at nt 1816 and U at nt 3148 remained significantly more prevalent among the patients with fulminant hepatitis than among those with acute hepatitis ($P = 0.0268$ and $P = 0.0018$, respectively). Of note, U3148 was found to have a closer relationship with fulminant hepatitis than C1816.

Comparison of the Nucleotides at Nt 3148 in HEV Isolates of All Four Genotypes

Figure 2A depicts the genomic organization of the four genotypes of HEV and the location of nt 3148. Nt 3148 is located within the RNA helicase domain of ORF1, and is the third base of a triplet codon encoding valine. The consensus sequence of each of the four genotypes of HEVs whose entire or nearly entire sequence was known was determined. When the consensus sequences of the 4 genotypes were aligned, a 22-nt sequence including nt 3148 was found to be well conserved (Fig. 2B). To compare the 22-nt sequence among additional isolates, the nucleotide sequence of nt 2995–3469 was determined for 20 additional isolates from patients infected with genotype 3 HEV, including that from a fulminant hepatitis patient (HE-JF2). Comparison of the 22-nt sequence among 86 isolates including the 20 genotype 3 and 16 genotype 4 isolates, whose partial nucleotide sequence was determined in the present study, disclosed that there are some minor substitutions in this conserved area (Fig. 2C). Notably, genotype 4 isolates had a much higher prevalence of U3148 than isolates of the other three genotypes (39.5% [15/38] vs. 4.2% [2/48], $P < 0.0001$), and U3148 was associated significantly more frequently with fulminant hepatitis than other nucleotides at nt 3148 (C or G) (35.3% vs. 2.9%, $P = 0.0006$). On the other hand, at nt 1816, no particular nucleotide was seen significantly more frequently among the HEV isolates from patients with fulminant hepatitis than among those from patients with acute hepatitis when the 86 isolates of four distinct genotypes were compared (data not shown).

Comparison of Demographic Characteristics and Laboratory Parameters Among the Hepatitis E Patients According to the Presence of U3148

The demographic features and laboratory parameters were compared in relation to the presence or absence of U3148 among 48 patients with type E acute or fulminant hepatitis for whom such data were available [Mizuo et al., 2002, 2005; Suzuki et al., 2002; Kuno et al., 2003; Yajima et al., 2003; Yazaki et al., 2003; Sainokami et al., 2004; Saitoh et al., 2004; Yamamoto et al., 2004; Hijioka et al., 2005]. Twenty-seven patients were infected with genotype 4 HEV and the remaining 21 patients with genotype 3 HEV. A patient who was infected with both genotypes 3 and 4 HEV isolates [Takahashi et al., 2002]

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TABLE II. Differences in the Nucleotide Sequence of Genotype 4 HEV Genomes Obtained From Patients With Fulminant or Acute Hepatitis E

		Nucleotide no.[b]																				
Isolate name[a]	Diagnosis	37	370	421	1345	1816	1963	2101	2128	2224	2725	3148	3185	3796	3856	4579	4888	5071	5907	5943	6126	6216
HE-JF3	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JF4	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JF5	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JSN-Sap-FH02	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JSF-Tot03	FH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JA2	AH	U	U	G	C	C	A	C	C	A	U	C	U	U	C	C	A	U	A	U	U	U
HE-JA19	AH	C	C	G	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	C	C	C
HE-JA28	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
HE-JA36	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
HE-JA37	AH	C	C	A	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	C	C	C
HE-JA41	AH	C	U	C	C	U	U	C	C	C	C	C	C	U	C	C	A	C	U	U	U	G
T1	AH	C	U	G	C	G	C	C	C	U	C	C	C	C	C	C	C	C	A	U	U	U
CCC220	AH	U	C	U	C	C	U	C	C	U	C	U	C	C	C	C	A	C	A	C	U	G
HE-JA1	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
HE-JK4	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JAK-Sai	AH	U	U	A	U	U	A	C	U	C	C	U	C	U	C	C	G	C	U	U	U	C
JSM-Sap95	AH	C	C	G	U	U	C	U	U	C	C	C	C	U	U	U	G	C	U	U	C	C
JKK-Sap00	AH	C	C	G	U	U	C	U	U	U	U	C	U	C	U	U	G	U	C	U	C	C
JYN-Sap01	AH	U	C	G	U	C	C	U	U	C	U	U	U	C	U	U	G	U	C	C	C	C
JYW-Sap02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JTS-Sap02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
JYN-Nii02	AH	U	C	G	U	C	C	U	U	U	U	U	U	C	U	U	G	U	C	C	C	C
		U/C	C/U	G/H	U/C	C/K	C/W	U/C	U/C	U/M	U/C	U/C	U/C	C/U	U/C	U/C	G/M	U/C	C/W	C/U	C/U	C/K
	FH	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0	5/0
	AH	9/8	11/6	11/6	11/6	8/9	11/6	10/7	11/6	10/7	10/7	8/9	10/7	11/6	10/7	10/7	11/6	10/7	9/8	9/8	10/7	11/6
	P value[c]	0.076	0.166	0.166	0.166	**0.049**	0.166	0.114	0.166	0.114	0.114	**0.049**	0.114	0.166	0.114	0.114	0.166	0.114	0.076	0.076	0.114	0.166

FH, fulminant hepatitis; AH, acute hepatitis; H = A or C or U; K = G or U; W = A or U; M = A or C.
Nucleotide substitutions in which the prevalence of a particular nucleotide at a nt position was higher among the HEV genomes from FH patients than among those from AH patients with $P < 0.2$ are shown.
[a]The isolate names whose entire sequences have been determined in this study are indicated in bold face.
[b]Nucleotide numbers are in accordance with HE-JA1 (AB097812).
[c]*P* values (Fisher's exact probability test) that are significant are indicated in bold face.

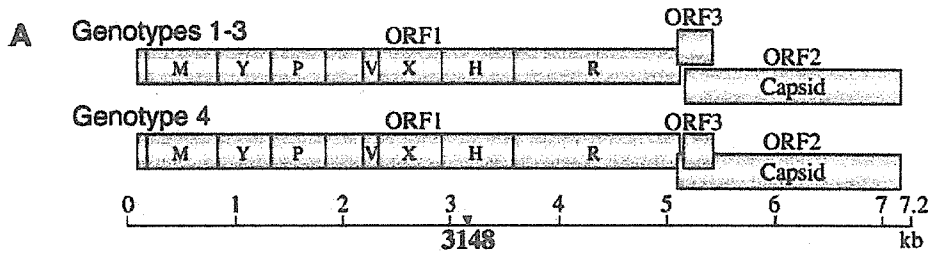
TABLE III. Comparison of Nucleotides at Nt 1816 and 3148 Among 38 Isolates of Genotype 4 HEV Recovered From Patients With Fulminant or Acute Hepatitis E

	Nt 1816		Nt 3148	
Patients with	C	G or U	U	C
Fulminant hepatitis	5	1	6	0
Acute hepatitis	10	22	9	23
P value	0.0268		0.0018	

was excluded. The patients infected with HEV with U3148 tended to have a higher peak level of total bilirubin than those with C3148 or G3148 ($P = 0.0721$) (Table IV). Of note, the value of the lowest prothrombin activity was significantly lower in patients with U3148 than in those with C3148 or G3148 ($P = 0.0293$).

DISCUSSION

It is generally considered that the severity of hepatitis E depends on host factors of the infected patients such as



B

```
Genotype 1  UGGUCCCGACCCGGGAGUUGCGUAAUGCCUGGCGCCGCCGCGGCUUCGCUGCUUUCACCCCGCACAC
Genotype 2  -U--G--C--U--C---C-U--G--C--U-----G-----G-----U--G--A-----U--------
Genotype 3  -U--G--C---------C-U------AGU-----U-----G--U--U--G--C-----A--U-----
Genotype 4  ----A--A--U--U---C-------CU-G-----A--------G--U--A--C-A---A--C-----

Genotype 1  UGCCGCUAGAGUCACCCAGGGGCGCCGGGUUGUCAUUGAUGAGGCCCCAUCCCUUCCCCCUCACCUG
Genotype 2  ---G--CC-U-----UAGC--C--UA--------------------U--G--C-----A---U--
Genotype 3  G--G--CC-U--U--UAUA--C-----C-----G--------------------C--A--G------
Genotype 4  C--G--CC-U--U----GU--C--UA--------------------G--A-----A--A---U--

Genotype 1  CUGCUGCUCCACAUGCAGCGGGCCGCCACCGUCCACCUUCUUGGCGACCCGAAUCAGAUCCCAGCCA
Genotype 2  -----UU-A--U--------U--U--AU-U--G-----C-----G----------------C----
Genotype 3  ------U-A--------------U--U-G-----U--C--C--U-----A----------U--U-
Genotype 4  --UU----G--------------U-GU-G--------C----------C--C--A-----U----
```

C

22-nt sequence with nt 3148	HEV genotype 1	2	3	4
GGGUUGUCAUUGAUGAGGCCCC	7	1	0	20
----------U--	6	0	0	1
----------A--U--	1(1)	0	0	0
----------C-----U--	1	0	0	0
-U--C-----C-----A-----	1	0	0	0
-C-----G----------U--	0	0	14	0
-U-----G-----C--------	0	0	7(1)	0
-C-----G----------	0	0	3	0
-U-----G----------	0	0	3	0
-U-----U-----C--------	0	0	2	0
-C-----G-----C--------	0	0	1	0
-U-----G--------A--A--	0	0	1	0
-------U-----C--------	0	0	0	6(2)
-------U----------	0	0	0	5(2)
--A----U-----C--------	0	0	0	2(1)
-------U--C--C--------	0	0	0	1(1)
----C--U--C--C-----U--	0	0	0	1
----C----------	0	0	0	1
-AA-------C--C--------	0	0	0	1
Total	16(1)	1(0)	31(1)	38(6)

Fig. 2. Characteristics of nt 3148 in HEV and its surrounding sequence. **A**: Organization of the HEV genome of genotypes 1–3 and that of genotype 4 showing the location of nt 3148. M, methyltransferase; Y, Y domain; P, papain-like protease; V, proline-rich hinge domain; X, X domain; H, RNA helicase; R, RNA polymerase. **B**: Comparison of consensus sequences of the region including nt 3148 (201 nt) in each of the four distinct genotypes of HEV. The inverted triangle indicates the position of nt 3148, and the box indicates a conserved area including nt 3148. The consensus sequence of each genotype was deduced from nucleotides common to each of the four genotypes (genotype 1, 16 isolates; genotype 2, 1 isolate; genotype 3, 11 isolates; and genotype 4, 22 isolates). **C**: Comparison of a 22-nt sequence including nt 3148 among 86 HEV isolates of four distinct genotypes. The inverted triangle indicates nt 3148. The numbers on the right of the sequences indicate the number of isolates with the indicated sequences, and the number in parentheses indicate the number of isolates obtained from patients with fulminant hepatitis. The genotype 1 isolate from a patient with fulminant hepatitis (I1, X98292 [Donati et al., 1997]), and the genotype 3 isolate from a patient with fulminant hepatitis (HE-JF2 [Suzuki et al., 2002]) are shown.