Comparison of nucleotide sequences in the basic core promoter and precore region between HBV/Ae and HBV/Aa isolates

The double mutation (T1762/A1764) was significantly more frequent in HBV/Aa than in HBV/Ae isolates (11/19 or 58 % vs 5/20 or 25 %, $P<0\cdot01$). Point mutations for T1809 and T1812, which were not known in HBV isolates of genotypes other than A, were found frequently in HBV/Aa isolates (18/19 or 95 % and 16/19 or 84 %, respectively). Sequences of the pregenome encapsidation (ε) signal in the precore region are compared between HBV/Ae and HBV/Aa isolates in Fig. 4. Remarkably, the point mutation from G to A or T at nt 1862 and that from G to A, C or T at nt 1888 occurred frequently in HBV/Aa isolates (16/19 or 84 % and 17/19 or 89 %, respectively); these point mutations were seen only in HBV/Aa isolates. The precore stop mutation (A1896), accompanied by a C-to-T mutation at nt 1858 making a pair with it, was found in a single HBV/Ae isolate from Europe (accession no. AF090838).

DISCUSSION

Genotype A is different from the other genotypes of HBV in that it has a C at nt 1858 that prohibits the G-to-A point mutation at nt 1896 for creating a stop codon in the precore region (Li *et al.*, 1993); C1858 and T1896 make a pair in the lower stem of the pregenome encapsidation (ε) signal. The G1896A mutation prohibits the translation of the HBeAg precursor (Carman *et al.*, 1989; Okamoto *et al.*, 1990) and, by doing so, prevents the seroconversion from HBeAg to the corresponding antibody. Despite the presence of C1858, individuals infected with HBV/A in Africa seroconvert to anti-HBe very frequently, and only 5 % of them possess HBeAg in their serum when they reach adulthood (Dusheiko *et al.*, 1985). Furthermore, HBV/A strains in Africa seem to be different from those in Western countries in that they induce hepatocellular carcinoma very often (Attia, 1998; Olweny, 1984). HBV accounts for most cases of hepatocellular carcinoma in Africa, but only for 15–20 % of cases in the USA where HBV/A is prevalent (Di Bisceglie *et al.*, 1998).

Bowyer *et al.* (1997) reported a subgroup of HBV/A isolates from Africa which clustered on a branch separate from that harbouring isolates from Europe and the USA, based on a phylogenetic analysis of preS2/S sequences. They designated genotype A prevalent in Africa as A′ to distinguish it from the original genotype A. Differences between A and A′ have been corroborated by comparison of the entire genomic sequences (Kramvis *et al.*, 2002). These two subgroups of genotype A are also serologically different. The African genotype A′ encodes HBsAg of serotype *adw* or *ayw*, unlike the original genotype A that encodes HBsAg of serotype *adw* (Bowyer *et al.*, 1997; Sugauchi *et al.*, 2003b). Although A and A′ belong to the same genotype, they seem to be very different epidemiologically and in their capacity to encode HBeAg and induce hepatocellular carcinoma.

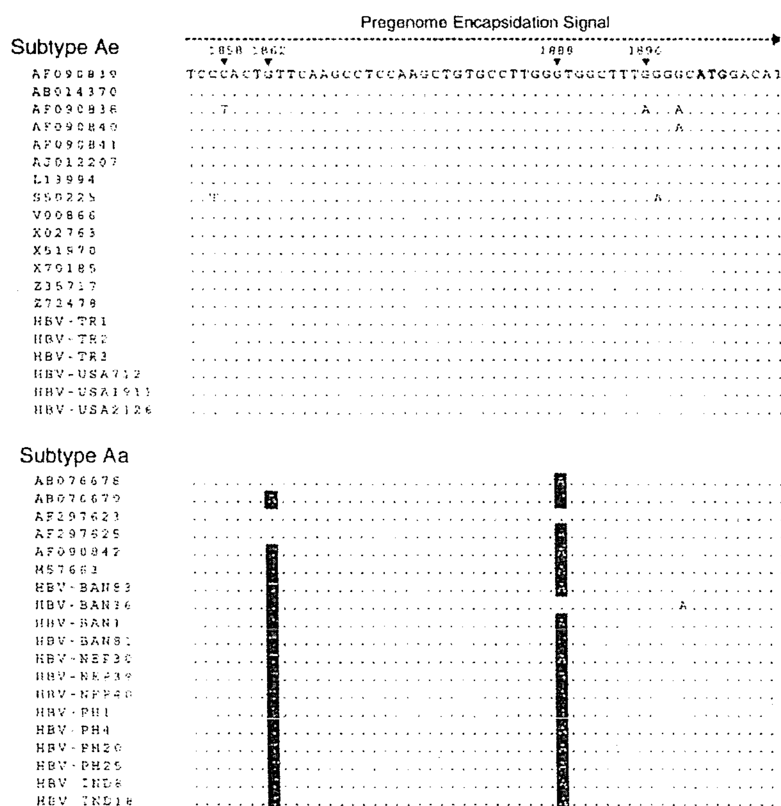

Fig. 4. Nucleotide sequences of the pregenome encapsidation (ε) signal in HBV/Ae and HBV/Aa isolates. Positions of C1858 making a pair with G1896 and those of the subtype-specific mutations at nt 1862 and nt 1888 are indicated by ▼. The start codon in the C gene is highlighted.

In the present study, 19 HBV/A isolates from various countries in Asia and from the USA were sequenced in full-length and, along with 20 sequences retrieved from the DDBJ/EMBL/GenBank databases, were examined phylogenetically. The 39 HBV/A isolates clustered on two separate branches for A and A′, as Bowyer *et al.* (1997) observed in their analysis of partial genomic sequences. It came as a surprise that the three HBV/A isolates from Japan were of subtype Ae, confirming previous reports. Hence, most HBV/Ae infections in Japan would have been imported from Western countries. They are definitely different, however, from HBV/Aa infections prevailing in other Asian countries, albeit subtype Aa was found in a minor population of HBV/A isolates from Japan in this study and is reported (accession no. AB014370) (Takahashi *et al.*, 1998).

Amino acids specific to subtype A′ isolates from South Africa clustering in the preS region and the P gene have been reported (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002). In the present study, also, there were amino acids in the preS region and the P gene that were unique to subtype Aa isolates from Asian countries. They were not found in other genotypes of HBV (Ae and B–H), while some of them were shared by subtype Aa isolates from African countries (54Q, 74V, 86A and 91V in the preS1 region; 32L in the preS2 region; 91A, 236T, 256C and 268G in the P gene).

Recombination was not responsible for differences between subtypes Ae and Aa, because recombinants of genotype A with another genotype (Owiredu *et al.*, 2001) were excluded from our phylogenetic analyses of the 39 HBV/A isolates. The comparison of subtype Aa with Ae revealed many differences, some of which have been described previously, while others have not. Sequence variation greater in HBV/Aa than HBV/Ae isolates reported in the preS/S gene (Bowyer *et al.*, 1997) was confirmed and extended to the entire genomic sequence as well as to the other reading frames (Table 1). The divergence in the preS1/preS2 sequence was found to be greatest between HBV/Aa and HBV/Ae isolates.

Probably of the most important virological relevance, nt 1862 was invariably G in the 20 HBV/Ae isolates, while it was frequently found to be T in the HBV/Aa isolates and was detected in 15/19 (79 %) of them; A at this position was found in a single (5 %) HBV/Aa isolate. Furthermore, nt 1888 was exclusively G in HBV/Ae isolates, but it was replaced by A ($n=14$), C ($n=2$) or T ($n=1$) in 17/19 (89 %) HBV/Aa isolates. These two nucleotides are positioned in the 6 nt bulge and upper stem, respectively, that make essential elements in the pregenome encapsidation (ε) signal (Fig. 5). Nt 1862 is a G in wild-type HBV and occupies the third position in the 6 nt bulge. The conversion of G1862 to any of the other three nucleotides does not interfere with the encapsidation of pregenomic RNA (Rieger & Nassal, 1995), but it does seem to affect the replication of HBV (Nassal & Rieger, 1996). How G1862 in HBV/Aa isolates is involved in hepatocarcinogenesis in Africa, where these isolates are prevalent, is a matter of clinical concern (Kramvis *et al.*, 1998).

It has been proposed that the G-to-T missense mutation at nt 1862 would interfere with the processing of the HBeAg precursor by its position close to the cleavage site of signal peptidase (Kramvis *et al.*, 1997). It creates phenylalanine two positions upstream of the signalase cleavage site in the amino acid sequence of the precore region, which makes it difficult for signalases to act properly (Kramvis *et al.*, 1997), and has been shown to reduce the production of HBeAg by *in vitro* transfection studies (Hou *et al.*, 2002). This could be a reason for suppressed production of HBeAg in African individuals infected with HBV/Aa (Dusheiko *et al.*, 1985), and might be implicated in fulminant hepatitis B in Chinese patients who were infected with HBV/B with G1862T in the absence of any mutations that abrogate or down-regulate the production of HBeAg (Hou *et al.*, 2002). Fulminant hepatitis B is caused by HBV variants with mutations in the precore region or core promoter that abort or reduce the synthesis of HBeAg (Kosaka *et al.*, 1991; Liang *et al.*, 1991; Omata *et al.*, 1991; Sato *et al.*, 1995).

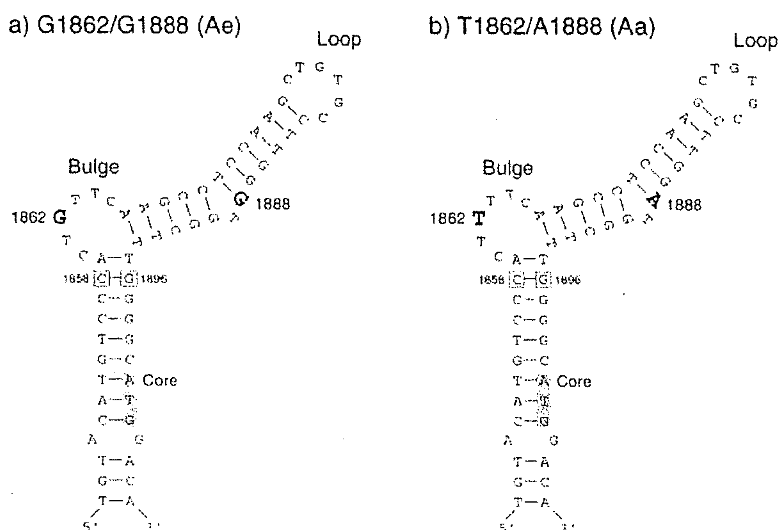

Fig. 5. Conformation of the pregenome encapsidation (ε) signal for HBV genomes of subtypes Ae and Aa. All 19 isolates of subtype Ae possess G1862 and G1888 (a), in contrast to T1862 and A1888 in 15 (79 %) and 14 (74 %), respectively, of the 19 HBV/Aa isolates (b). A Watson–Crick pair between C1858 and G1896, which is characteristic of genotype A (Li *et al.*, 1993), is boxed and the initiation codon of the C gene is shaded.

Although the G1862T mutation was prevalent and detected in 15/19 (79 %) HBV/Aa isolates for which the full-length sequences were known, in remarkable contrast to 0/20 HBV/Ae isolates, it was not exclusive to subtype Aa of genotype A. T1862 is present in 7/27 (26 %) HBV/B isolates from patients with fulminant hepatitis in China (Hou *et al.*, 2002), as well as in two isolates of HBV genotype C [accession nos D23683 (Horikita *et al.*, 1994) and X85262 from Italy]. Moreover, it was detected in the full-length sequences of HBV isolates from gibbons [accession nos AJ131574 (Grethe *et al.*, 2000) and AY077735 (Noppornpanth *et al.*, 2003)]. In addition, G1862 has been documented in many HBV isolates of unspecified genotypes from patients with chronic hepatitis (Carman *et al.*, 1995; Horikita *et al.*, 1994; Kramvis *et al.*, 1997; Loriot *et al.*, 1995; Santantonio *et al.*, 1991; Tran *et al.*, 1991; Valliammai *et al.*, 1995), fulminant hepatitis (Hou *et al.*, 2002; Laskus *et al.*, 1993) and hepatocellular carcinoma (Kramvis *et al.*, 1998). The prevalence of G1862T in African HBV/Aa isolates needs to be surveyed on a large scale; however, in the four African HBV/Aa isolates whose full-length sequences are available, nt 1862 is G in three and A in one (accession no. AB076679).

In conclusion, a comparison of 20 HBV/Ae and 19 HBV/Aa isolates over their entire genomic sequences has disclosed many previously reported and unknown differences between them. Inasmuch as these differences may affect the replication of HBV as well as the translation of HBeAg, and can modify the clinical courses of acute and chronic infections, the prevalence of HBV/Ae and HBV/Aa would need to be determined in a number of epidemiological and clinical settings. The classification of genotype A into Ae and Aa subtypes would be more appropriate than the A/A′ grouping in which A′ tends to sound subordinate to A. It may turn out that isolates of A′ are more frequent than those of A on a worldwide basis and that they are also much older phylogenetically. This view would be supported by sequence variation in the entire genome significantly wider in HBV/Aa than HBV/Ae isolates.

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YMDD Mutants in Patients With Chronic Hepatitis B Before Treatment Are Not Selected by Lamivudine

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Hepatitis B virus (HBV) mutants with mutations in the YMDD motif of viral DNA polymerase/reverse transcriptase are described in patients infected with HBV who have not received lamivudine therapy, but their pathogenetic potential is not clear. These mutants were detected by the polymerase chain reaction with peptide nucleic acid clamping in pretreatment sera from two patients who later received lamivudine. One patient developed acute exacerbation with hepatic encephalopathy and received lamivudine along with plasma exchange, which were effective on his illness. YIDD mutants were detected in all three pretreatment sera and both posttreatment sera from him. HBV DNA clones from pretreatment and posttreatment sera, however, did not have the same amino acid sequence. In the other patient who developed severe breakthrough hepatitis after receiving lamivudine, YIDD mutants were detected in two pretreatment and two posttreatment sera. When amino acid sequences of HBV DNA clones with the YIDD mutation were compared before and after he received lamivudine, however, they were not in accord. Hence, YIDD mutants in both patients with chronic hepatitis B before treatment were not selected by lamivudine after they had been placed on it. Numerous amino acid conversions were detected in HBV DNA clones with YIDD mutations, and some of them created stop codons in the overlapping *S* gene sequence. In Conclusion, HBV mutants with mutations in the YMDD motif in patients before treatment would not be selected by lamivudine or induce breakthrough hepatitis, and some of these would not be replication-competent due to stop codons in the *S* gene. ***J. Med. Virol. 74:361–366, 2004.*** © 2004 Wiley-Liss, Inc.

KEY WORDS: breakthrough hepatitis; chronic hepatitis B; hepatitis B virus; lamivudine; YMDD mutants

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INTRODUCTION

Lamivudine is an oral nucleoside analogue with a chemical structure of (−)-b-L2′,3′-dideoxy-3′thiacytidine (ATC) [Coates et al., 1992]. Due to its potent antiviral activity, lamivudine has been used widely for treatment of patients with chronic hepatitis B and led to an excellent response without much untoward effects [Lai et al., 1997; Nevens et al., 1997; Dienstag et al., 1999; Suzuki et al., 1999; Liaw et al., 2000]. Mutants of hepatitis B virus (HBV) with mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif in the domain C of viral DNA polymerase/reverse-transcriptase can develop, however, in most patients who receive lamivudine and increase in parallel with the duration of therapy [Honkoop et al., 1997; Allen et al., 1998; Chayama et al., 1998; Liaw et al., 1999; Suzuki et al., 2003]. Because breakthrough hepatitis elicits in some patients in whom YMDD mutants develop, a long-term use of lamivudine is hampered in these patients.

It has become increasingly evident that YMDD mutants occur naturally and exist in HBV carriers who have not received lamivudine therapy, preferentially in those with antibody to hepatitis B e antigen (anti-HBe) in serum [Kobayashi et al., 2001; Kirishima et al., 2002]. It is not known, however, whether or not pretreatment YMDD mutants are selected by lamivudine and induce breakthrough hepatitis in patients with hepatitis B during treatment with it.

Recently, a highly sensitive method to detect YMDD mutants has been developed by means of the polymerase chain reaction (PCR) with peptide nucleic acid clamping (PNA) [Kirishima et al., 2002], which is a 3–5 log order

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more efficient than the conventional PCR with restriction fragment length polymorphism (RFLP) [Chayama et al., 1998]. By means of the PCR-PNA method, YMDD mutants were detected in pretreatment sera from two patients with chronic hepatitis B who later received lamivudine and developed YMDD mutants. Furthermore, HBV DNA clones with mutations in the YMDD motif were propagated from their pretreatment sera and followed after they had received lamivudine, to see if YMDD mutants are selected by it and can induce breakthrough hepatitis.

MATERIALS AND METHODS

Patients With Chronic Hepatitis B

In the Department of Gastroenterology at Toranomon Hospital in Tokyo, patients with chronic hepatitis B have been given oral lamivudine 100 mg daily, and 90% are continued on this drug. Two groups of patients with chronic hepatitis B were studied. Group I comprised 20 patients who were started with lamivudine during November 1998 through January 2000 and had been continued on it for 2 years or longer. They had the median age of 46 years (range: 25–71 years) and 15 (75%) of them were men. Hepatitis B e antigen (HBeAg) was detected in pretreatment sera from ten (50%) of them. YMDD mutants in HBV DNA samples from their sera were tested before and during lamivudine therapy by three different methods (see below). Group II consisted of 51 patients who were started with lamivudine during September 1995 through July 2001, and in whom YMDD mutants were detected before December 2002 by the PCR-RFLP method [Chayama et al., 1998]. They had the median age of 45 years (range: 25–71 years) and included 41 (80%) men; none of them overlapped with patients in Group I. YMDD mutants in patients in Group I detectable by enzyme-linked minisequence assay (ELMA) have been reported elsewhere [Matsuda et al., 2003]. HBeAg was detected in sera from 35 (67%) of the patients before treatment. Pretreatment sera from the 51 patients in Group II were tested for YMDD mutants by the PCR-PNA method.

Determination of YMDD Mutants

YMDD mutants were determined by three different methods in nucleic acids extracted from 100 μl of serum with a commercial kit (SMITEST EX R&D; Genome Science, Tokyo, Japan). They were sought by PCR-RFLP after the method of Chayama et al. [1998], ELMA with a commercial assay kit (PCR-ELMA; Genome Science) and PCR-PNA followed by RFLP by a modification of the method of Kirishima et al. [2002]. In the modified PCR-PNA method, the first round of PCR was performed with F1/R1 primers for 20 cycles (94°C, 1 min; 60°C, 1 min; 72°C, 1 min [10 min in the final cycle]). The amplification products were digested with *Nde*I (TaKaRa Shuzo, Co., Ostu, Japan) and subjected to the second round of PCR with F2/R1 primers in the presence of PNA primer under the same conditions as the first-round PCR. The products were amplified by the third round of PCR with either F3/R2 primers for detecting the YIDD sequence or F2/R2 primers for the YVDD sequence for 35 cycles (94°C, 1 min; 56°C, 1 min; 72°C, 1 min [10 min in the last cylce]). Products of the third-round PCR were digested with *Ssp*I (TakaRa Shuzo) for the detection of YIDD sequence and with *Nla*III (New England BioLabs, Beverly, MA) for that of YVDD sequence, run on electrophoresis in 4% (wet/vol) agarose, and stained with ethidium bromide. Thereafter, these two YMDD mutants were detected by the size of digests characteristic for each of the mutants.

Cloning and Sequencing HBV DNA

Amplification products of the second-round PCR, spanning nucleotides 739–883 (amino acids 550–598) in the viral DNA polymerase/reverse transcriptase, were ligated with plasmid and transformed in *Esherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). Colonies were stained with Big Dye Terminator (Applied Biosystems, Tokyo, Japan) and their sequences were determined by the direct method in a sequencer (310 Genetic Analyzer; Applied Biosystems).

Determination of HBV DNA, Genotypes, and Subtypes

HBV DNA was determined by transcription-mediated amplification and hybridisation assay (TMA; Chugai Diagnostics, Tokyo, Japan) and the results were expressed as log genome equivalents (LGE) per millilitre of serum, over a detection range from 3.7 LGE/ml (corresponding to 5,000 copies/ml) to 8.7 LGE/ml. Seven major genotypes of HBV (A–G) were determined by the combination of preS2 epitopes on hepatitis B surface antigen by enzyme-linked immunosorbent assay, which is specific for each of them [Usuda et al., 1999, 2000; Kato et al., 2001], by commercial assay kits (HBV GENOTYPE EIA; Institute of Immunology, Co., Ltd., Tokyo, Japan). Subtypes of genotype B designated Ba and Bj were determined by the presence and absence, respectively, of the recombination with genotype C over the precore region plus the *C* gene [Sugauchi et al., 2002].

RESULTS

YMDD Mutants in Patients With Chronic Hepatitis B Before and During Lamivudine Therapy

YMDD mutants detected in the 20 patients in Group I are listed in Table I. Overall, YMDD mutants were found in only one of the 20 (5%) patients in Group I before they were started with lamivudine therapy. Before lamivudine, YMDD mutants with the YIDD sequence were detected in one of the 10 (10%) patients with HBeAg by the PCR-PNA method alone; YMDD mutants were not detected by PCR-RFLP or PCR-ELMA method in this patient. YMDD mutants, in contrast, were not detected in pretreatment sera from any of the 10 patients without HBeAg at the baseline. While they

TABLE I. YMDD Mutants Before and During Lamivudine in Prospectively Followed Patients With Chronic Hepatitis B in Group I

			The second amino acid in the YMDD motif[a]					
			Before lamivudine			During lamivudine		
Case no.	Age/sex	HBeAg	PCR-RFLP	PCR-ELMA	PCR-PNA	PCR-RFLP	PCR-ELMA	PCR-PNA
1	25/F	+	M	M	M	(−)[b]	(−)	(−)
2	26/M	+	M	M	M	(−)	(−)	(−)
3	28/M	+	M	M	**I**	M	M	(−)
4	30/M	+	M	M	M	**I/V**	**I**	**I/V**
5	34/M	+	M	M	M	(−)	M	(−)
6	44/M	+	M	M	M	(−)	M	(−)
7	45/M	+	M	(−)	M	(−)	(−)	(−)
8	47/M	+	M	M	M	**I**	**I**	**I**
9	50/M	+	M	M	M	**I**	**I**	**I**
10	59/F	+	M	M	M	**I**	**I**	**I**
11	30/F	−	M	M	M	(−)	M	(−)
12	40/M	−	M	M	M	**I**	**I**	**I**
13	45/M	−	M	M	M	M	(−)	(−)
14	51/M	−	M	M	M	(−)	**I**	**I**
15	52/M	−	M	M	M	**I**	**I**	(−)
16	52/M	−	M	M	M	**V**	**V**	**V**
17	53/F	−	M	M	M	M	M	**I**
18	63/M	−	M	M	(−)	**V**	**V**	**V**
19	67/F	−	M	M	M	(−)	(−)	(−)
20	71/M	−	M	M	M	(−)	M	(−)
Total			0	0	1 (5%)	8 (40%)	9 (45%)	9 (45%)

[a]Amino acid changes by mutations are indicated in the boldface.
[b]Negative for YMDD mutants by PCR-PNA.

were receiving lamivudine, YMDD mutants were detected in eight (40%) patients by PCR-RFLP, nine (45%) by PCR-ELMA and nine (45%) by PCR-PNA. YMDD mutants were detected in four of the ten (40%) patients with HBeAg and six of the ten (60%) without HBeAg at the baseline.

In pretreatment sera from the 51 patients in Group II, who developed YMDD mutants during lamivudine, YMDD mutants were detected in only one (2%). Pretreatment YMDD mutants were detected in none of the 35 patients with HBeAg and one of the 16 (6%) without HBeAg at the baseline.

Of the 20 patients in Group I, one (5%) was infected with HBV genotype A, three (15%) with genotype B, and the remaining 16 (80%) with genotype C. Whilst of the 51 patients in Group II, three (6%) were infected with genotype A, three (6%) with genotype B, and the remaining 45 (88%) with genotype C. The both patients with YMDD mutants before lamivudine treatment, one in Group I and the other in Group II, were infected with HBV genotype B. Subtypes of genotype B, in terms of Ba and Bj [Sugauchi et al., 2002], were different between the two patients, however. The patient in Group I was infected with subtype Bj, while that in Group II with subtype Ba.

YMDD Mutants in the Patients in Group I Before and During Lamivudine Therapy

The patient under Case 3 (Table I) was a 28-year-old man who was infected with HBV of subtype Bj and possessed HBeAg in Serum. The clinical course and development of YMDD mutants in him are illustrated in Figure 1. During 3 months toward the end of 1998 when he presented with chronic hepatitis B, he exhibited YMDD mutants with the YIDD sequence in all three serum samples. Alanine aminotransferase (ALT) levels in his serum gradually increased and, accompanied by abruptly elevated of bilirubin levels, he lapsed into the grade II coma for the diagnosis of hepatic encephalopathy. Plasma exchange was performed on him immediately, followed by oral lamivudine 100 mg daily. After the commencement of lamivudine, HBV DNA levels in serum decreased sharply and became undetectable by the TMA method. Serum levels of ALT and bilirubin decreased gradually thereafter, and he recovered

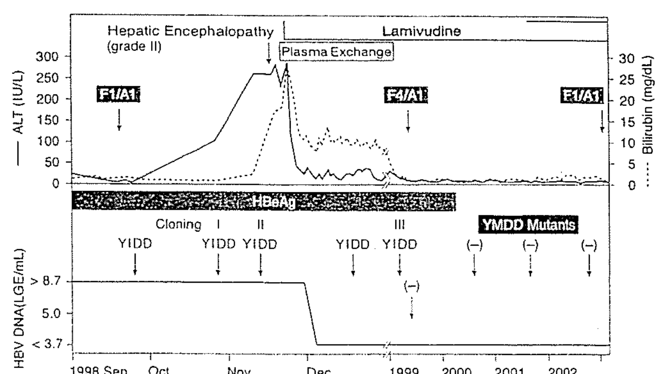

Fig. 1. Clinical course of the patient with chronic hepatitis B (Case 3 in Table I) who developed hepatic encephalopathy and in whom YIDD mutants were detected by PCR with peptide nucleic acid (PNA) clamping before he received lamivudine.

completely within 1 month on treatment. Lamivudine was continued in this patient, and he lost HBeAg from serum in the early 2000 after he had received it for 16 months. YMDD mutants became undetectable by PCR-PNA since the midst of 1999 in him, and he never experienced virological breakthroughs. Liver histology aggravated in the fibrosis stage after he developed an acute exacerbation and then returned to the baseline value while the activity grade remained the same throughout the course.

YMDD mutants were detected by PCR-PNA in the initial five serum samples recovered from him. HBV DNA clones spanning nucleotides 739–883 (amino acids 550–598) were propagated in two pretreatment sera and one posttreatment serum amongst the five samples, and they are compared in Figure 2 for the amino acid sequence of DNA polymerase/reverse-transcriptase from the YMDD motif downstream. From his pretreatment serum, a variety of HBV DNA clones were recovered including the one with the wild-type sequence (Clone I-6). Notably, some mutations in the YMDD motif and downstream gave rise to stop codon mutations in the overlapping *S* gene sequence (underlined in Fig. 2).

YMDD Mutants in the Patient in Group II Before and During Lamivudine Therapy

This patient in Group II was a 42-year-old man who was infected with HBV of subtype Ba. Clinical, histological, and virological courses are illustrated in Figure 3 for him. His serum ALT levels fluctuated during the course and significant fibrosis and moderate inflammation were detected in the first liver biopsy taken soon after the admission in 1995. Lamivudine was started on him and suppressed HBV DNA levels below the detection limit of the TMA method (<3.7 LGE/ml) through almost 3 years. In the midst of 1998, however, he developed breakthrough hepatitis with sharply increased ALT levels and reappearance of HBV DNA detectable by TMA. Thereafter, bouts of HBV DNA continued to recur accompanied by fluctuating ALT and bilirubin levels throughout 4 years of follow-up.

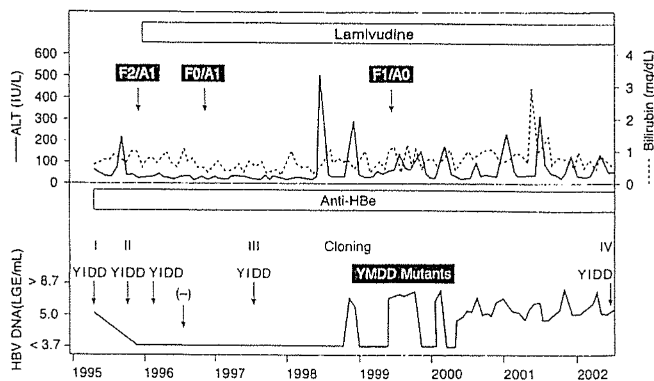

Fig. 3. Clinical course of the other patient with chronic hepatitis B in whom YIDD mutants were detected by PCR with PNA clamping before and after lamivudine therapy and who later developed breakthrough hepatitis.

YMDD mutants with the YIDD sequence were detected at the outset and remained in his serum, except when he lost them temporarily in the year 1996, and were present at the last examination after 7 years of follow-up. HBV DNA clones were propagated from his serum four times, twice each before and after he was placed on lamivudine, and their amino acid sequences are compared in Figure 4. All clones from his serum at any time points possessed numerous mutations for amino acid changes, and some of them created stop codons in the overlapping *S* gene (underlined in Fig. 4). Unlike in the patient in Case 3 in Group I (Fig. 2), there were no HBV DNA clones of the wild-type in this patient. Although some HBV DNA clones detected in pretreatment sera (Clone I-3 and Clone II-1) remained after lamivudine therapy (Clone III-1), they did not prevail after the patient had developed breakthrough hepatitis.

DISCUSSION

YMDD mutants are detected in some asymptomatic HBV carriers and patients with chronic hepatitis B who

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                   PNA
                   YMDDVVLGAKSVQHLESLYAAVTNFLLSLGIHLNPHKTKRWGYSLNFMGY
Pretreatment I                                                          (n = 9)
  Clone I-1         I----------------FTSI-------------N--------------   3
  Clone I-2         I----------------FTSI-------------N---------D----   1
  Clone I-3         V----------------FTSI-------------N--------------   1
  Clone I-4         V------------------------------------------------   1
  Clone I-5         T------------------------------------------------   2
  Clone I-6         -------------------------------------------------   1
Pretreatment II                                                         (n = 18)
  Clone II-1        I----------------FTSI-------------N--------------   3
  Clone II-2 †      I-NII---------K---TTI-------N---------K-R-----IK-   2
  Clone II-3 †      INN---R-----------TTI--S----S----------*----D-IK-   1
  Clone II-4        T------------------------------------------------   3
  Clone II-5        R------------------------------------------------   2
  Clone II-6        -------------------------------------------------   7
Posttreament III                                                        (n = 11)
  Clone III-1       I----------------FTSI-------------N--------------   1
  Clone III-2 †     I-----K---I------FTSI-------N-----N-----------IK-   3
  Clone III-3 †     IN---------------FST--------------N-----D-----IK-   5
  Clone III-4       V------------------------------------------------   2
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Fig. 2. Amino acid sequences of HBV DNA clones propagated from sera of the patient in Figure 1. Clonings were performed three times at the time points indicated in Figure 1. The sequence of amino acids 549–598 in the wild-type HBV DNA is shown at the top with the position of PNA primer. An asterisk indicates the stop codon, and a dagger at the shoulder of clones represents a stop codon in the amino acid sequence of the overlapping *S* gene in them. Amino acid conversions that created stop codons in the *S* gene are underlined.

```
                   PNA
                   YMDDVVLGAKSVQHLESLYAAVTNFLLSLGIHLNPHKTKRWGYSLNFMGY
Pretreatment I                                                          (n = 13)
  Clone I-1         I---------A------FTSIN------------N--------------   4
  Clone I-2 †       I--------------------------------------*-------R-   3
  Clone I-3         I----------------FTSI-------------N--------------   1
  Clone I-4 †       INN----------------T------F-------------K--------   2
  Clone I-5 †       INN----------------T--------------------K--------   1
  Clone I-6         T------------------------------------------------   2
Pretreatment II                                                         (n = 10)
  Clone II-1        I----------------FTSI-------------N--------------   9
  Clone II-2        I---------------------------------N-------------*   1
Posttreament III                                                        (n = 6)
  Clone III-1       I----------------FTSI-------------N--------------   3
  Clone III-2       I-----------------T------------------------------   3
Posttreament IV (breakthrough hepatitis)                                (n = 9)
  Clone IV-1        I-----------------T------------------------------   5
  Clone IV-2        I-----------------T------M-----------------------   4
```

Fig. 4. Amino acid sequences of HBV DNA clones propagated from sera of the patient in Figure 3. Clonings were performed four times at the time points indicated in Figure 3. The sequence of amino acids 549–598 in the wild-type HBV DNA is shown at the top with the position of PNA primer. An asterisk indicates the stop codon, and a dagger at the shoulder of clones represents a stop codon in the amino acid sequence of the overlapping *S* gene in them. Amino acid conversions that created stop codons in the *S* gene are underlined.

have never received lamivudine treatment [Kobayashi et al., 2001; Kirishima et al., 2002]. Hence, YMDD mutants in patients with chronic hepatitis B before treatment, even in a minor population, would be selected after they receive lamivudine and might prevail due to the drug-resistance. Such a scenario is very convenient for explaining the emergence and predominance of YMDD mutants in patients during lamivudine therapy. Should YMDD mutants occur in some patients with chronic hepatitis B before treatment, therefore, they would have to be identified for special care while they receive lamivudine or, better still, indicated for the other treatment.

In order to justify such a therapeutic strategy for treating patients with chronic hepatitis B, YMDD mutants need to be surveyed in patients with chronic hepatitis B before treatment and followed after they receive lamivudine, to see if they prevail due to drug-resistance and cause breakthrough hepatitis after they receive lamivudine. The results obtained in this study on 20 patients who were followed-up before and after they received lamivudine and the other 51 patients who developed YMDD mutants during lamivudine therapy, however, do not support such a strategy in improving the efficacy of lamivudine therapy by anticipating virological breakthroughs in patients who receive it.

First, YMDD mutants in pretreatment serum are not frequent, being detected in only one of the 20 (5%) patients with chronic hepatitis B, who corresponded to one of the ten (10%) with HBeAg at the baseline; they were not detected in any of the ten patients without HBeAg. Likewise, YMDD mutants were detected in pretreatment serum from only one of the 51 (2%) patients who developed them during lamivudine therapy, representing a single one of the 16 (6%) patients without HBeAg before therapy. These results corroborated a rare occurrence of YMDD mutants in our previous study in which they were not detected in any of 20 HBV carriers or 30 patients with chronic hepatitis B who had not received lamivudine [Matsuda et al., 2003].

The results obtained in the present study, however, are at a substantial variance with the detection of YMDD mutants in 5 of the 18 (28%) symptom-free HBV carriers [Kobayashi et al., 2001] by PCR-ELMA, as well as in 4 of the 18 (22%) patients with chronic hepatitis B before lamivudine treatment [Kirishima et al., 2002] by PCR-PNA; they all possessed anti-HBe in serum. None of the 18 patients with chronic hepatitis B having HBeAg in serum revealed YMDD mutants at the baseline [Kirishima et al., 2002]. Infrequent detection of YMDD mutants in HBV carriers in our studies is not attributed to poor sensitivity of the method, because they were determined by PCR with PNA clamping, which is by five logs more sensitive than the previous PCR-RFLP method [Kirishima et al., 2002]. The detection of YMDD mutants in an HBeAg-positive patient with chronic hepatitis B before lamivudine treatment (Fig. 1) indicates that pretreatment YMDD mutants are not restricted to symptom-free carriers and patients with anti-HBe as is inferred [Kobayashi et al., 2001; Kirishima et al., 2002].

Of the two patients with YMDD mutants before lamivudine, the mutants disappeared along with the clearance of HBV DNA from serum in one, while they grew in the other accompanied by chronic active hepatitis B through years on lamivudine. Taken at the face value, the latter patient allows for a possibility that YMDD mutants existing before therapy would be selected by lamivudine and induce breakthrough hepatitis. In order that such a consequence is valid, the identity needs to be established between YMDD mutants pretreatment and during lamivudine therapy.

Sequence comparison between HBV DNA clones before and after lamivudine therapy in the patient who developed breakthrough hepatitis along with the emergence of YMDD mutant (Fig. 4), however, did not support such a scenario. Of a variety of HBV DNA clones existing pretreatment, none prevailed even when the patient developed breakthrough hepatitis during a long-term lamivudine. Although there still remains a possibility that YMDD mutants in a minor population that had existed before lamivudine escaped detection even by PCR-PNA, some of them appeared to be defective and replication-incompetent.

Outside the YMDD motif, a number of anonymous mutations for amino acid changes were detected downstream of it; there might be more such mutations along the entire sequence of DNA-polymerase/reverse-transcrtiptase gene should they have been sought for. Furthermore, some amino-acid conversions in the YMDD motif and downstream may involve the overlapping genes in a different reading frame and prohibit their proper transcription. In actuality, stop codons were created in the *S* gene overlapping the polymerase gene in some HBV DNA clones with YMDD mutations from the two patients, thereby making them replication-incompetent. There is a possibility that HBV virions carrying YMDD mutations, which create stop codons in the overlapping *S* gene, might replicate by trans-complementation by the wild-type HBV coinfecting hepatocytes with them. This view would be in line with replication capacity and pathogenic potential of YMDD mutants that are found lower than the wild-type [Melegari et al., 1998].

It should be noted that pretreatment YMDD mutants occurred only in two of the six patients infected with HBV genotype B, in remarkable contrast to none of the four patients with genotype A infection or the 61 with genotype C infection. Two subtypes of genotype B have been reported, one of which possesses the recombination with genotype C over the precore region plus core gene (Ba) while the other does not (Bj) [Sugauchi et al., 2002]. The two patients with YMDD mutants in pretreatment serum, both of whom were infected with genotype B, differed in that one was infected with subtype Ba while the other with subtype Bj. In view of marked geographical differences in the distribution of HBV genotypes [Magnius and Norder, 1995; Miyakawa and Mizokami, 2003], it would be worthwhile to see if pretreatment

YMDD mutants occur more frequently in HBV infections of some genotypes that the others. In addition, YMDD mutants would need to be evaluated for replication capacity in transfection experiments, with special reference to mutations in the *S* gene that they may induce.

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Effect of Acute Self-Limited Hepatitis C Virus (HCV) Superinfection on Hepatitis B Virus (HBV)-Related Cirrhosis. Virological Features of HBV–HCV Dual Infection

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We investigated the virological impact of acute hepatitis C virus (HCV) superinfection on two patients with hepatitis B virus (HBV)-related cirrhosis. In both patients, chronic HBV-infection persisted while acute HCV infection resolved spontaneously. HBV DNA was transiently suppressed in both patients but increased with HCV resolution. In Case 1 (HBeAg-positive; wild type of basic core promoter [BCP] and precore [PreC]), fluctuations of HBV DNA and HBeAg state were accompanied by mutations of the BCP and PreC. In Case 2 (HBeAg-negative; mutant type of the BCP and PreC), changes in HBV DNA levels were associated with mutations of PreC. In both cases, mutant PreC changed to the wild type upon HCV resolution, and no nucleotide A insertion at position 193 of the HCV 5′-untranslated region, which influences HCV spontaneous clearance, was detected. The putative DNA-binding motif in the HCV core was SPRG (amino acids 99–102). HCV infection was associated with changes in the nucleotide sequences of the binding site for the nuclear receptor family in HBV enhancer 2 (Enh2) including the BCP rather than Enh1. Our results suggest that the impact of acute HCV infection on chronic HBV infection varies according to HBV virological state.

KEY WORDS: chronic hepatitis B; acute hepatitis C; enhancer 1/2; precore; basic core promoter; 5′-untranslated region.

Understanding of the impact of acute hepatitis C virus (HCV) superinfection on chronic hepatitis B virus-(HBV) infection is still incomplete. Previous reports showed that HCV superinfection in HBsAg carriers is associated with severe and progressive liver disease, including fulminant/subfulminant hepatitis (1–5). It is also reported that HCV superinfection could exert a suppressive effect on HBV replication and enhance seroclearance of HBV antigens (HBeAg seroconversion and HBsAg clearance) (6–8). Others stated that HCV could potentially take over the role of HBV in causing persistent chronic hepatitis (8).

With regard to HBV viral markers, it is reported, though with some controversy, that secretion of hepatitis B e antigen (HBeAg) might be influenced by mutations in the precore region (PreC)—typically a G-to-A mutation at nucleotide (nt) 1896 (A1896) (9, 10) and double mutations in the basic core promoter region (BCP) (T1762/A1764) (11–14)—and that the levels of HBV viral replication in vitro could be modulated by double mutation in the BCP (T1762/A1764) (15, 16) and enhancer 1 (Enh1) (17). However, it is not clear whether the nucleotide sequences

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in HBV Enh1, enhancer 2 (Enh2) including the BCP, and PreC affect fluctuations of HBeAg state and HBV viral load in the unusual state of acute HCV superinfection on chronic HBV infection.

The molecular mechanism(s) of the suppressive effect of HCV on HBV is(are) also still unclear. A recent study showed that nt A insertion at position 193 of the 5′-prime untranslated region (UTR) could affect HCV spontaneous clearance by acute hepatitis B superinfection during liver transplantation (18). Furthermore, in an in vitro study, Shih and coworkers reported that HCV core protein suppressed HBV replication by affecting the process of transcription and encapsidation of HBV pregenomic RNA, which contains the putative DNA-binding motif (SPRG; amino acids 99–102) (19). In addition, Schuttler *et al.* demonstrated that HCV core protein suppressed the activity of Enh1 and Enh2, which contain the binding sites for COUP-TF1, HNF4, PARP, and RXR of the nuclear receptor family (20).

The aims of the present study were the following: (a) to determine the mechanisms that influence fluctuations of HBV viral load and HBeAg state during acute HCV superinfection on chronic HBV infection—for this purpose, we determined the nucleotide sequences of HBV in the regions of Enh1, BCP (nt 1762/1764), and PreC (nt 1896); (b) to determine the mechanisms that influence the outcome of acute HCV superinfection by examining the nucleotide sequences of HCV 5′-UTR (nt 193); and (c) to determine the mechanisms of HCV core protein-induced suppression of HBV by defining the nucleotide sequences of Enh1 and Enh2, which contain the binding sites for members of the nuclear receptor family, and amino acid sequences of the HCV core region that contain the putative DNA-binding motif.

MATERIALS AND METHODS

Patients

Between 1991 and 2002, two inpatients of Toranomon Hospital with HBV-related liver cirrhosis were serologically confirmed to have acute HCV superinfection. The latter was assumed to have been acquired iatrogenically during treatment of hepatocellular carcinoma (HCC). These two cases were enrolled in the present study to analyze the impact of acute HCV infection on chronic HBV infection. Clinical and laboratory assessments were performed at least once every month, and imaging studies were conducted for early detection of HCC at least once every 3 months. In both cases, de novo seroconversion of antibody against HCV (HCV-Ab) and de novo seropositivity of HCV-RNA in HBsAg-positive and IgM-anti-HBc-negative cirrhosis patients indicated acute HCV superinfection on a chronic HBV state (21–23). Coinfection or superinfection with hepatitis A, delta viruses, TT virus, cytomegalovirus, Epstein–Barr virus, herpes simplex virus, or human immunodeficiency virus was excluded serologically or genomically using commercially available kits or conventional polymerase chain reaction (PCR)-based assays. Both patients were also confirmed to be free of other chronic liver diseases including alcohol-related liver disease (lifetime cumulative alcohol intake, <500 kg), metabolic disease, hemochromatosis, autoimmune liver disease, primary biliary cirrhosis, α-antitrypsin deficiency, Wilson disease, and hepatic venous outflow obstruction.

In both cases, nucleotide sequences of HBV of previously reported regions, i.e., nt 1136–1148 of Enh1 (17, 20), nt 1755–1768 of Enh2 (20) including nt 1762/1764 of BCP (11, 15, 16), and nt 1896 of PreC (9, 10), were determined at four stages as follows: phase 1, negative HCV-Ab and negative HCV-RNA before HCV infection; phase 2, high HCV-RNA titer during HCV infection (early phase of HCV infection); phase 3, low HCV-RNA titer during HCV infection (late phase of HCV infection); and phase 4, positive HCV-Ab and negative HCV-RNA, accompanied by spontaneous resolution of HCV infection (resolution phase of HCV infection). At early phase 2 of HCV infection, we also investigated the existence of nt A insertion at position 193 of the HCV 5′-UTR (18). We also determined the amino acid sequences of the HCV core region including the reported putative DNA-binding site (amino acids 99–102) (20).

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

Laboratory Investigations

Laboratory assessments were performed at least once every month. Serum samples were analyzed for serum transaminase, HBsAg; HBeAg/e Ab, and HBV-DNA levels. HBsAg were determined by radioimmunoassay (Abbott Diagnostics, Chicago, IL), and HBeAg/e Ab were determined by enzyme-linked immunoassay (Abbott Diagnostics). Antibody against HCV (HCV-Ab) was detected with a third-generation enzyme-linked immunoassay (Ortho Diagnostic Japan, Tokyo), The serum samples were stored in aliquots at −80°C until use. HBV-DNA and HCV-RNA kinetics were determined at least once every month using the stored samples for 24 months before and after the incidence of acute HCV superinfection and were measured at least once every week when severe ALT flare-up was accompanied by HCV infection. Levels of HBV-DNA were measured using a quantitative PCR assay (Amplicor HBV Monitor Test; Roche Molecular Systems, Inc., USA) and the results were log-transformed. The lower limit of this assay is 2.6 log copies/ml. Levels of HCV-RNA were measured using a quantitative PCR assay (Cobas Amplicor HCV Monitor Test, v2.0; Roche Molecular Systems). The lower limit of this assay is 0.5 kIU/ml. Samples in which the results of the HCV quantitative PCR assay were below the lower limit (<0.5 kIU/ml) were also evaluated using HCV-RNA qualitative analysis with PCR (Amplicor HCV, v2.0; Roche Diagnostic System). The results of this assay were expressed as positive or negative, and the lower limit of the assay is 100 copies/ml. HCV genotype was determined by PCR using a mixed primer set derived from nucleotide sequences of NS5 region (24). HBV genotype was determined as described previously (25, 26).

Nucleotide Sequencing of HBV and HCV

Nucleotide sequences of HBV were compared with the prototype sequences of the HBV genotype C (25), and those of HCV

TABLE 1. CLINICAL FEATURES OF TWO PATIENTS WITH HBV-RELATED CIRRHOSIS AND ACUTE HCV SUPERINFECTION

Case No.	Age (yr)	Sex	Histopathology	HBV history	HBeAg	HBV genotype	Etiology of HCV infection	HCV genotype	Peak level AST/ALT	Outcome	
										HBV	HCV
1	53	M	LC	>20 yr	Positive	C	Iatrogenic	1b	126/115	Persistence	Clearance
2	83	F	LC	>30 yr	Negative	C	Iatrogenic	1b	1116/940	Persistence	Clearance

Note. LC, liver cirrhosis; AST, aspartate aminotransferase (IU/L); ALT, alanine aminotransferase (IU/L).

were compared with the prototype sequences of HCV genotype 1b (27).

HBV-DNA was extracted with a Smitest EX&R kit (Genome Science, Tokyo). HCV-RNA was extracted with a SepaGene RV-R kit (Sanko Junyaku, Tokyo) and reverse transcribed with random primer and MMLV reverse transcriptase (Takara Syuzo, Tokyo). Nucleic acids were amplified by PCR using the following primers.

(a) Nucleotide Sequences of HBV Enh1. The first-round PCR was performed with B1F (sense, 5′-GGG CCA AGT CTG TAC AAC ATC-3′ [nt 758–778]) and B1R (antisense, 5′-GAA GAA GGG GAC GGT AGA G-3′ [nt 1498–1480]) primers, and the second-round PCR with B2F (sense, 5′-GTC TTT GGG TAT ACA TTT AAA CCC-3′ [nt 816–839]) and B2R (antisense, 5′-GAC GTA GAC AAA GGA CGT C-3′ [nt 1431–1413]) primers.

(b) Nucleotide Sequences of HBV Enh2 (Including BCP [nt 1762/1764]) and PreC (nt 1896). The first-round PCR was performed with BCP-F7 (sense, 5′-TGC ACT TCG CTT CAC CTC TG-3′ [nt 1580–1599]) and BCP-R8 (antisense, 5′-TAA GCG GGA GGA GTG CGA AT-3′ [nt 2295–2276]) primers, and the second-round PCR with BCP-F5 (sense, 5′-GCA TGG AAA CCA CCG TGA AC-3′ [nt 1606–1625]) and BCP-R6 (antisense, 5′-ATA CAG AGC AGA GGC GGT AT-3′ [nt 2014–1995]) primers.

(c) Nucleotide Sequences of the HCV 5′-UTR. The single-round PCR was performed with NC2 (sense, 5′-CCT GTG AGG AAC TAC TGT C-3′ [nt 32–50]) and NC1 (antisense, 5′-CAA CAC TAC TCG GCT AGC AGT C-3′ [nt 254–233]) primers.

(d) Nucleotide Sequences of the HCV Core Region. The first-round PCR was performed with CC11 (sense, 5′-GCC ATA GTG GTC TGC GGA AC-3′ [nt 125–144]) and e14 (antisense, 5′-GGA GCA GTC CTT CGT GAC ATG-3′ [nt 953–933]), and the second-round PCR with CC9 (sense, 5′-GCT AGC CGA GTA GTG TT-3′ [nt 237–253]) and e14 (antisense) primers. (a and b, nested PCR; c, single-round PCR; d, heminested PCR.)

All samples were initially denatured at 95°C for 4 min. Thirty-five cycles of amplification were as follows: denaturation for 1 min at 94°C, annealing of primers for 2 min at 55°C, and extension for 3 min at 72°C, with an additional 7 min for extension. Then 1 μl of the first-round PCR product was transferred to the second-round PCR reaction. Other conditions for the second-round PCR were the same as for the first-round PCR, except that the second-round PCR primers were used instead of the first-round PCR primers. The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen, Tokyo) after agarose gel electrophoresis and then used for direct sequencing. Dideoxynucleotide termination sequencing was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Perkin–Elmer, Chiba, Japan).

To avoid false-positive results, the procedures recommended by Kwok and Higuchi (28) to prevent contamination were strictly applied to these PCR assays. No false-positive results were observed in this study.

RESULTS

Case Histories

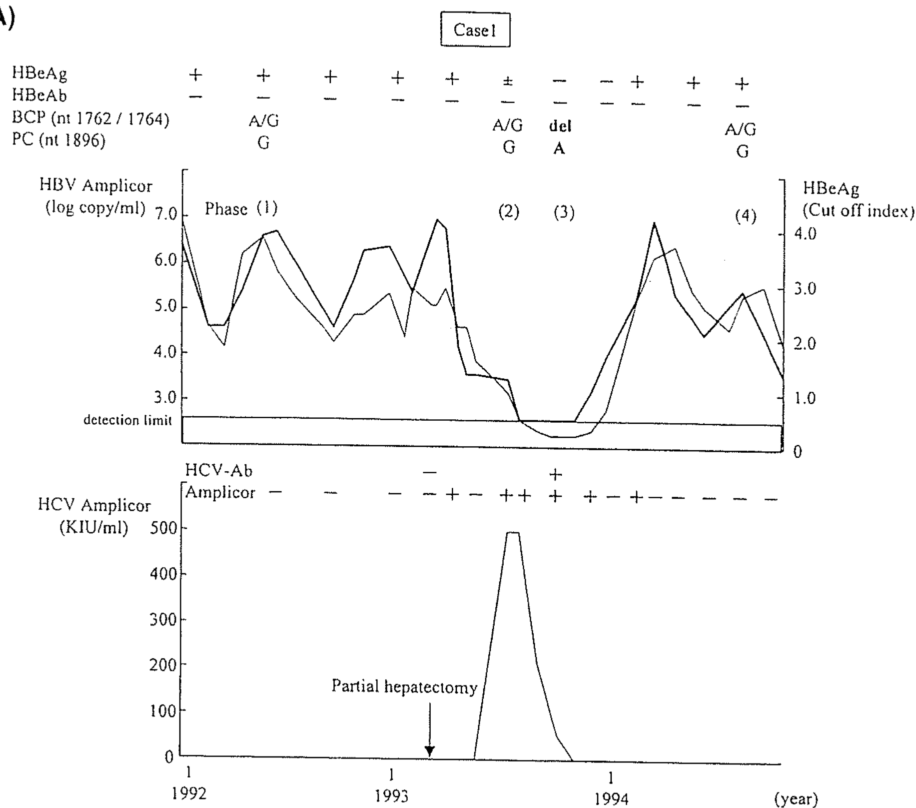
The clinical features of the two patients with HBV-related liver cirrhosis and acute HCV superinfection are summarized in Table 1. Both patients were HBV carriers infected with genotype C and were followed up at least once every month and remained negative for HCV-Ab and HCV-RNA. However, both became positive for HCV-Ab and HCV-RNA of genotype 1b after treatment for HCC (Case 1, partial hepatectomy; Case 2, transcatheter arterial embolization [TAE]). In both cases, chronic HBV infection was transiently suppressed by HCV coinfection but later persisted while acute HCV superinfection resolved spontaneously. Viral kinetics of HBV and HCV indicated reciprocal changes.

Case 1 showed a more active state of HBeAg positively, with high levels of HBV-DNA and serum transaminase. Acute HCV superinfection was associated with transient suppression of HBV-DNA levels and conversion from HBeAg positive to negative. However, spontaneous resolution of HCV infection was accompanied by conversion of negative HBeAg to positive and increased HBV-DNA and serum transaminase levels, which became equal to the levels noted before HCV infection. On the other hand, Case 2 showed a less active state of HBeAg negativity, with low levels of HBV-DNA and serum transaminase. Acute HCV superinfection in this patient was associated with a transient suppression of HBV-DNA levels. However, spontaneous resolution of HCV infection was accompanied by increases in HBV-DNA and serum transaminase to levels higher than those measured before HCV infection, while HBeAg remained negative. The clinical courses of both patients are shown in Figures 1A and B.

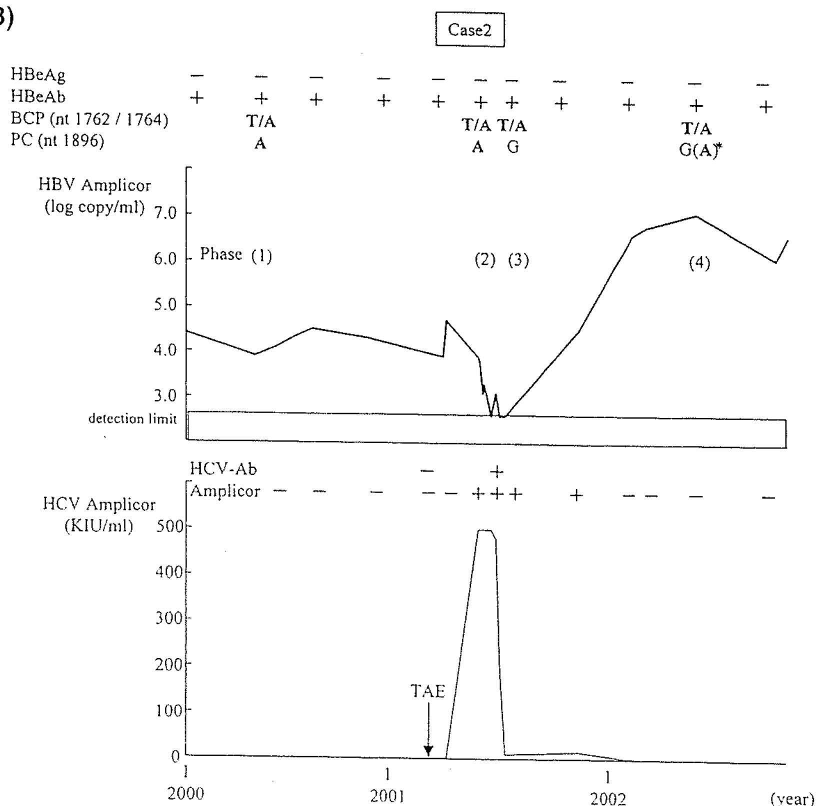
Relations Between Viral Markers and Mutations of Enh1, BCP, and PreC in HBV

Case 1, who was HBeAg-positive, with the wild types of PreC (G1896) and BCP (A1762/G1764), transiently changed with conversion to HBeAg-negative, and mutation of BCP (8-base pair [bp] deletion of nt 1758–1765

(A)



(B)



[del]) and PreC (A1896) accompanied HCV infection. However, this patient became HBeAg-positive, and the appearance of the wild types PreC and BCP at phase 4 accompanied the resolution of HCV infection, together with a transient increase in the suppressed HBV viral load to become equal to the pre-HCV infection level. Case 2, who was HBeAg-negative, had mutation of PreC (A1896) and double mutations of BCP (T1762/A1764). Interestingly, HCV infection was accompanied in this patient by the appearance of PreC wild type (G1896) from phase 3 and an increase in the transiently suppressed HBV viral load to a level higher than that determined before HCV infection. Thus, the change in HBeAg state in Case 1 was associated with changes in BCP and PreC sequences, while in Case 2, there were no changes in HBeAg state and BCP. In both cases, nucleotide sequences of PreC changed in parallel with fluctuations of HBV viral loads but those (nt 1136–1148) of Enh1 did not change in spite of fluctuations in HBV viral loads. Figure 2 shows the nucleotide sequences of Enh1, Enh2 including BCP, and PreC.

HCV 5′-UTR and HCV Spontaneous Clearance

In both cases, acute HCV superinfection resolved spontaneously, but no nt A insertion at position 193 of HCV 5′-UTR was detected.

Nucleotide Sequences of HBV Enh1 and Enh2, and Amino Acid Sequences of HCV Core Region

In both cases, nucleotide sequences (nt 1136–1148) of the binding site for members of the nuclear receptor family in Enh1 did not change during HCV infection, but nucleotide sequences (nt 1755–1768) in Enh2 changed into mutant type only at phase 3. Case 1 showed 8-bp deletions of nt 1758–1765, and Case 2 showed T-to-A mutation at nt 1768. The amino acid sequences of the putative DNA-binding motif site in the HCV core were SPRG, as reported previously (19).

DISCUSSION

The impact of acute HCV superinfection on chronic HBV infection remains poorly understood. Previous reports showed that HCV superinfection in HBsAg carries could result in severe and progressive liver disease, including fulminant/subfulminant hepatitis (1–5). Furthermore, it is also reported that HCV superinfection might exert a suppressive effect on HBV replication and enhance seroclearance of HBV antigens (HBeAg seroconversion and HBsAg clearance) (6–8), and HCV could potentially take over the role of HBV in causing persistent chronic hepatitis (8). However, the virological impact of HCV superinfection on HBV infection is still not clear. The aim of our study was to analyze the virological impact of acute HCV superinfection on chronic HBV infection. For this purpose, we investigated the nucleotide sequences of HBV Enh1, Enh2 including BCP, PreC, and HCV 5′-UTR and amino acid sequences of the HCV core.

Whether the nucleotide sequences of BCP and PreC affect the HBeAg state during acute HCV superinfection on chronic HBV infection is debatable. Mutation in PreC (A1896) is associated with loss of HBeAg (9, 10). Double mutation in BCP (T1762/A1764) is also associated with reduced synthesis of HBeAg through the suppression of transcription of precore mRNA (11–14). On the other hand, the effects of BCP mutation are not as thorough as those of PreC mutation (12, 13), although BCP mutation is implicated in seroconversion to HBeAb in some carriers whose PreC of HBV-DNA is of the wild type (G1896) (11, 29). In the present study, the HBeAg state of Case 1 (who was HBeAg-positive at the time of HCV infection) changed along with nucleotide sequences of BCP and PreC, whereas that of Case 2 (who was HBeAg-negative at the time of HCV infection) did not change along with those of PreC. These findings suggest that BCP and PreC sequences might change in different manners by HBeAg state (positive or negative) at the time of HCV infection. It should be cautioned, however, that the present results are based on only two cases, and further large-scale studies should be conducted to confirm our findings.

The mechanisms that influence HBV viral kinetics during HCV superinfection are poorly understood. Bock and coworkers reported that mutants of Enh1 have a major impact on HBV replication and that these mutations might determine the switch from high to low levels of viral replication (17). However, our results showed that nucleotide sequences of the binding site for COUP-TF1,

Fig 1. Clinical courses of two patients with HBV related-cirrhosis and acute HCV superinfection. Both patients were infected with HBV genotype C and HCV genotype 1b. In both patients, chronic HBV infection was transiently suppressed by HCV infection but persisted later while acute HCV infection resolved spontaneously. Viral kinetics of HBV and HCV indicated reciprocal change. (A) A 53-year-old male with active hepatitis (HBeAg-positive, high levels of HBV-DNA). (B) An 83-year-old female with less active hepatitis (HBeAg-negative, lower levels of HBV-DNA), in comparison with Case 1. In both cases, nucleotide sequences of precore (PreC), but not those of basic core promoter (BCP), influenced HBV viral load. The shaded area represents HBV-DNA level below the detection limit. The cutoff index of HBeAg is shown by the light line at the top of A. HBV Amplicor is shown as the heavy line at the top of A and B. del, deletion; TAE, transcatheter arterial embolization. *PreC (nt 1896) in phase 4 of Case 2 was mixed wild (G1896)- and mutant (A1896)-type virus, and wild type was more dominant than mutant type.

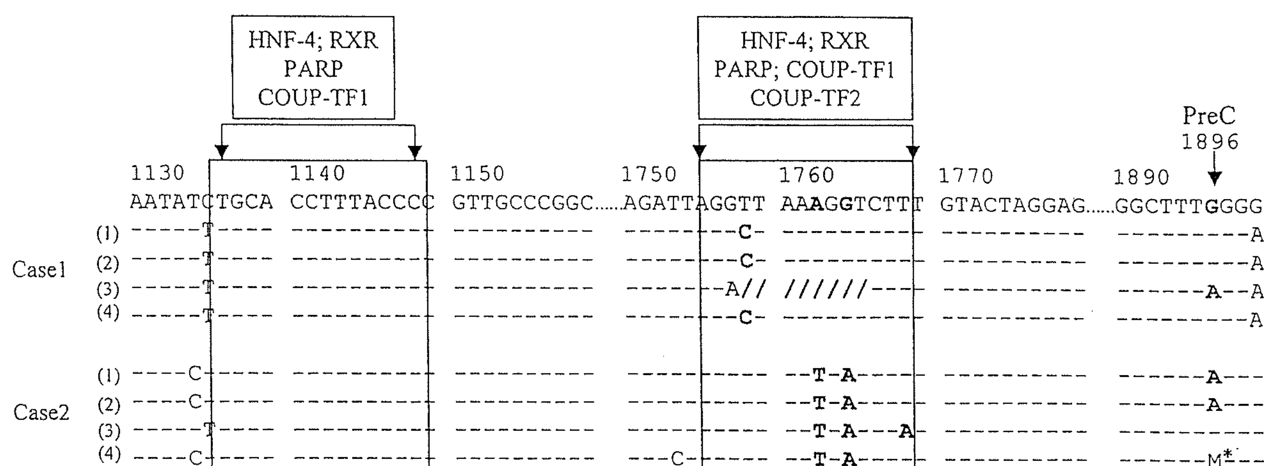


Fig 2. Nucleotide sequences of HBV enhancer 1 (Enh1), enhancer 2 (Enh2), including basic core promoter (BCP), and precore (PreC). The binding sites for COUP-TF1, HNF4, PARP, and RXR are shown in gray shading (nt 1136–1148 of Enhl, nt 1755–1768 of Enh2). The source of the binding sites is Schuttler *et al.* (20). Phase 1; HCV-Ab-negative and HCV-RNA-negative before HCV infection. Phase 2: High titer of HCV-RNA during HCV infection (early phase of HCV infection), Phase 3: Low titer of HCV-RNA during HCV infection (late phase of HCV infection), Phase 4: HCV-Ab-positive and HCV-RNA-negative accompanied by spontaneous resolution of HCV infection (resolution phase of HCV infection). Nucleotide sequences of the binding sites in Enh1 did not change with the development of acute HCV infection, but those in Enh2 changed to mutant type at phase 3. M* of PreC (nt 1896) in phase 4 of Case 2—mixed wild (G1896)- and mutant-(A1896)-type virus (wild type was more dominant than mutant type).

HNF4, PARP, and RXR in Enh1 did not change with fluctuations in HBV-DNA levels. The present results are based on only two cases and the exact reason(s) for this discrepancy is not known at present. Recently, Haushofer and colleagues suggested that there might be mutual influence on HBV and HCV replication at coinfection and that HBV viral loads might be different according to HBeAg state and existence of HCV-RNA (30). In our study, viral kinetics of HBV and HCV certainly indicated reciprocal changes, and the HBV viral load following spontaneous resolution of HCV infection was equal to (Case 1, who was HBeAg-positive and had wild-type PreC at the time of HCV infection) or greater than (Case 2 who was HBeAg-negative and had mutant PreC at the time of HCV infection) the HBV viral load before HCV superinfection. Thus, our results showed that HBV viral kinetics also differ according to HBeAg state at the time of HCV infection and existence of HCV-RNA. Interestingly, the PreC mutant reversed (Case 1) or changed (Case 2) into wild type along with resolution of HCV infection, suggesting that nucleotide sequences of PreC are another viral factor that could affect the HBV viral load. Considered collectively, these results suggest that HBV viral load at HCV superinfection might be influenced by a variety of factors, including HBeAg state, sequences of PreC, and existence of HCV-RNA. Further large-scale study should be conducted to confirm not only the virological but also the clinical impact of acute HCV superinfection on chronic HBV infection.

Acute HCV infection becomes chronic in more than 80% of infected individuals (31, 32), while less than 5% of those with acute HBV infection will progress into chronic infection (33, 34). Hence, the spontaneous clearance of HCV noted in our study is very unusual. Furthermore, this course is in contrast to the findings of earlier studies that HCV usually suppresses HBV in patients with HBV/HCV dual infection (7, 8, 19), especially the stronger suppression of HBV Enh1 by the HCV core from genotype 1b (20). A recent report showed that nt A insertion at position 193 of the 5′-UTR might affect spontaneous HCV clearance by acute hepatitis B superinfection during liver transplantation (18). However, our analysis showed no nucleotide A insertion at position 193 despite spontaneous HCV resolution. The discrepancies between our results and the above studies may be due to one or more factors. The first is probably related to the different setting used for investigating acute HCV superinfection on chronic HBV infection. The second is probably due to the virological difference in participating patients including genotype of HBV (genotype C)/HCV (genotype 1b). The third reason is related to differences in subjects with respect to the severity of liver disease and duration of HBV infection. Both our cases had suffered from HBV infection for more than 20 years, and they advanced to the stage of liver cirrhosis. Hence, while chronic HBV infection could be transiently suppressed, it might not be taken over by HCV infection. Further studies of a larger population are important to confirm these findings.

The molecular mechanisms responsible for the suppressive effect of dual HBV/HCV infection are still unclear. Shih *et al.* reported that in vitro HCV core protein suppressed HBV replication by inhibiting the process of transcription and encapsidation of HBV pregenomic RNA, which contained the putative DNA-binding motif (SPRG; amino acids 99–102) (19). Schuttler and coworkers reported in vitro that HCV core protein could suppress Enh1 up to 11-fold and Enh2 up to 3- to 4-fold, but not 5′-terminally truncated constructs of Enh1 and common mutants (8-bp deletion or double mutation of BCP) of Enh2 (20). Based on these results, they concluded that HCV core protein suppressed the activity of Enh1/2 to contain binding sites for COUP-TF1, HNF4, PARP, and RXR, members of the nuclear receptor family (20). In both cases reported in the present study, the amino acid sequences of the putative DNA-binding motif site in HCV core were SPRG, as reported previously (19). Nucleotide sequences of the binding site for members of the nuclear receptor family in Enh1 did not change but those in Enh2 did change during HCV infection. Specifically, sequences in Enh2 changed at phase 3 (late phase of acute HCV infection), suggesting that suppression of HBV by HCV at this phase might be milder than that of phase- 2 (early phase of acute HCV infection). We could not determine, in this clinical study of only two cases, whether these changes in Enh2 might occur as part of the process of escape from HBV suppression by HCV or the process of HCV elimination by HBV or whether they occur for other reasons. Further investigations should be conducted to confirm the clinical significance of these binding sites.

To our knowledge, our study is the first to investigate clinically the virological impacts of acute HCV superinfection on chronic HBV infection. Further large-scale studies should be conducted to confirm the virological and clinical impacts of HCV infection on HBV infection.

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HEPATOLOGY

Clinical and pathological characteristics of the autoimmune hepatitis and primary biliary cirrhosis overlap syndrome

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Abstract

Background and Aims: The defining of the autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) overlap syndrome as a separate clinicopathological entity has been controversial and temporally and geographically subjective.

Methods: From 1979 until 2000, 227 patients diagnosed with AIH, PBC or the overlap thereof were treated. Cases with genuine AIH/PBC overlap syndrome were sorted out using close clinical follow up and serial liver biopsies.

Results: Of the 227 patients, 19 (8.4%) were diagnosed with the AIH/PBC overlap syndrome. They all cleared a score >10 for the diagnosis of AIH, and tested positive for antimitochondrial antibodies during their courses. Long-term follow up with frequent histological examinations, however, established the diagnosis of AIH/PBC overlap syndrome in only two (0.8%) patients. The most powerful factor distinguishing AIH from PBC was acidophilic bodies in lobules that were detected significantly more frequently in patients with AIH than PBC or spurious overlap syndrome (39/46 [85%] *vs* 3/85 [4%], $P < 0.001$). It was more reliable than bile-duct lesions for the distinction of PBC from AIH.

Conclusions: Although AIH/PBC overlap syndrome does exist, it is infrequent and needs to be diagnosed carefully using close clinical and histological follow up to enable timely and effective treatment.
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Key words: acidophilic body, autoimmune hepatitis, overlap syndrome, primary biliary cirrhosis.

INTRODUCTION

Autoimmune liver disease has an uneven geographic distribution, and is much less frequent in Japan than in Western countries. There have been increasing registrations of autoimmune liver disease in the Ministry of Health, Labor and Welfare of Japan. It is not certain, however, whether this increase is attributable to a real increase as a result of changes towards a more Western lifestyle, or to the inclusion of more patients previously unrecognized before the advent of modern diagnostic techniques. In fact, the diagnostic criteria for autoimmune hepatitis (AIH) has been modified often, in 1993[1] and 1999[2] by International Committees, as well as in 1992 and 1996 in Japan.[3] The lack of specific markers for AIH makes it difficult to establish a clear-cut and indisputable consensus for its diagnosis.

By contrast, antimitochondrial antibodies (AMA) are widely accepted as the hallmark of primary biliary cirrhosis (PBC),[4–6] although doubts have been cast on their being essential to diagnosis.[7,8] In addition, there are characteristic bile-duct lesions and granuloma that help diagnose PBC. Although there are many features in common between AIH and PBC,[7–10] they are at the opposite ends of the spectrum of autoimmune liver disease and differ remarkably with respect to pathological findings and therapeutic interventions.

During the 1970s, there were scattered reports on the co-occurrence of AIH and PBC in the same patients.[11–14] Since then, development of methods to

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distinguish between AIH and PBC, along with specific therapies for treating each condition using immunosuppressants and ursodeoxycholic acid (UDCA), respectively, appeared to temporarily draw a clear line between them.[15,16] At present, however, there is much argument either for the common occurrence (7%) of an AIH/PBC overlap syndrome,[17] for which a combination therapy with immunosuppressants and UDCA is indicated, or for disproving the concept of the overlap syndrome by defining it as a hepatic form of PBC.[18,19] In Japan, where AIH and PBC are much less common than in Western countries, the overlap syndrome, should it exist, would be taken more seriously in evaluating the existence of the 'true' AIH/PBC overlap syndrome. A close survey with long-term follow up has been conducted on patients with autoimmune liver disease in a city hospital in Japan to verify the authenticity of the AIH/PBC overlap syndrome.

METHODS

Patient populations

From 1979 to 2000, 227 patients with autoimmune liver disease were treated in the Gastroenterology Department of Toranomon Hospital in Tokyo, Japan. The diagnosis of autoimmune liver disease, including AIH, PBC and the overlap thereof, was made based on clinical symptoms, biochemical and serological tests and markers of hepatitis virus infections in sera taken at the first liver biopsy before the start of therapy. They were followed for biochemical and serological findings, as well as pathological changes in the liver. The study design conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the institution. A written informed consent was obtained from each patient.

Diagnostic criteria

The patients with AIH fulfilled the score for 'definite AIH' (>15) or 'probable AIH' (10–15), using the criteria of the International Autoimmune Hepatitis Group as proposed in 1999; they satisfied the Japanese diagnostic criteria agreed upon in 1994, also. The diagnosis of PBC was based on the criteria implemented by the Japanese Ministry of Health and Welfare in 1992. The patients who satisfied criteria for both AIH and PBC at presentation or during follow up were deemed to have the AIH/PBC overlap syndrome.

Autoantibodies

The presence of antinuclear antibodies (ANA), antismooth muscle antibodies (ASMA) and AMA was determined using indirect immunofluorescence on rat liver tissues with the standard methods. AMA of M2 category[20] was determined using a standard ELISA method incorporating beef pyruvate dehydrogenase.

Histological examinations

Liver specimens were obtained with a Silverman needle under laparoscopy. They were fixed with formalin, embedded in paraffin, cut into 2-μm slices and stained with HE, D-periodic acid Schiff (D-PAS) and silver. Specimens from patients suspected of the overlap syndrome were cut serially, and three serial 2-μm slices were each stained with either HE, D-PAS or silver, and the maneuver was performed 10 times for coverage of a total thickness of 120 μm. In addition, sections were stained with Evan's van Gieson and chromotrim-aniline blue as well as for iron. Histological assessments were made by a single pathologist who was unaware of the biochemical and serological profiles of patients. Pathological evaluation was made using the METAVIR score on liver specimens from patients with AIH and using the Scheuer classification on those with PBC; specimens from patients suspected of AIH/PBC overlap syndrome were evaluated using both methods.

The number of acidophilic bodies was counted in a high-power field (×400), and graded high for ≥6, middle for 2–5 and low for ≤1 per field.

Statistical analysis

Categorical and continuous variables were compared between groups using the two-tailed Mann–Whitney *U*-test. *P*-values <0.05 were considered significant.

RESULTS

Patients suspected of having the autoimmune hepatitis/primary biliary cirrhosis overlap syndrome

Of the 227 patients with autoimmune liver disease in Japan, 19 (8.4%) were diagnosed with the AIH/PBC overlap syndrome. Table 1 compares the features of the 19 patients with the overlap syndrome against those of the 52 patients with AIH as well as the 156 patients with PBC. Women predominated in the group with AIH/PBC overlap syndrome. Decrease in the transaminase levels occurred was greatest in the AIH group, followed by the overlap and the PBC groups, while immunoglobulin (Ig)M increased in the same order.

The biochemical, serological and histological profiles of the 19 patients with AIH/PBC overlap syndrome, along with the evolution of AMA in them, are shown in Table 2. Of these patients, seven (cases 2, 3, 7, 13, 15, 18 and 19), met the score for definite AIH (>15), and the remaining 12 for probable AIH (10–15). A ratio of alanine aminotransferase (ALT) over alkaline phosphatase (ALP) >3.0 was found in 10 patients (cases 1, 2, 5–8, 12–14 and 16). AMA persisted in six (cases 1–6), turned negative in 12 (cases 7–18) and became positive during follow up in the remaining one (case 19).