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Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A

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Complete nucleotide sequences of 19 hepatitis B virus (HBV) isolates of genotype A (HBV/A) were determined and analysed along with those of 20 previously reported HBV/A isolates. Of the 19 HBV/A isolates, six including three from Japan and three from the USA clustered with the 14 HBV/A isolates from Western countries. The remaining 13 isolates including four from The Philippines, two from India, three from Nepal and four from Bangladesh clustered with the six HBV/A isolates reported from The Philippines, South Africa and Malawi. Due to distinct epidemiological distributions, genotype A in the 20 HBV isolates was classified into subtype Ae (e for Europe), and that in the other 19 into subtype Aa (a for Asia and Africa) provisionally. The 19 HBV/Aa isolates had a sequence variation significantly greater than that of the 20 HBV/Ae isolates (2·5±0·3% vs 1·1±0·6%, $P<0·0001$); they differed by 5·0±0·4% (4·1–6·4%). The double mutation (T1762/A1764) in the core promoter was significantly more frequent in HBV/Aa isolates than in HBV/Ae isolates (11/19 or 58% vs 5/20 or 25%, $P<0·01$). In the pregenome encapsidation (ε) signal, a point mutation from G to A or T at nt 1862 was detected in 16 of the 19 (84%) HBV/Aa isolates but not in any of the 20 HBV/Ae isolates, which may affect virus replication and translation of hepatitis B e antigen. Subtypes Aa and Ae of genotype A deserve evaluation for any clinical differences between them, with a special reference to hepatocellular carcinoma prevalent in Africa.

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INTRODUCTION

Hepatitis B virus (HBV) has been classified into seven genotypes based on a sequence divergence over the entire genome exceeding 8% (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000), and they are designated by upper-case letters from A to G. A possible eighth genotype is proposed with a tentative designation of H that is closely related to genotype F phylogenetically (Arauz-Ruiz *et al.*, 2002). The six major HBV genotypes (A–F) have distinct geographical distributions (Lindh *et al.*, 1997; Magnius & Norder, 1995). HBV genotypes A and D are predominant in Europe, North America and Africa, while genotypes B and C are prevalent in east and south Asia. On clinical fronts, there have been increasing lines of evidence to indicate influences of HBV genotypes on the outcome of liver diseases in hosts and the

The sequences reported in this article have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB116076–AB116094.

response to antiviral therapies, especially between genotypes A and D prevalent in Western countries as well as B and C common in Asia (Chu *et al.*, 2002; Kao *et al.*, 2002; Kobayashi *et al.*, 2002; Mayerat *et al.*, 1999; Orito *et al.*, 2001; Sugauchi *et al.*, 2002a; Wai *et al.*, 2002). Information is limited for geographical distribution or clinical relevance of genotypes G and H which were discovered recently (Arauz-Ruiz *et al.*, 2002; Kato *et al.*, 2002a, b; Stuyver *et al.*, 2000).

Virological characteristics and clinical manifestations may differ, however, even amongst HBV isolates of the same genotype. We have reported two subtypes of genotype B, designated Ba (a for Asia) and Bj (j for Japan), of which Ba has the recombination with genotype C over the precore region plus core gene, while Bj does not (Sugauchi *et al.*, 2002b). Response to antiviral therapies and the prevalence of hepatitis B e antigen (HBeAg) differed amongst patients with chronic liver diseases who were infected with HBV/Ba and HBV/Bj (Akuta *et al.*, 2003; Sugauchi *et al.*, 2003b). Likewise, amongst isolates of HBV genotype A (HBV/A), two subtypes have been reported, one of which distributes widely in European countries and the USA, while the other prevails in sub-Saharan Africa (Bowyer *et al.*, 1997; Kramvis *et al.*, 2002; Sugauchi *et al.*, 2003a). The subtype of genotype A, designated A′ by Bowyer *et al.* (1997), seems to be virologically distinct from the original genotype A and associated with reduced serum levels of HBV DNA and a low prevalence of HBeAg in serum (Kramvis *et al.*, 1997, 1998). In addition, subtype A′ may have an association with hepatocellular carcinoma prevalent in Africa (Attia, 1998; Edman *et al.*, 1980; Olweny, 1984).

Complete nucleotide sequences were determined for 19 HBV/A isolates recovered from the USA and Asian countries. Including the sequences of 20 HBV/A isolates retrieved from the DNA databases, 20 isolates of the original genotype A and 19 isolates of subtype A′ were compared phylogenetically and for unique mutations in their nucleotide sequences. Due to distinct epidemiological distributions, together with marked virological differences, we would like to propose the classification of the original genotype A prevalent in European countries into subtype Ae (e for Europe) and A′ common in African and Asian countries into subtype Aa (a for Africa/Asia).

METHODS

Serum samples. Nineteen serum samples containing HBV/A were collected from native HBV carriers in various countries [Bangladesh, $n=4$; India, $n=2$; Japan, $n=3$; Nepal, $n=3$; The Philippines $n=4$; USA, $n=3$ (none of African ethnicity)]. Samples from India, Nepal and The Philippines were submitted by doctors who attended the Second Workshop on Hepatocellular Carcinoma in Asia held on 21 February 2002 in Tokyo by the Miyakawa Memorial Research Foundation. HBV genotypes were determined by ELISA with a commercial kit (HBV GENOTYPE EIA, Institute of Immunology Co., Ltd) involving monoclonal antibodies to type-specific epitopes in the preS2 region product (Kato *et al.*, 2002b; Usuda *et al.*, 1999, 2000) as well as by RFLP on the small-S gene sequence amplified by PCR with nested primers (Mizokami *et al.*, 1999). The entire nucleotide sequences of the HBV/A isolates in the 19 serum samples, which had been stored at −20 °C, were determined. The study protocol was approved by the Ethics Committees of the institutions, in accordance with the 1975 Declaration of Helsinki, and an informed consent was obtained from each HBV carrier.

Determination of the full-length sequence of HBV. Nucleic acids were extracted from serum (100 µl) using a DNA extractor kit (Genome Science Laboratory). HBV DNA fragments covering the entire genome sequence in 19 samples were amplified by the method reported previously (Sugauchi *et al.*, 2001). Amplified HBV DNA fragments were sequenced directly by the dideoxy method using a *Taq* Dye Deoxy Terminator cycle sequencing kit and a fluorescent 3100 DNA sequencer (Applied Biosystems).

Phylogenetic analysis. Complete genome sequences of 46 HBV isolates were aligned using the CLUSTAL W software program (Thompson *et al.*, 1994), and the alignment was confirmed by visual inspection. The genetic distances were calculated with the 6-parameter method (Saitou & Nei, 1987), and the phylogenetic tree was constructed by the neighbour-joining method using the ODEN program of the National Institutes of Genetics (Mishima, Japan) (Ina, 1994). To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times.

Statistical analyses. Frequencies between groups were compared by the chi-square test or by Fisher's exact test. Differences were considered significant for P values less than 0·05.

RESULTS

Phylogenetic relatedness and genetic diversity of the two subtypes of HBV/A

Complete nucleotide sequences of 19 HBV/A isolates were determined. Of the 19 HBV/A genomes, 18 had a genome length of 3221 bp and one (HBV-NEP40) possessed a deletion of 21 nt in the preS1 region, as did three HBV/A isolates retrieved from the DDBJ/EMBL/GenBank databases (accession nos AF297623, AF297625 and V00866). The insertion of 6 nt characteristic of genotype A was present in the core region in all 19 HBV/A isolates. Together with the 20 complete genome sequences of HBV/A isolates retrieved from the databases, the 19 determined in the present study were subjected to phylogenetic analysis along with seven HBV isolates representative of genotypes B, C, D, E, F, G and H (Fig. 1). Three recombinant HBV strains of genotypes A and D [AF297621 (Kramvis *et al.*, 2002); AF418674 and AF418682 (unpublished)], as well as a single recombinant strain of genotypes A and A′ (Bowyer *et al.*, 1997), were excluded from the phylogenetic analysis.

Of the 19 HBV/A isolates for which full-length sequences were determined in the present study, 13 including four from The Philippines, two from India, three from Nepal and four from Bangladesh were classified into subtype Aa and clustered with the HBV isolates from The Philippines, South Africa and Malawi retrieved from the databases; they differed from one another in 2·5 ± 0·3 % (range 1·1–4·6 %) of the entire genome sequence by pairwise comparison. The remaining six HBV/A isolates including three from the USA and three from Japan were classified into subtype Ae, and clustered with the 14 HBV isolates from Western countries

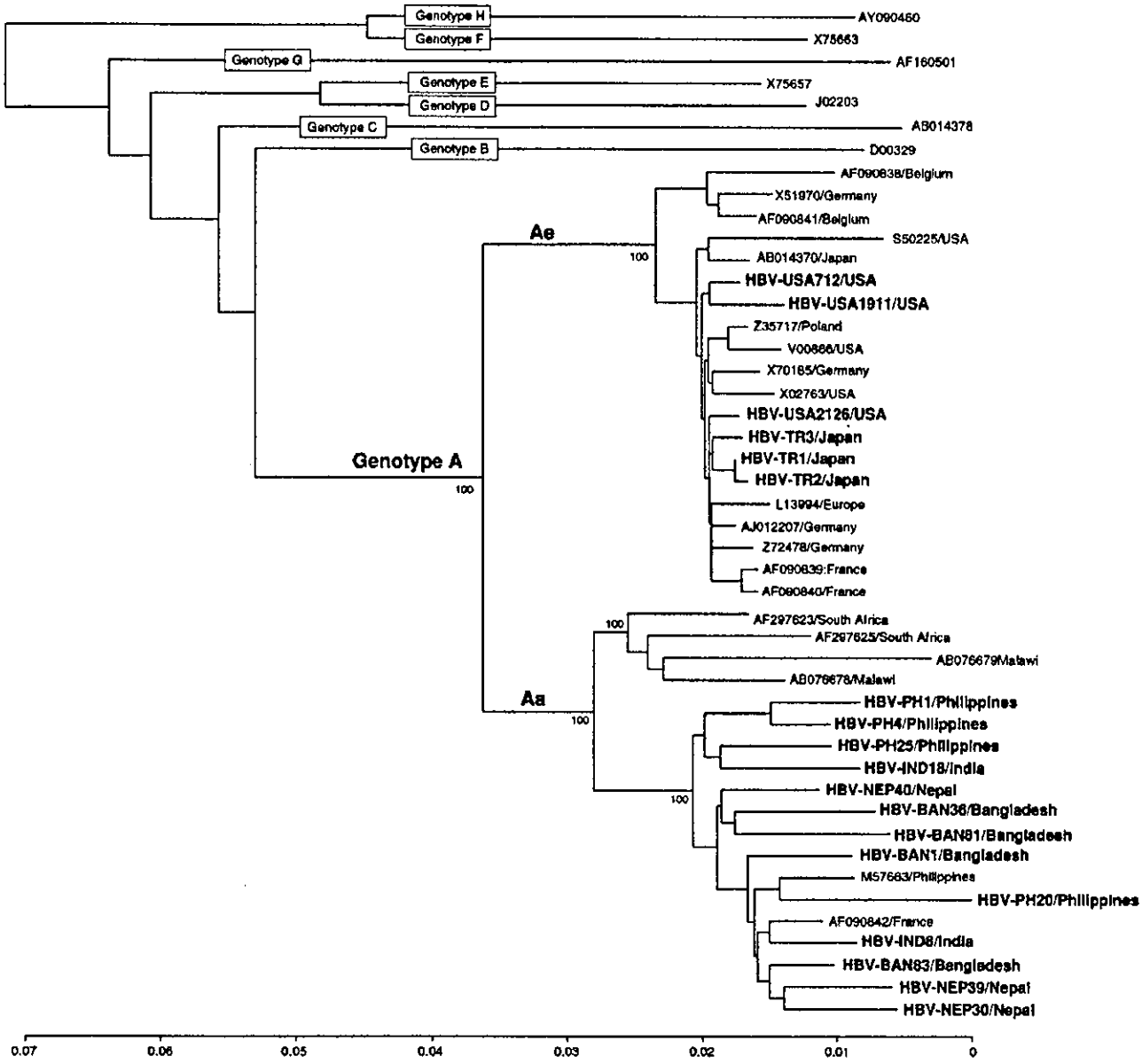

Fig. 1. Phylogenetic tree constructed using the entire nucleotide sequences of 46 HBV isolates. The 39 HBV/A isolates were compared with seven HBV isolates representing genotypes B–H. HBV/A isolates clustered on two branches, Ae (the original European genotype A) and Aa (the new African/Asian genotype A corresponding to A′ proposed by Bowyer *et al.*, 1997). The 19 HBV/A isolates, the sequences of which were determined in this study, are shown in boldface; accession numbers are given for sequences of the other 27 HBV isolates. The country of origin is indicated after a solidus for each HBV/A isolate. Genetic distance is indicated below the tree. Bootstrap values are shown at the nodes of the main branches.

and Japan whose sequences were retrieved from the databases. These 20 HBV/Ae isolates had a sequence variation of $1{\cdot}1 \pm 0{\cdot}6$ %, which was significantly smaller than the $2{\cdot}5 \pm 0{\cdot}3$ % in the 19 HBV/Aa isolates ($P < 0{\cdot}0001$) (Table 1). The inter-group sequence divergence between the 20 and 19 isolates of genotypes Ae and Aa, respectively, was $5{\cdot}0 \pm 0{\cdot}4$ % (4·1–6·4 %) by pairwise comparison.

Phylogenetic analyses were performed on 19 HBV/Aa isolates and six HBV/Ae isolates, the sequences of which were determined in the present study and retrieved from DNA databases, within four reading frames, i.e. the preS1/preS2 region, the S gene, the X gene and the precore/core region (Fig. 2). A clear separation of subtype Ae from Aa is seen in the tree topology for the preS1/preS2 region, the X gene and

Table 1. Mean number of differences in nucleotide sequences of the entire genome and its reading frames within 20 HBV/Ae and 19 HBV/Aa isolates as well as between them

The mean ± SD values are shown with the ranges in parentheses.

Reading frames	Differences within Ae or Aa isolates (%)		Differences between Ae and Aa isolates (%)
	Ae	Aa	
Entire genome	1·1 ± 0·6 (0·1–3·6)	2·5 ± 0·3 (1·1–4·6)	5·0 ± 0·4 (4·1–6·4)
preS1/preS2	1·4 ± 0·2 (0–4·6)	3·3 ± 0·4 (0·8–6·9)	6·6 ± 0·9 (5·0–8·6)
S gene	0·5 ± 0·4 (0–2·1)	1·4 ± 0·6 (0·3–2·9)	1·9 ± 0·5 (0·9–3·7)
X gene	0·9 ± 0·2 (0–1·9)	2·4 ± 0·4 (0·4–4·1)	4·1 ± 0·7 (2·2–5·6)
Precore/core	1·3 ± 0·2 (0–5·4)	3·0 ± 0·3 (0·6–6·4)	4·6 ± 0·6 (2·8–7·9)
Polymerase gene	1·0 ± 0·1 (0·1–2·4)	2·5 ± 0·2 (1·2–4·3)	5·0 ± 0·4 (4·1–6·0)

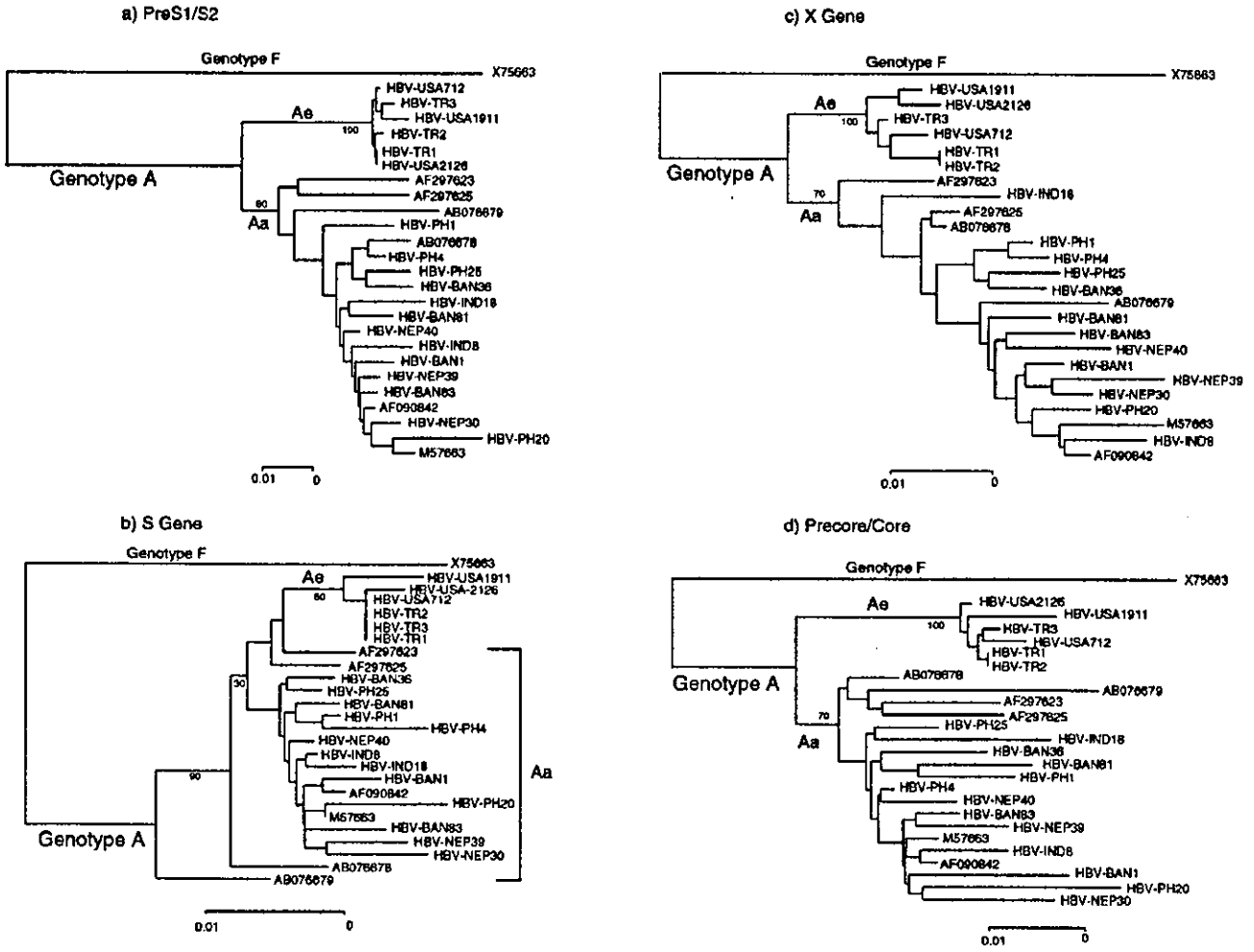

Fig. 2. Phylogenetic trees constructed using partial genome sequences of 25 HBV/A isolates. Nineteen full-length sequences were determined in the present study. Four trees representing (a) the preS1/preS2 region, (b) the S gene, (c) the X gene and (d) the precore region plus the core gene are shown with the sequence of genotype F serving as an outgroup. Genetic distance is indicated below each tree. Bootstrap values are shown at the nodes of the main branches.

the precore/core region. Phylogenetic trees constructed from S gene sequences, however, revealed no significant bootstrap values at the bifurcation of Ae and Aa.

With the availability of many sequences of the preS2 region and the S gene for genotype A isolates, 49 preS2/S sequences of genotype A were retrieved from the DNA databases, and a phylogenetic tree was constructed from them along with those of the 19 genotype A isolates sequenced in the present study (Fig. 3). Genotype A isolates from African countries (South Africa, Malawi and Zimbabwe) clustered with those from Asian countries (Bangladesh, India, The Philippines and Nepal) that were classified into subtype Aa, and they were separated from subtype Ae isolates from Western countries.

Pairwise genetic distances within 20 HBV/Ae and 19 HBV/Aa isolates, as well as between them, in the complete genome and each reading frame are shown in Table 1. The genetic divergence between HBV subtypes Ae and Aa was largest in the preS1/preS2 region amongst all the reading frames compared. Furthermore, HBV/Aa isolates had greater genetic divergence than HBV/Ae isolates in the complete genome as well as in all reading frames.

The serotype of hepatitis B surface antigen (HBsAg) was *adw* in all 20 HBV/Ae isolates and in 16 of the 19 HBV/Aa isolates. It was *ayw* in three HBV/Aa isolates including two (HBV-PH1 and HBV-PH4) from the present study and AB076678 from Malawi. Serotypes were deduced by codons 122 and 160 for either lysine or arginine (Okamoto *et al.*, 1987).

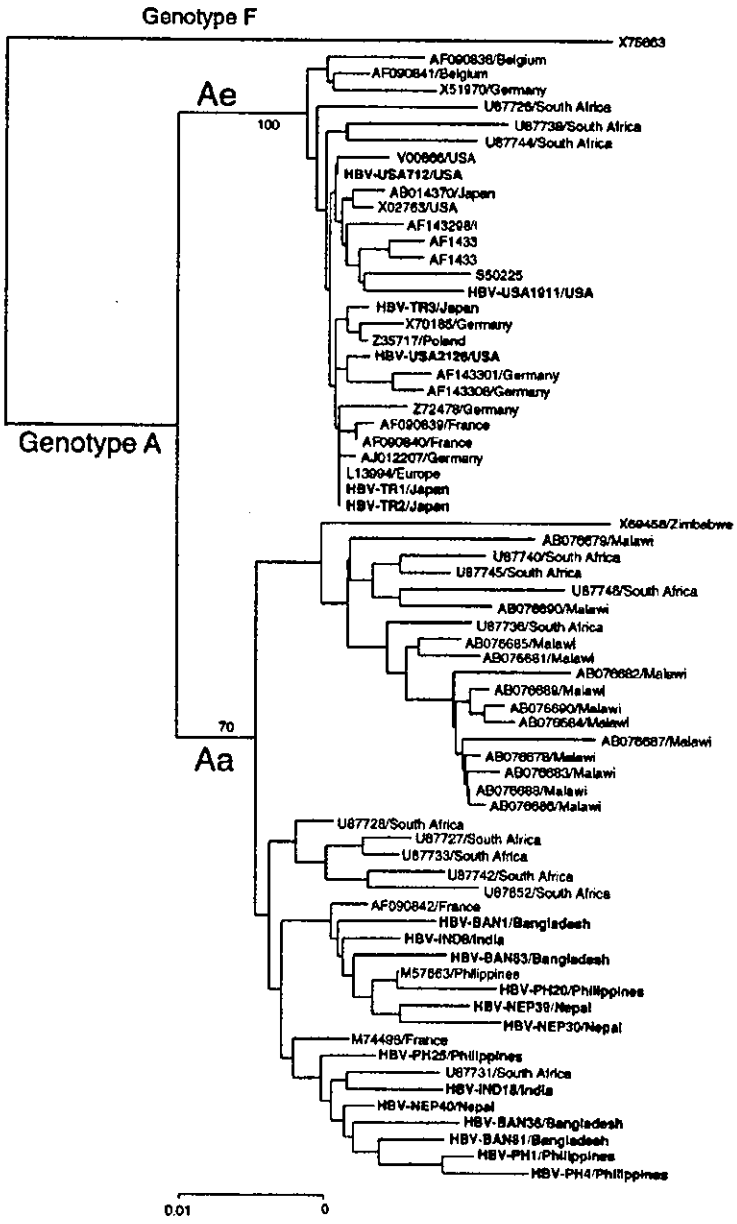

Fig. 3. Phylogenetic tree constructed using the preS2 and S gene sequences of 68 HBV/A isolates. They clustered on two separate branches, Ae (European genotype A) and Aa (African/Asian genotype A corresponding to A′ proposed by Bowyer *et al.*, 1997). The 19 HBV/A isolates, the sequences of which were determined in this study, are shown in boldface; accession numbers are given for sequences of the other 49 HBV isolates. The country of origin is indicated after a solidus for each HBV/A isolate. Genetic distance is indicated below the tree. Bootstrap values are shown at the nodes of the main branches.

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 %, $P<0{\cdot}01$). 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.

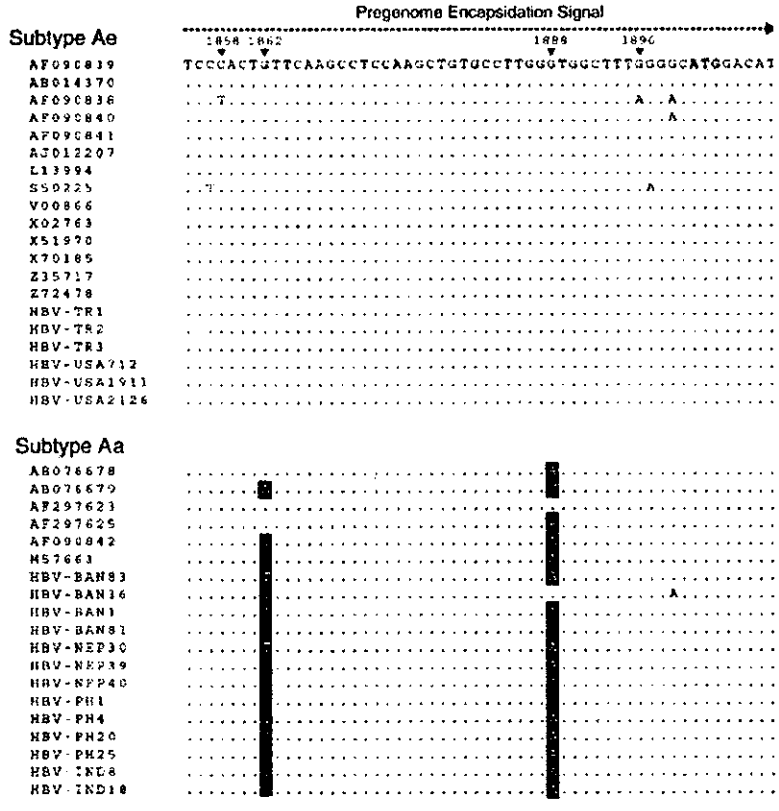

Fig. 4. Nucleotide sequences of the pregenome encapsidation (ε) signal in HBV/Ae and HBV/Aa isolates. Positions of C1858 making a pair with G1896 and those of the subtype-specific mutations at nt 1862 and nt 1888 are indicated by ▼. The start codon in the C gene is highlighted.

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).

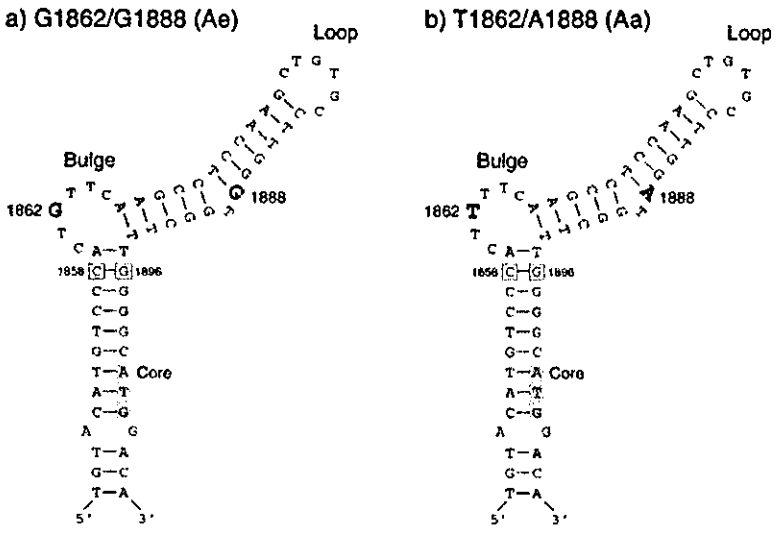

Fig. 5. Conformation of the pregenome encapsidation (ε) signal for HBV genomes of subtypes Ae and Aa. All 19 isolates of subtype Ae possess G1862 and G1888 (a), in contrast to T1862 and A1888 in 15 (79%) and 14 (74%), respectively, of the 19 HBV/Aa isolates (b). A Watson–Crick pair between C1858 and 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

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 *Escherichia 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

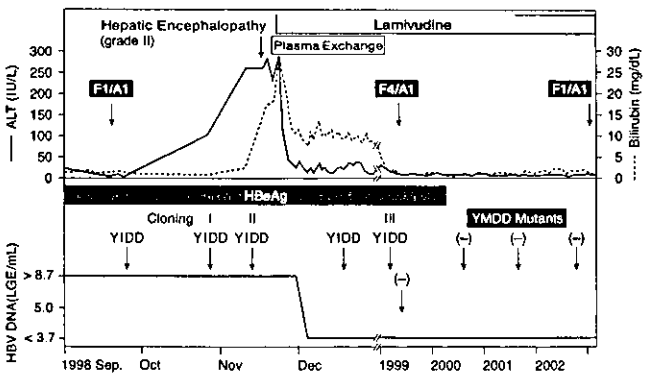

Fig. 1. Clinical course of the patient with chronic hepatitis B (Case 3 in Table I) who developed hepatic encephalopathy and in whom YIDD mutants were detected by PCR with peptide nucleric acid (PNA) clamping before he received lamivudine.

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.

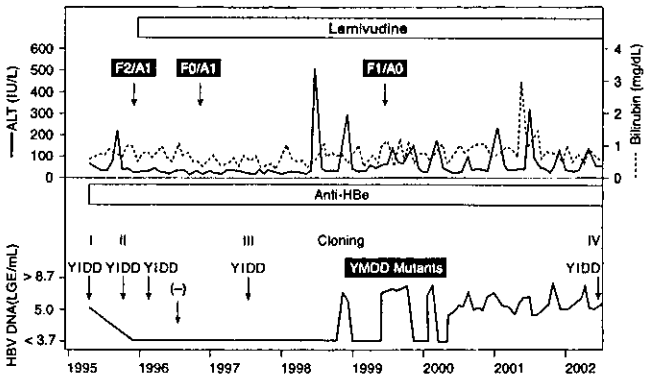

Fig. 3. Clinical course of the other patient with chronic hepatitis B in whom YIDD mutants were detected by PCR with PNA clamping before and after lamivudine therapy and who later developed breakthrough hepatitis.

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

	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

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

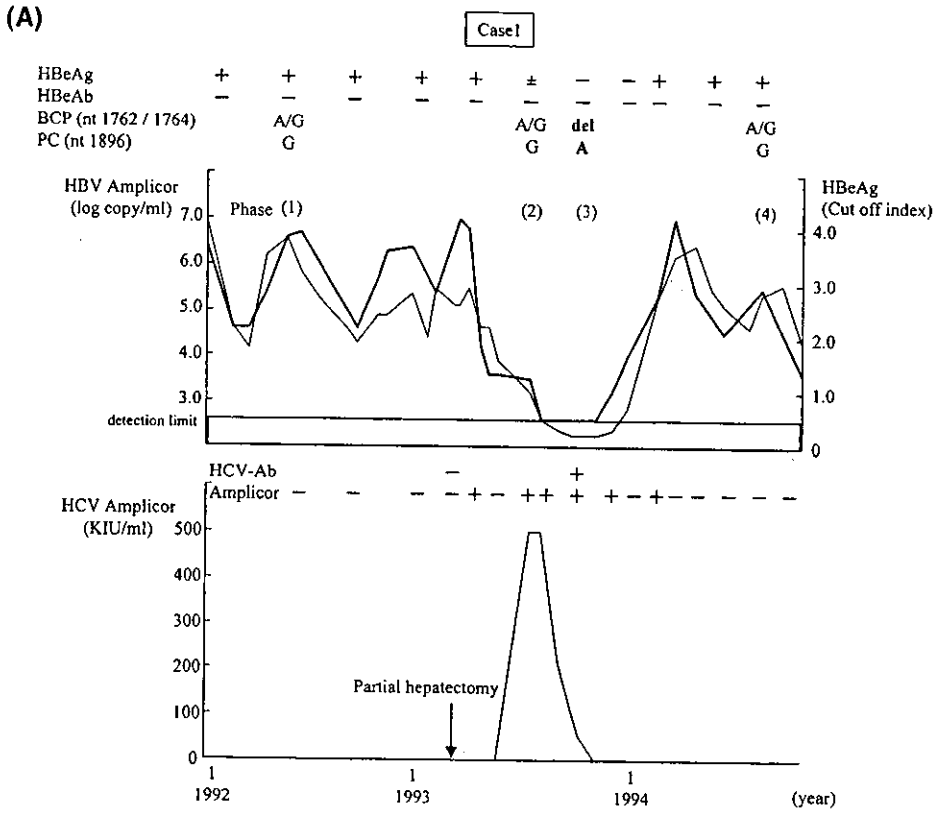
Case Histories

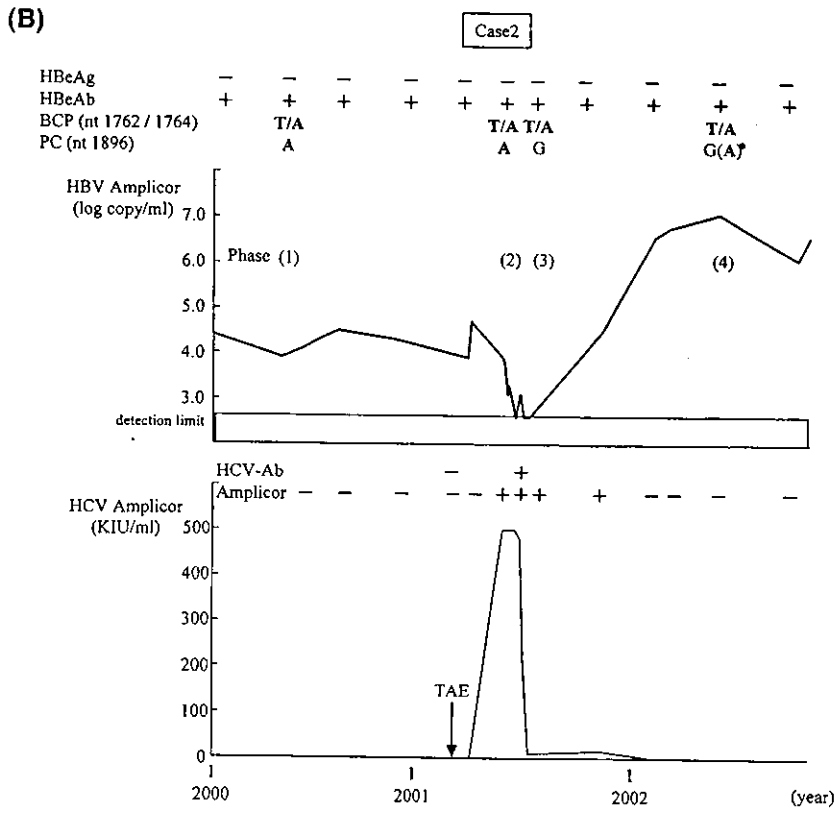
The clinical features of the two patients with HBV-related liver cirrhosis and acute HCV superinfection are summarized in Table 1. Both patients were HBV carriers infected with genotype C and were followed up at least once every month and remained negative for HCV-Ab and HCV-RNA. However, both became 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)
Case1
HBeAg
HBeAb
BCP (nt 1762 / 1764)
PC (nt 1896)
A/G
G
A/G
G
del
A
A/G
G
HBV Amplicor
(log copy/ml)
Phase (1)
(2)
(3)
(4)
HBeAg
(Cut off index)
detection limit
HCV-Ab
Amplicor
HCV Amplicor
(KIU/ml)
Partial hepatectomy
1992
1993
1994
(year)


(B)
Case2
HBeAg
HBeAb
BCP (nt 1762 / 1764)
PC (nt 1896)
T/A
A
T/A
A
T/A
G
T/A
G(A)
HBV Amplicor
(log copy/ml)
Phase (1)
(2)
(3)
(4)
detection limit
HCV-Ab
Amplicor
HCV Amplicor
(KIU/ml)
TAE
2000
2001
2002
(year)