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Completely or Nearly Identical Hepatitis B Virus Strains Replicate Between Patients With Acute or Fulminant Hepatitis B and Their Respective Infectious Sources

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Five patients with acute hepatitis B and four with fulminant hepatitis B were selected for sequencing of the precore/core gene of the virus strains. Furthermore, identical sequencing was done with the HBV of the infectious sources, i.e., the sexual partner in eight cases and a natural child (chronic carrier) infecting the mother in one case. Of the subjects responsible for the infection, four were healthy HBV carriers, three suffered from chronic hepatitis B, and one from acute and one from fulminant hepatitis B. The nucleotide sequences of HBV from both the patients and the implicated sources of infection exhibited perfect identity of the precore region and perfect or high identity of the core region. The completely or nearly identical strain of virus seemed to proliferate successively in the patients following the transmission from the infecting individuals regardless of sequence variations and infectious status. In two cases a peculiar pattern of infection and disease was found: In one married couple the husband, during the incubation period of acute hepatitis B, infected his wife, who developed fulminant hepatitis. In another married couple, both partners ultimately developed fulminant hepatitis (the wife being the source of the infection).

KEY WORDS: hepatitis virus, mutation, virus gene, virus transmission

INTRODUCTION

Recent sequencing of the precore region of hepatitis B virus (HBV) has revealed that seroconversion of hepatitis B e antigen (HBeAg) to anti-HBe in chronic HBV infection corresponds to the occurrence and prevalence of precore-defective mutations [Carman et al., 1989; Okamoto et al., 1990], and that the HBV responsible for fulminant hepatitis B frequently has such precore mutations [Kosaka et al., 1991; Carman et al., 1991; Kojima et al., 1991; Omata et al., 1991; Liang et al., 1991]. The precore gene encodes the precursor of HBeAg, and the precore-defective mutant, which usually results from a G-to-A point mutation at nucleotide 1896 from the *Eco*R1 site [Ono et al., 1986], converting tryptophan (TGG) to a stop codon (TAG), is not capable of producing HBeAg.

In previous sequence analysis it was found that HBVs isolated from patients with fulminant hepatitis showed nucleotide sequences with the precore-defective mutation identical with that of the infecting individuals who were responsible for HBV transmission [Terazawa et al., 1991; Yotsumoto et al., 1992]. Viruses with identical precore sequences seemed to multiply both in the patients and in the infectious source, despite the completely different clinical pictures.

In the present investigation we selected nine pairs of individuals with HBV infection, and sequenced not only the precore region but also the core region of HBV in order to confirm that the same virus strain proliferated successively in both the newly infected and infecting individuals. The patients included two married couples. In one couple, the husband, during the incubation period of acute hepatitis B, infected his wife, who subsequently developed fulminant hepatitis. In the other couple, both partners developed fulminant hepatitis B, the woman infecting the man. In both of these cases there was an interval of a few months between the onsets of the disease in the two partners. These rare cases provide important information about genetic variations in HBV and their relationship with the onset of fulminant infection.

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SUBJECTS AND METHODS

Patients

Eleven patients (including two married couples) were initially admitted to the Kin-ikyo Hospital with a diagnosis of acute (six cases) or fulminant (five cases) hepatitis B between February 1984 and June 1992. All the patients except for one (BV150) had hepatitis B surface antigen (HBsAg) (radioimmunoassay) and anti-hepatitis B core (HBc) IgM (radioimmunoassay). All the patients were negative for anti-hepatitis D (radioimmunoassay) and anti-hepatitis C (second generation, enzyme-linked immunosorbent assay). The diagnosis of fulminant hepatitis was made as defined previously [Trey and Davidson, 1970]. A list of the patients is shown in Table I.

The patients included two married couples (BV24 and BV149; BV151 and BV150). In the first couple, severe acute hepatitis B developed in the husband (BV149) after blood transfusion for a ruptured esophageal varix owing to alcoholic liver cirrhosis, and his wife (BV24) developed fulminant hepatitis B 3 months later. In the second couple, the wife (BV150) was admitted twice with jaundice with a 2-month interval. She had severe acute hepatitis at the first admission. On the second admission the hepatitis took a fulminant course. This patient showed a transient improvement of bilirubin and aminotransferases in the period between the two admissions. Although this case took a subacute course and did not correspond strictly to a diagnosis of fulminant hepatitis, it was classified as fulminant hepatitis for simplicity in this investigation. Her infectious source was unclear, and the serum samples taken at the time of both admissions were negative for HBsAg and anti-HBc IgM. Consequently the first diagnosis was non-A, non-B hepatitis. Her husband (BV151) developed fulminant hepatitis B 2 months after his wife's second admission.

Investigation of the sources of infection of the other seven patients revealed sexual partners except for BV139, where the infectious source appeared to have been a chronic carrier child. In the six cases involving sexual transmission between unmarried individuals, the patients had started to have sexual relationships with the respective implicated subjects shortly before admission. Among the six implicated subjects, three were considered to be healthy HBV carriers and three were considered to have chronic hepatitis B, a diagnosis later proven to be chronic active hepatitis B by liver biopsy. In the case involving the child, the husband of BV139 and her first son were negative for HBsAg, while her second son (BV140) was positive for HBsAg and was thought to be a healthy carrier. The patient had been negative for HBsAg prior to the delivery of BV140. The source responsible for infection of BV140 was unclear.

The relationship between the patients and their respective sources of infection was confirmed by the HBsAg subtype, except for the two couples, where the subtype was not detectable in BV24 and BV150. Patients BV147, BV24, BV151, and BV150 died of hepatic failure, while the others survived. A clinical summary of the patients and their respective sources of infection is shown in Table I. The sera were additionally examined for HBeAg/anti-HBe status (radioimmunoassay) and HBsAg subtype (enzyme-linked immunosorbent assay). All the HBV-related antigens and antibodies were examined by commercial laboratories.

Extraction, Amplification, and Sequencing of the Precore/Core Gene of HBV DNA

For the present study, the patients' serum samples obtained on admission (first admission serum for BV150) were selected. All the patients were admitted to the Kin-ikyo Chuo Hospital within 7 days after the onset of symptoms. Then, after an interview, blood was taken from the probable infectious sources.

A 100-μl aliquot of each serum sample was mixed with 300 μl of lysis buffer consisting of 12.0 mM Tris hydrochloride buffer pH 8.0/10.0 mM EDTA/0.6% sodium dodecyl sulfate/120 μg/ml proteinase K and incubated at 70°C for 3 hours. DNA was extracted with phenol/chloroform and was dissolved in 20 μl of water after ethanol precipitation.

Two pairs of oligonucleotide primers were synthesized by a 380B DNA synthesizer (Applied Biosystems Japan, Tokyo) to amplify the precore/core gene by nested polymerase chain reaction (PCR). One pair was P142 5′-GACTGGGAGGAGTTGGGGGA-3′ (at nucleotide 1728 to 1747 from the *Eco*R1 site of HBV DNA [Ono et al., 1986]) and P144 5′-GGATTAAAGACAGGTACAGT-3′ (at nucleotide 2508 to 2527); the other pair was P86 5′-GGAGATTAGGTTAAAGGTCT-3′ (at nucleotide 1748 to 1767) and P89 5′-AGACAGGTACAGTAGAAGAA-3′ (at nucleotide 2501 to 2520). The first PCR used P142 and P144 and the second PCR used P86 and P89.

Each PCR was carried out using 5 μl of serum DNA solution or the first PCR product as a template, each of the primers at 0.25 μM and 1 unit of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT) with the PCR buffer (50 mM KCl/10 mM Tris-HCl, pH 8.3/1.5 mM $MgCl_2$/0.002% gelatin) and each dNTP at 100 μM in a total volume of 100 μl. The mixtures were processed through 40 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes in a programmable heat block (Perkin-Elmer Cetus).

The PCR products of the second PCR were electrophoresed through a 2% agarose gel and were visualized by ethidium bromide fluorescence. The specific bands were excised from the gel and purified using a Geneclean II kit (BIO 101 Inc., La Jolla, CA) following the manufacturer's instructions.

The purified DNAs were then dissolved in 20 μl of water and a 5-μl aliquot was directly sequenced by the dideoxy sequencing method [Sanger et al., 1977] using a Sequenase kit (United States Biochemical Corp., Cleveland, OH). P86, P89, and an additionally synthesized internal oligonucleotide P141 5′-TTGCCTTCT-

TABLE I. Patients With Acute and Fulminant Hepatitis B and the Individuals Responsible for the Infection; Clinical Summary, Precore Status, and Numbers of Nucleotide Differences of the Precore/Core Genes Compared With the Consensus Sequence (Subtype adr)*

Subject No.	Age (years)	Sex	Diagnosis	Bilirubin (mg/dl)	ALT[a]	HBeAg/anti-HBe[b]	Subtype	Precore status	Non-synonymous substitution	Synonymous substitution
BV133	20	F	Acute hepatitis	4.7	1,315	+/−	adw	Intact	11	34
BV134	21	M	Healthy carrier	0.3	23	+/−	adw	Intact	11 (0)[c]	34 (0)
BV135	20	M	Acute hepatitis	19.3	1,356	+/−	adr	Intact	0	16
BV136	20	F	Healthy carrier	0.6	10	+/−	adr	Intact	0 (0)	17 (1)
BV137	47	M	Acute hepatitis	32.8	987	+/−	adr	Intact	0	8
BV138	33	F	Chronic hepatitis	0.5	42	+/−	adr	Intact	0 (0)	6 (4)
BV139	23	F	Acute hepatitis	2.9	1,692	+/−	adr	Intact	0	16
BV140	4 mo	M	Healthy carrier	0.4	18	+/−	adr	Intact	0 (0)	16 (0)
BV141	23	F	Acute hepatitis	3.3	1,749	+/−	ayw	Intact	10	45
BV142	22	M	Healthy carrier	0.4	16	+/−	ayw	Intact	10 (0)	46 (1)
BV145	22	F	Fulminant hepatitis	17.4	3,882	+/−	adr	Intact	9	47
BV146	28	M	Chronic hepatitis	0.5	79	+/−	adr	Intact	9 (0)	46 (1)
BV147	24	F	Fulminant hepatitis	9.3	6,860	+/−	adr	Defective	4	13
BV148	40	M	Chronic hepatitis	0.4	75	−/+	adr	Defective	4 (0)	13 (0)
BV 24	42	F	Fulminant hepatitis	19.2	2,670	−/+	ND[d]	Defective	9	13
BV149	41	M	Acute hepatitis	18.2	539	−/−	adr	Defective	10 (1)	14 (2)
BV151	50	M	Fulminant hepatitis	42.2	1,068	+/−	ar	Intact	2	14
BV150	47	F	Fulminant hepatitis	18.9	821	−/+	ND	Intact	4 (2)	12 (6)

*In each pair the lower subject infected the upper patient. Peak values of bilirubin and ALT in acute or fulminant hepatitis B are shown.
[a]ALT; serum alanine aminotransferase (international units).
[b]Data at the time of admission.
[c]Nos. in parentheses indicate nucleotide differences of precore/core gene between the patient and the corresponding source of infection in each pair.
[d]Not detected.

GACTTCTTT-3′ (at nucleotide 1955 to 1972) were used as the sequencing primers.

RESULTS

The nested PCR amplified successfully the HBV precore/core genes from all the patients and their implicated sources of infection including BV150. The amplified DNA was sequenced. The nucleotide sequences of all 18 precore/core genes differed from the subtype adr-consensus sequence [Ono et al., 1986] to a variable degree, ranging from six to 56 substitutions. Some of the nucleotide variations may have been due to subtype differences of HBsAg. Nucleotide variations classified into non-synonymous and synonymous substitutions are shown in Table I.

The predicted amino acid sequences are given in Figure 1. The consensus amino acid sequence of adr [Ono et al., 1986] is shown on the top line. Precore-defective mutants were found in two pairs of patients (BV147 and BV148; BV24 and BV149), while the other seven pairs had precore-intact HBV genomes. The precore regions of the patients and their respective sources of infection were perfectly identical at the nucleotide level.

Although the nucleotide sequences of all the HBV genomes differed from the consensus sequence to a variable degree, the amino acid sequences of 3 pairs (BV135 and BV136; BV137 and BV138; BV139 and BV140) were identical with the consensus sequence in the precore/core gene. The other six pairs had between two and 11 amino acid substitutions compared with the consensus sequence.

On the other hand, the sequences for the patients and their respective sources of infection showed complete or nearly complete identity at both the amino acid and nucleotide levels. The variations between the examined pairs ranged from 0 to 8 at the nucleotide level. Most of the nucleotide substitutions were synonymous, i.e., without amino acid change. Of the non-synonymous substitutions one was found between BV24 and BV149 and two between BV151 and BV150. These were the married couples, who had all developed acute or fulminant hepatitis B. It is noteworthy that the precore region in BV151 and BV150 was intact, allowing the synthesis of HBeAg.

DISCUSSION

The present results show that the HBV genomes isolated from the patients with acute or fulminant hepatitis had nucleotide and amino acid sequences perfectly identical in the precore region and perfectly or nearly identical in the core region with those isolated from the respective infecting individuals. Regardless of the precore/core gene sequence and the infectious state of

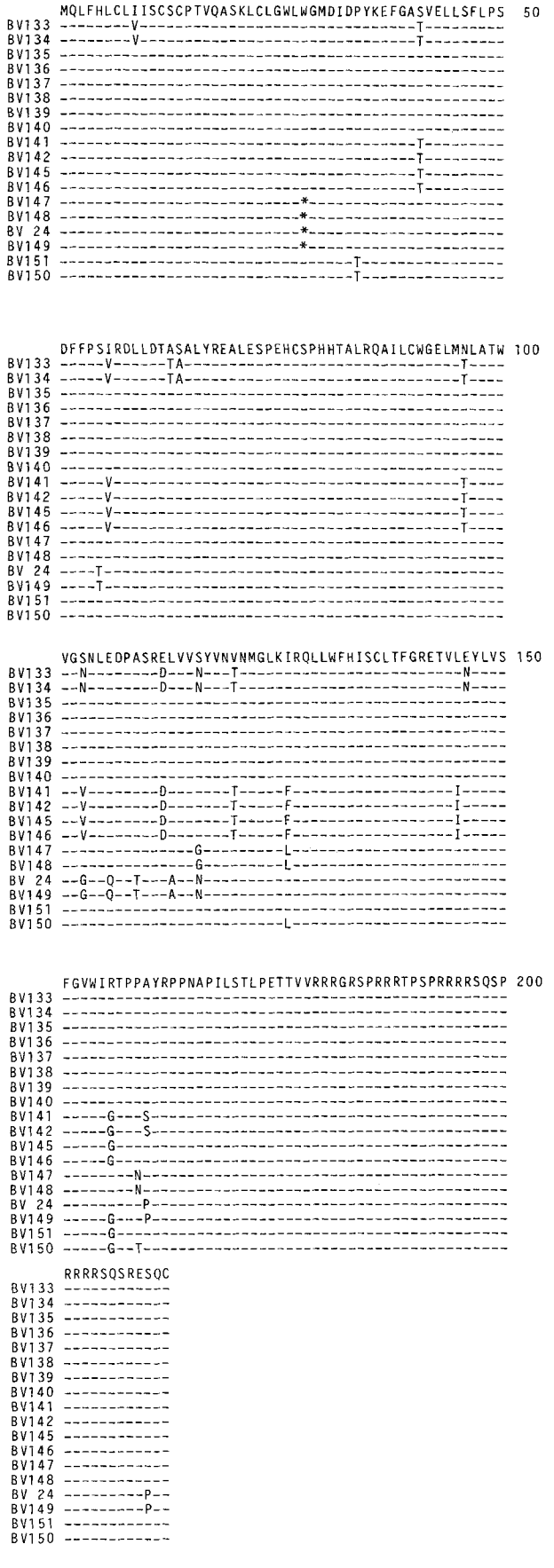

Fig. 1. Predicted amino acid sequences of the precore/core genes of the patients and infecting individuals. The top line shows the consensus amino acid sequence (subtype adr) in single-letter code. The sequences from patients and their sources of infection are shown below. Dots represent amino acid identity. * indicates the stop codon.

the patients and their implicated sources of infection, the virus of each pair was of the same, or a closely similar, strain.

The immune pressure of chronic HBV infection may force escape mutants to emerge over time in the HBV genomes. The precore/core gene is thought to be an immune target [Penna et al., 1991; Bertoletti et al., 1991], thus allowing frequent escape mutations to occur in this gene in chronic hepatitis. In such chronic infection, the HBV genome is thought to be heterogeneous; several virus strains with different sequences co-circulate in the sera of patients [Okamoto et al., 1987; Kaneko and Miller, 1989; Tran et al., 1991], and PCR may amplify the strains predominant among several variants. The present direct sequencing method can further determine the most predominant strain. Sequencing after cloning into plasmids may have revealed heterogeneous sequences in the present patients. In fact, positive serum HBeAg of BV147 with a precore-defective mutation suggests co-circulating precore-intact strains in the minor population.

HBV with the consensus sequence is probably a virus not exposed to immune pressure, because the virus isolated from HBeAg-positive healthy carriers, who showed immunological tolerance, always revealed the consensus sequence with an intact precore sequence [Ehata et al., 1992]. Although HBV can replicate without the precore function [Chang et al., 1987; Schlicht et al., 1987], a virus with an intact precore may have a replication advantage over a precore-defective mutant, as suggested by previous immunostaining of HBcAg and measurement of HBV DNA in sera [Suzuki et al., 1987]. The same assumption may be applied to the core gene mutation.

If this assumption is true, we would expect the replication of precore-non-defective and core-non-variable (precore/core-intact) HBV genomes rather than precore-defective and/or core-variable mutants in patients who were infected by individuals with presumably co-circulating heterogeneous HBV strains. However, the results were exactly the opposite, and the strains were concordant between the partners of each couple.

Three explanations are possible. First, the precore/core-intact HBV strain might have been completely eliminated from the infecting individuals by the time of transmission to other individuals. Second, the precore/core-intact variants, which were transmitted and proliferated together with the precore-defective and/or core-variable mutants in the successive patients, had been cleared before blood was taken for sequencing. Third, the precore-defective and/or core-variable mutants had a replication advantage equal to the precore/core-intact genome.

Although there were a few nucleotide sequence differences in the respective core regions of the viruses from couples we examined, most of them were synonymous substitutions. Non-synonymous substitutions were seen only in the two married couples, where both husband and wife developed acute or fulminant hepatitis. In these cases, the genome variations between the couples may have resulted from the fact that transmission occurred during the acute infectious state, when the circulating HBV genomes might have been more heterogeneously variable.

Precore-defective mutants were isolated in two (BV147 and BV24) out of five patients with fulminant hepatitis. The serine-to-proline mutation of the third-to-last codon of BV24 is frequently recognized in precore-defective fulminant hepatitis [Uchida et al., 1993]. In the other three patients (BV145, BV151, and BV150) with fulminant hepatitis, while the precore region was intact, we found nine, two, and four amino acid substitutions, respectively, in the core gene. In these three patients the core mutations or some mutations outside the precore/core gene may have played an important role in the development of fulminant hepatitis, although we were unable to specify them in the present investigation. In any event, cases BV151 and BV150 suggested the importance of the HBV strain with respect to fulminant onset.

HBVs with precore-defective mutations and/or some critical mutations outside the precore region may be capable of continued proliferation in subjects with chronic infection, because such mutants are adapted to the host's immune reaction. However, if such mutant viruses infect susceptible subjects, the patients will frequently develop fulminant hepatitis with an accelerated immune clearance.

Strangely, BV150 did not have any serological markers for acute hepatitis B infection, i.e., HBsAg and anti-HBc IgM, at either of the two admissions. Only PCR was able to prove this infection. We have experienced the same phenomenon in seven additional patients in another investigation [Uchida et al., 1993]. Although these seven patients had precore-defective mutations, the present patient BV150 exhibited an intact precore.

There were many variations in the core genes at the amino acid level in some HBVs in the absence of precore-defective mutation. These HBVs may be transmitted vertically from mothers to infants without provoking fulminant hepatitis. Such accumulations of mutations might occur either in the period of infection of a single subject or through several generations of transmission.

Finally, two (BV134 and BV142) of the four healthy HBV carriers had seven and two amino acid substitutions in the core gene compared with the sequence of the respective subtype, *adw* [Ono et al., 1986] and *ayw* [Galibert et al., 1979]. BV134 was followed for more than 5 years and was confirmed to be a healthy carrier. This finding is contrary to a previous report [Ehata et al., 1992]. On the other hand, one (BV138) of the three patients with chronic active hepatitis did not display amino acid mutation in the HBV precore/core gene.

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