MUTATIONS IN THE PRECORE REGION OF HEPATITIS B VIRUS DNA IN PATIENTS WITH FULMINANT AND SEVERE HEPATITIS

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Abstract *Background.* The presence of the hepatitis B e antigen (HBeAg) in serum is known to be a marker of a high degree of viral infectivity. However, fulminant hepatitis may occur in persons who are negative for HBeAg. A single point mutation has been reported to produce a stop codon in the precore region of hepatitis B virus DNA and prevent the formation of the precore protein required to make HBeAg. To determine whether a precore-mutant virus is causally related to severe liver injury, we analyzed the entire precore region in viral strains isolated from patients with fatal cases and uncomplicated cases of hepatitis B.

Methods. Serum was obtained from 9 patients with fatal hepatitis B (5 with fulminant and 4 with severe exacerbations of chronic hepatitis) and 10 patients with acute, self-limited hepatitis B. Serum samples from a sex partner implicated as the source of the virus in one case of fulminant hepatitis were also studied. The 87 nucleotides in the precore region of the hepatitis B virus were amplified by the polymerase chain reaction and then directly sequenced.

Results. Of the nine patients with fatal hepatitis, seven had retrievable hepatitis B DNA. In all seven there was a point mutation from G to A at nucleotide 1896 of the precore region, converting tryptophan (TGG) to a stop codon (TAG). In contrast, this mutation was not found in the 10 patients with acute, self-limited hepatitis B. The hepatitis B DNA from the implicated source contained a sequence with the stop-codon mutation that was identical to the sequence in her partner, who had fulminant hepatitis.

Conclusions. The presence of a mutant viral strain is associated with and may be involved in the pathogenesis of fulminant hepatitis B and severe exacerbations of chronic hepatitis B. (N Engl J Med 1991; 324:1699-704.)

RECENT studies of hepatitis B virus (HBV) have shown that this small DNA virus has four open reading frames: the S gene coding for an envelope protein (hepatitis B surface antigen, HBsAg), the core gene coding for a core protein (hepatitis B core antigen, HBcAg), the P gene coding for a DNA polymerase, and the X gene. The core gene encodes 183 amino acid residues of a nucleocapsid protein and is preceded by the precore region, which starts with an initiation codon and encodes 29 amino acid residues.[1] Precore and core genes have one in-phase open reading frame, but separate initiation codons. Recently, hepatitis B e antigen (HBeAg) was shown to be derived from the cleavage of the translation product of the entire precore region and core gene.[2-4] Subsequent investigations of chronically infected patients with antibodies to HBeAg (anti-HBe) found one point mutation that produces a stop codon in the precore region of HBV DNA.[5-8] Earlier studies suggested that fulminant hepatitis was occasionally related to infection transmitted by persons positive for anti-HBe.[9-13] To determine whether the mutant virus is causally related to severe liver injury, we analyzed the nucleotide sequence of the entire precore region of HBV DNA from patients who died of either fulminant hepatitis B or a severe exacerbation of chronic hepatitis B.

Methods

Patients

Serum samples were taken from nine patients with fatal hepatitis B: five patients with fulminant hepatitis and four patients with severe exacerbations of chronic hepatitis B. Of the five patients with fulminant hepatitis, two had post-transfusion fulminant hepatitis and three had sporadic fulminant hepatitis (Table 1). Of the three patients with sporadic hepatitis, one (Patient 3) appeared to have been infected by his anti-HBe–positive female sex partner. Serum samples were obtained from the 34-year-old woman during the patient's hospitalization. She had only mild increases in serum levels of liver enzymes and had been a carrier of HBV for at least three years. All five patients had antibodies to hepatitis B core antigen (anti-HBc IgM). Although Patient 4 was seronegative for HBsAg at admission, the diagnosis of fulminant hepatitis B was made on the basis of the strong positive result of the assay for anti-HBc IgM (Table 1).

Four patients (Patients 6, 7, 8, and 9) (Table 1) were given a diagnosis of severe exacerbation of chronic hepatitis B for the following reasons: all were known to have been seropositive for HBsAg at least 5 to 36 months before the exacerbation of the disease; all had an increase in serum aminotransferase levels that coincided with markers of active HBV replication (HBV DNA in serum and a conversion to positive results in the assay for anti-HBc IgM); none had superinfection with hepatitis A or C virus, as indicated by serologic tests (see below); and none had taken hepatotoxic drugs or had been exposed to other possible causes of hepatic injury. An autopsy was performed in eight of the nine cases (Table 1).

Serum samples were also taken from 10 patients with typical, self-limited, acute hepatitis B. These patients had high serum aspartate aminotransferase levels (mean, 8.86 μmol per second per liter) and were seropositive for HBsAg and anti-HBc IgM. Liver-biopsy samples were obtained from 7 of these 10 patients and had the typical pathological features of acute hepatitis. All these patients recovered quickly, and HBsAg cleared from the serum. To establish whether there was a mutation of the precore region of HBV DNA in five patients with nonfatal acute hepatitis B, two samples were obtained from each patient, one taken during the HBeAg-positive phase and one during the anti-HBe–positive phase. These five pairs of samples were obtained an average of 28 days apart (minimum, 16 days; maximum, 42 days). In the remaining five patients, serum samples were already negative for HBeAg when they were examined. Thus, only one sample was examined for these five patients.

Serologic Analysis of Hepatitis Virus Markers

HBsAg, HBeAg and anti-HBe, IgM antibodies to the hepatitis A virus, and antibody to delta antigen were assayed with solid-phase radioimmunoassays (Ausria II, HBe radioimmunoassay kit, Havab-M, and Anti-Delta, respectively; Abbott, North Chicago). Anti-HBc IgM was assayed by radioimmunoassay (IgM class anti-HBc radioimmunoassay kit, Dainabbott, Tokyo). Anti–hepatitis C virus was assayed by enzyme immunoassay (Ortho Diagnostics, Tokyo).

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Table 1. Clinical and Pathological Characteristics of Nine Patients with Fatal Fulminant Hepatitis or Severe Exacerbations of Chronic Hepatitis B.*

Patient No.	Age/Sex	Type of Hepatitis B	Initial Serologic Results			HBV DNA§		Length of Known Carrier State	Pathological Findings
			HBsAg	HBeAg/anti-HBe†	anti-HBc IgM‡	spot	PCR		
								mo	
Fulminant									
1	77/F	Post-transfusion	+	−/−	+	−	+	0	Submassive necrosis
2	59/F	Post-transfusion	+	−/−	+	−	+	0	Massive necrosis
3	38/M	Sporadic	+	−/−	+	−	+	0	No autopsy done
4	50/M	Sporadic	−	−/−	+	−	−	0	Submassive necrosis
5	32/M	Sporadic	+	−/+	+	−	−	0	Massive necrosis
Severe exacerbation									
6	68/F	Chronic	+	−/+	−	2+	+	5	Submassive necrosis
7	64/M	Chronic	+	−/+	−	2+	+	27	Submassive necrosis
8	67/F	Chronic	+	−/+	+	3+	+	9	Cirrhosis and necrosis
9	64/F	Chronic	+	−/+	+	3+	+	36	Submassive necrosis

*None of these patients were seropositive for antibody to delta antigen, IgM antibodies to hepatitis A virus, anti–hepatitis C virus antibody, or hepatitis C virus RNA on the basis of PCR results.

†For the HBeAg assay a value of 2.1 or more was considered positive; the patients' values ranged from 0.2 to 1.5. For the anti-HBe assay, values above 50 percent were considered positive; the patients' values ranged from 28 to 97 percent.

‡Patients 8 and 9 were seronegative for anti-HBc IgM when they were first found to be seropositive for HBsAg.

§The amount of HBV DNA was graded semiquantitatively as 1+, 2+, or 3+ (see the Methods section). Spot denotes spot hybridization.

HBV DNA was extracted from 100 μl of serum and detected by spot hybridization as described previously.[14] Positive radioactive signals on nitrocellulose papers were graded semiquantitatively as 1+ (0.5 to 20 pg), 2+ (21 to 100 pg), and 3+ (more than 100 pg).

Amplification and Sequencing of the Precore Region of HBV DNA

To amplify a segment of HBV DNA, we prepared a pair of synthetic oligonucleotide primers according to the sequence reported by Ono et al.[15]: nucleotides 1744 to 1761 (5′GGGAGGAGATTAGGTTAA3′) and nucleotides 1940 to 1959 (5′GGCAAAAA(A/C)GAGAGTAACTC3′) (Fig. 1). With the use of these primers, a segment of HBV DNA spanning 216 nucleotides (from 1744 to 1959) that constitutes the entire precore region was amplified by the polymerase chain reaction (PCR).[16] These primers were synthesized with a DNA synthesizer (model 380A, Applied Biosystems, Foster City, Calif.) according to the phosphoramidite method and purified with a high-performance liquid-chromatographic separation system (model 152A, Applied Biosystems).

Amplification of HBV DNA was performed according to the method of Saiki et al.[16] In brief, 100-μl reaction mixtures containing 10 μl of specimen DNA, 50 mM potassium chloride, 10 mM TRIS–hydrochloric acid (pH 8.4), 2.5 mM magnesium chloride, 1 μM each of the two oligonucleotide primers, 200 μM dNTP, 200 μg of gelatin per milliliter, and 2 units of *Taq* polymerase (New England Biolabs, Beverly, Mass.) were overlaid with 100 μl of mineral oil. Samples were heated at 95°C for five minutes (to denature the DNA), cooled to 55°C for two minutes (to anneal primers to the template DNA), and heated to 70°C for two minutes (to activate the enzyme and extend annealed primers). These steps were repeated for 30 to 50 cycles. Beginning with the second cycle the samples were heated at 95°C for two minutes. After the last cycle all the samples were incubated for an additional five minutes at 70°C to ensure that the final extension step was complete.

After the final step of amplification, each 10-μl sample was applied to an 8 percent acrylamide gel or to a composite gel of 3 percent Nusieve (FMC Bioproducts, Rockland, Me.) mixed with 1 percent Seakem agarose (FMC Bioproducts) in TRIS–borate buffer (pH 8.0) and electrophoresed beside pBR322 DNA digested with *Alu*I as a molecular-size marker. After electrophoresis the gels were stained with ethidium bromide for 10 minutes and observed under ultraviolet light. Southern blot hybridization was performed as described elsewhere[14] to confirm the presence of a homologous sequence in the amplified product.

For direct sequencing of a portion of HBV DNA, the PCR products were centrifuged in a microconcentrator (Centricon 30, Amicon, Danvers, Mass.). The sequence was determined according to a modification of the procedure of Sanger et al.[17] To sequence the amplified segment biodirectionally, we prepared two oligonucleotide primers: nucleotides 1774 to 1792 (5′TAGGAGGCTGTAGGCATAA3′) and nucleotides 1916 to 1932 (5′GCTCCAAATTCTTTATA3′). One sequencing primer was radiolabeled with [^{32}P]ATP and T_4 polynucleotide kinase (Fig. 1). After purification in the microconcentrator, 1 to 10 pmol of PCR product and 5 pmol of ^{32}P-labeled sequencing primer were combined in 12 μl of a mixture consisting of 50 mM potassium chloride, 50 mM TRIS (pH 8.0), 5 mM magnesium chloride, and 10 mM dithiothreitol. The direct sequencing of the PCR products was performed as previously described.[18]

Detection of Hepatitis C Virus RNA

To exclude the possibility of superinfection with hepatitis C virus in the patients with fatal hepatitis, RNA was extracted from 1 ml of serum, and the presence or absence of hepatitis C virus RNA was determined by the PCR, as described previously.[19]

Results

Detection of HBV Markers

The assay for serum HBeAg or anti-HBe was strongly positive in only one of the five patients with fulminant hepatitis (Patient 5) (Table 1). In contrast, all four patients with fatal exacerbations of chronic hepatitis B were positive for anti-HBe.

Of the nine patients with fatal hepatitis, the assay for anti-HBc IgM was strongly positive in all five patients with fulminant hepatitis. Furthermore, during the course of their illness two of the four patients with fatal exacerbations of chronic hepatitis B seroconverted from negative to positive (Table 1). HBV DNA was not detected by the spot hybridization technique in any of the 5 patients with fulminant hepatitis (Table 1) or in the 10 patients with acute, self-limited hepatitis. In contrast, it was detected in all four patients with severe exacerbations of chronic hepatitis B (Table 1). HBV DNA could be detected only by PCR in three of the five patients with fulminant hepatitis. In the other two patients (Patients 4 and 5), both of whom had

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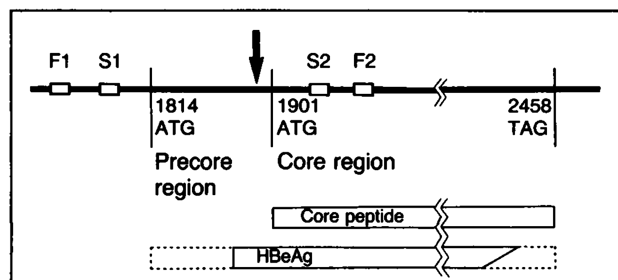

Figure 1. Schematic Representation of the Precore and Core Regions of the HBV Genome.

The core region (nucleotides 1901 to 2458) encodes 183 amino acid residues of a nucleocapsid protein and is preceded by the precore region (nucleotides 1814 to 1900), which encodes 29 amino acid residues.[15] Since the precore and core genes have an in-phase open reading frame, the open reading frame starting at nucleotide 1814 could encode the precore and core peptide that is processed to make HBeAg in the endoplasmic reticulum. Since the hydrophobic domain of the precore peptide functions as a leader sequence to anchor the peptide to the membrane of the endoplasmic reticulum, the stop-codon mutation (nucleotide 1896) in the precore region (arrow) will disturb the formation of HBeAg.

To amplify the precore region of HBV DNA, we prepared two pairs of synthetic oligonucleotide primers: F1 (nucleotides 1744 to 1761) and F2 (nucleotides 1940 to 1959) to amplify a precore segment of the HBV genome, and S1 (nucleotides 1774 to 1792) and S2 (nucleotides 1916 to 1932) to sequence the amplified precore segment of the HBV genome bidirectionally.

sporadic fulminant hepatitis B, HBV DNA was undetectable even with the use of this sensitive method. HBV DNA was detected by this technique in all 10 patients with acute, self-limited hepatitis.

Precore Sequence in Fatal Hepatitis

The entire nucleotide sequence (87 nucleotides) of the precore region was determined by direct sequencing in all seven patients with fatal hepatitis who were positive for HBV DNA by PCR (Fig. 2). Mutations were found in several locations of the region. A G-to-A nucleotide substitution converting tryptophan (TGG) to a stop codon (TAG) at nucleotide 1896 was found in all seven patients (two with post-transfusion hepatitis, one with sporadic fulminant hepatitis, and four with severe exacerbations of chronic hepatitis B) (Fig. 2). (The mutation at nucleotide 1896 corresponds to that reported elsewhere in this issue by Liang et al.[20] at nucleotide 1898.) In addition to this mutation, which introduced a stop codon into the precore region, an A-to-G missense mutation at nucleotide 1838, converting isoleucine (ATC) to valine (GTC), and an A-to-T mutation at nucleotide 1846 were found in all seven of these fatal cases (Fig. 2). The nucleotide sequence in the viral DNA from Patient 3 was identical to that in the viral DNA from his sex partner (Fig. 2).

The course of a patient with a severe exacerbation of chronic hepatitis B is shown in Figure 3. Patient 9 was a 64-year-old woman who had been known to be positive for HBsAg and anti-HBe since 1980. In 1982, her serum levels of alanine aminotransferase increased slightly, but she recovered from the episode (Fig. 3). Thereafter, she was asymptomatic until November 1983, when jaundice developed and she was admitted to our hospital. Hepatic failure developed gradually, and she died in February 1984. A postmortem examination revealed submassive necrosis of the liver. HBV DNA sequences in precore regions were studied in serum samples taken on five different occasions (October 27, 1980; February 22, 1982; December 29, 1983; and January 24 and 25, 1984) (Fig. 3). The stop-codon mutation at nucleotide 1896 and a missense mutation from isoleucine to valine at nucleotide 1838 were found in all five samples.

Precore Sequence in Acute, Self-Limited Hepatitis

The entire nucleotide sequence was also determined in 10 patients with acute hepatitis B that resolved. A mutation at nucleotide 1896 that would introduce a stop codon was not found in any of the 10 patients (Fig. 2). In all these patients liver function eventually returned to normal, and HBsAg was cleared from serum. The A-to-G missense mutation at nucleotide 1838, which converted isoleucine (ATC) to valine (GTC), and the A-to-T mutation at nucleotide 1846 were found in two of the patients.

To assess the possibility of a late appearance of a stop-codon mutation in the precore region, serum was obtained from five patients during both the HBeAg-positive and the anti-HBe–positive phases of acute hepatitis B. No differences in the nucleotide sequence were noted in the five pairs of samples.

Discussion

The clearance of the hepatitis B virus may be rapid in patients with fulminant hepatitis.[21] Thus, serum HBsAg is occasionally undetectable as patients progress to hepatic failure. This rapid clearance of the virus makes it difficult to obtain amounts sufficient to study the nucleic acid structures. Using PCR to amplify small amounts of nucleic acids,[16] we were able to study the gene structure of the hepatitis B virus in the serum. We studied nine patients whose course was fulminant or severe and who died of hepatic failure in our facility or an affiliated hospital at some time during the past six years. Of these, five patients had no known history of liver disease. An assay for serum anti-HBc IgM was strongly positive in all five patients. Postmortem examination of the liver revealed massive or submassive necrosis. Thus, these five patients appear to have had typical fulminant hepatitis B. The other four patients had a history as carriers of the hepatitis B virus, with severe exacerbation of hepatitis and hepatic failure. A precore mutation at nucleotide 1896 was found in all seven patients from whom we could retrieve HBV DNA by PCR amplification. In contrast, this mutation was not found in any of the 10 patients with typical acute hepatitis B.

Our finding that two patients who had fatal hepatitis after transfusion had the mutant virus is of particular importance. Currently, the Japanese Red Cross

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A

	Met ATG	Gln CAA	Leu CTT	Phe TTT	His CAC	Leu CTC	Cys TGC	Leu CTA	Ile ATC	Ile ATC	Ser TCA	Cys TGT	Ser TCA	Cys TGT	Pro CCT
Fulminant hepatitis															
Patient 1	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Patient 2	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Patient 3	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Source	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Severe exacerbation															
Patient 6	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Patient 7	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Patient 8	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Patient 9	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Acute hepatitis															
Patient 10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 12	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 13	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Patient 14	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 15	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 16	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 17	---	---	---	---	---	---	---	---	Val G--	---	--T	---	---	---	---
Patient 18	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 19	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

B

	Thr ACT	Val GTT	Gln CAA	Ala GCC	Ser TCC	Lys AAG	Leu CTG	Cys TGC	Leu CTT	Gly GGG	Try TGG	Leu CTT	Trp TGG	Gly GGC
Fulminant hepatitis														
Patient 1	---	---	---	---	---	---	---	---	---	---	---	---	Stop -A-	---
Patient 2	---	---	---	---	---	---	---	---	---	---	---	---	Stop -A-	Asp -A-
Patient 3	---	---	---	---	---	---	---	---	---	---	---	---	Stop -A-	---
Source	---	---	---	---	---	---	---	---	---	---	---	---	Stop -A-	---
Severe exacerbation														
Patient 6	---	---	---	---	---	---	---	---	---	---	---	---	Stop -A-	---
Patient 7	---	---	---	---	---	---	---	---	---	---	---	---	Stop -A-	Asp -A-
Patient 8	---	---	---	---	---	---	---	---	---	---	---	---	Stop -A-	---
Patient 9	---	---	---	---	---	---	---	---	---	---	---	---	Stop -A-	---
Acute hepatitis														
Patient 10	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 11	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 12	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 13	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 14	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 15	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 16	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 17	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 18	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Patient 19	---	---	---	---	---	---	---	---	---	---	---	---	---	---

Figure 2. Precore Nucleotide and Deduced Amino Acid Sequences of the HBV Genome.

These 18 sequences for nucleotides 1814 to 1858 (Panel A) and 1859 to 1900 (Panel B) in 3 patients with fatal fulminant hepatitis (Patients 1, 2, and 3) and the sex partner of Patient 3 (Source), in 4 patients with fatal severe exacerbations of hepatitis B (Patients 6, 7, 8, and 9), and in 10 patients with acute, uncomplicated hepatitis (Patients 10 through 19) were compared with the precore sequence reported by Ono et al.[15] The nucleotide mutations and the stop codon are shown.

checks all donated blood for the presence of HBsAg by reverse passive hemagglutination, and blood infected with the hepatitis B virus is eliminated. However, there is still a chance that blood containing levels of the virus that are below the detection limits of this screening method may be given to patients. Such blood might have a very low titer of HBsAg and should be anti-HBe–positive. We and others have previously shown that some anti-HBe–positive patients with chronic liver disease still have circulating virions detected by DNA hybridization.[22,23] Fagan et al. have reported the occurrence of fulminant hepatitis B in successive sex partners of an anti-HBe–positive patient.[12] Krogsgaard et al. also reported that fulminant, fatal hepatitis developed in one recipient of blood positive for HBV DNA and anti-HBe, although interestingly, HBeAg was not detected in the serum of that patient.[9] Similarly, infection due to anti-HBe–positive maternal blood has often been incriminated in the occurrence of fulminant hepatitis in infants.[10,11,24] These observations lead to the speculation that a low infecting dose of HBV can cause an overwhelming reaction, resulting in massive or submassive necrosis through unknown mechanisms. The present study provides molecular biologic evidence that this overwhelming reaction is associated with the presence of a precore-mutant HBV, whereas resolving acute hepatitis B is associated with wild-type HBV. This observation indicates that a difference in the strain may be critical in determining the outcome of an acute infection with HBV.

This study also provides evidence that precore-mutant viruses may be responsible for fatal exacerbations of chronic hepatitis B. Seroconversion from HBeAg to anti-HBe has been thought to be indicative of the loss of viremia and activity of chronic hepatitis.[22,25] However, increases in serum aminotransferase levels have often been observed in patients with anti-HBe–positive chronic hepatitis.[22,26,27] Indeed, advanced liver disease occurs more often in patients who are positive for both anti-HBe and HBV DNA.[22] We reported previously that the elevation of serum alanine aminotransferase levels coincided with the increase in HBV DNA polymerase activity and HBV DNA levels.[22,27] In the present study of fatal hepatitis, we found that the activated HBV DNA was not the wild type, but rather the precore mutant.

One can argue that patients with the precore-mutant virus are superinfected with the wild type of HBV, the delta agent, or hepatitis C virus, all of which are responsible for severe hepatic injury. Infection with the delta agent is rare in Japan and was excluded by serologic analysis. Hepatitis C virus infection was also excluded by the reverse PCR method, as well as by antibody testing. Regarding superinfection with or the coexistence of wild-type HBV, the nucleic

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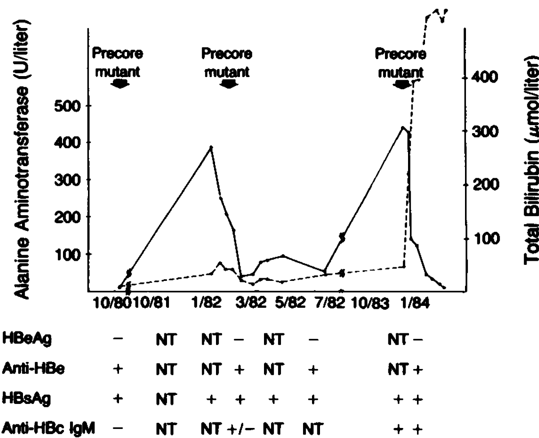

Figure 3. Clinical Course of a 64-Year-Old Patient with a Severe Exacerbation of Chronic Hepatitis B (Patient 9).

Serum total bilirubin (dashed line) and alanine aminotransferase (solid line) levels and the results of assays for HBeAg, anti-HBe, HBsAg, and anti-HBc IgM are shown. The patient had been known to be positive for HBsAg and anti-HBe since 1980. Jaundice and hepatic failure developed in November 1983, serum HBV DNA became detectable by spot hybridization, and the assay for anti-HBc IgM became positive. Postmortem examination revealed submassive necrosis of the liver. HBV DNA sequences in the precore regions were studied in serum samples taken on five occasions (October 27, 1980; February 22, 1982; December 29, 1983; and January 24 and 25, 1984; the last three of these five occasions are represented by one arrow because they were close together). The stop-codon mutation at nucleotide 1896 and a missense mutation from isoleucine to valine at nucleotide 1838 were found in all five samples. NT denotes not tested.

acids retrieved from our patients with fatal hepatitis were all precore mutants. In addition, none of the patients had serologic reactivation of HBeAg. These data argue against the involvement of the wild-type virus in fatal hepatitis.

The question could be raised whether the infecting strain was in fact the precore mutant or whether the mutation occurred during the course of hepatitis B infection. We observed the change from the wild-type virus to the mutant virus during chronic infection, but it usually took several years for the wild type to be completely replaced by the mutant (unpublished data). In this study, we tested the possibility of the late appearance of precore mutations in acute hepatitis B, which usually has a course of several weeks. In contrast to our observations in chronic hepatitis, in acute hepatitis we could not detect a shift of the viral population from the wild type to the mutant. Furthermore, we obtained samples from the apparent source of an acute fatal infection in one of our patients (Patient 3). The donor and the recipient had identical nucleotide sequences with a stop codon in the precore region. These data indicate that the precore-mutant virus was the infecting strain in this patient with fulminant hepatitis. Since it was impossible to obtain samples from the probable source in all the cases studied, other possibilities, such as rapid clearance of one of two initial strains, cannot be totally ruled out. In this regard, we recently succeeded in infecting one-day-old ducklings with wild-type virus only, the precore-mutant virus only, or both viruses (duck HBV). Only the precore-mutant virus produced pathological evidence of severe liver injury (unpublished data). In addition, in ducks infected with both strains, the mutant rather than the wild strain was eliminated first. These experimental data also support the contention that the precore-mutant strain is responsible for severe hepatic injury.

The mechanism by which these mutants induce hepatocyte injury is not known. HBcAg and HBeAg have been implicated as targets of cytotoxic T cells.[28,29] If HBcAg is produced by the mutants and expressed on the cell surface,[29] the absence of circulating HBeAg may divert a stronger immunologic attack by lymphocytes against HBcAg on hepatocytes, resulting in more severe hepatic injury. Another possibility is that the truncated peptide derived from the precore region with a stop codon may act as an immunologic target. In that context, a missense mutation at nucleotide 1838 may play an important part in the pathogenesis of severe hepatic injury. The mutation at the ninth codon of the precore region (nucleotide 1838) was documented in only 2 of the 18 reported nucleotide sequences.[30-39] The importance of this nucleotide needs to be examined in various liver diseases. A third possibility is that the mutant virus is directly cytopathic to the hepatocytes. In vitro, the deletion of the precore sequence resulted in a dramatic increase in the synthesis of HBcAg.[40] In contrast, cells containing the precore region predominantly express and secrete HBeAg in the culture medium.[40] Thus, the precore-mutant type may synthesize a much larger amount of HBcAg than the wild type and therefore act as a cytopathic virus.

Clinically, there is still a lack of reliable markers that predict the outcome of acute hepatitis. The most important clinical implication of our findings is that we may be able to predict the outcome of liver disease in patients with acute hepatitis B or an exacerbation of chronic disease by analyzing the precore sequences of the viral genome.

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