Hepatitis B Virus Mutants with Precore-Region Defects in Two Babies with Fulminant Hepatitis and Their Mothers Positive for Antibody to Hepatitis B e Antigen

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ABSTRACT. Clones of hepatitis B virus (HBV) DNA were propagated from sera of two babies who developed neonatal fulminant hepatitis B, as well as from sera of their mothers who carried HBV with antibody to hepatitis B e antigen, and the precore-region sequences were determined. A point mutation from guanine to adenine, converting codon 28 for tryptophan (TGG) to a stop codon (TAG), was detected in 18 of 20 HBV DNA clones from mother and all 31 clones from baby in one family, and invariably in 55 clones from mother and three clones from baby in the other family. These results indicate that HBV mutants defective in the precore region in some carrier mothers with antibody to hepatitis B e antigen may transmit fulminant hepatitis B to their babies. (*Pediatr Res* 29: 5-9, 1991)

Abbreviations

ALT, alanine aminotransferase
anti-HBe, antibody to hepatitis B e antigen
anti-HBs, antibody to hepatitis B surface antigen
anti-HDV, antibody to hepatitis delta virus
HBeAg, hepatitis B e antigen
HBsAg, hepatitis B surface antigen
HBV, hepatitis B virus
IgM anti-HBc, antibody against hepatitis B core antigen of Ig M class
nt, nucleotide
PCR, polymerase chain reaction

By contrast, babies born to carrier mothers with serum anti-HBe, rarely, if ever, develop a persistent carrier state. Some of them, however, contract acute HBV infection with or without clinical symptoms (5, 6). Although infrequently, fulminant hepatitis B develops in babies born to asymptomatic carrier mothers (7-13), and anti-HBe in sera of implicated mothers has attracted increasing attention (11-13).

Recent advances in the molecular biology of HBV have established the biosynthetic pathway of HBeAg. In HBV DNA, the C gene coding for the nucleocapsid protein is preceded in phase by the precore region composed of 87 bp and capable of encoding 29 amino acid residues (14). The product of the precore region and the C gene forms the precursor of HBeAg. The precursor is directed to the endoplasmic reticulum membrane by means of the signal peptide that is encoded within the precore region. There the precursor receives proteolytic cleavage and loses amino-terminal and carboxy-terminal parts. Resulting HBeAg protein, made of 159 amino acid residues, is secreted from hepatocytes into the circulation (15, 16).

HBV DNA clones were propagated, after amplification by PCR (17), from sera of two babies with neonatal fulminant hepatitis B and their mothers who carried HBV with serum anti-HBe. The great majority of HBV DNA clones from mothers, as well as all clones from their babies, was found to possess a precore-region defect that inhibited the synthesis and secretion of HBeAg. On the basis of these observations, some HBV mutants with precore-region defects in carrier mothers positive for anti-HBe would be particularly virulent and transmit fulminant hepatitis B by the perinatal infection.

MATERIALS AND METHODS

Individuals infected with HBV have HBsAg, representing the viral envelope, in the circulation. In addition, they have either HBeAg, which is closely related to the viral nucleocapsid (1), or anti-HBe. The presence of HBeAg or anti-HBe in the serum is significant for the immunopathologic status of hosts and also for the biology of HBV infecting them (2).

Perinatal HBV infection, resulting in a persistent carrier state of babies, is transmitted by carrier mothers who are positive for HBeAg (3, 4). Most babies born to them harbor HBV without symptoms; some develop a spectrum of hepatic disease ranging from chronic hepatitis to hepatocellular carcinoma later in their lives.

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Subjects. Case 1 in family A was a boy who developed high fever, convulsion, and disturbance of consciousness 2 mo after birth. Liver was not palpable. ALT was 5200 IU/L and bilirubin 5.2 mg/100 mL. He had coagulopathy with prothrombin less than 10% and fibrinogen 39 mg/100 mL. His serum was negative for HBsAg, and positive for anti-HBs with a hemagglutination titer of 2⁶. Neither HBeAg nor anti-HBe was detected, and anti-HDV was negative. He was positive for IgM anti-HBc, and the diagnosis of fulminant hepatitis B was established (18). His mother carried HBV asymptotically without elevated levels of ALT. Her serum contained HBsAg with a hemagglutination titer of 2⁵ and HBV DNA at a level of 1-5 pg/50 µL, and was positive for anti-HBe. After admission, hepatic encephalopathy of the baby aggravated to coma with serum ammonium 300 µg/100 mL. He responded to exchange transfusion, 700 mL each

four times, and infusion with branched-chain amino acids. On the 8th d after the onset, consciousness started to improve with ammonium decreasing to 101 $\mu\text{g}/100\text{ mL}$. Liver biopsy performed on the 15th d disclosed submassive necrosis of hepatocytes. The absence of fine droplets of lipid in a pan-lobular distribution, taken along with increased matrix density of mitochondria on electron microscopic observations, ruled out Reye syndrome. ALT levels gradually decreased, and he recovered completely 3 mo after the admission.

Case 2, a boy in family B, developed signs and symptoms of fulminant hepatitis 2 mo after birth. On admission, he was generally icteric with serum bilirubin 10.6 mg/100 mL, and ALT 3130 IU/L. Coagulopathy was evident with prothrombin less than 10% and fibrinogen 36 mg/100 mL. Liver biopsy was not performed. He had disturbed consciousness with ammonium 104 $\mu\text{g}/100\text{ mL}$. He was negative for HBsAg, and positive for anti-HBs with a titer of 2^3 . He was positive for anti-HBe. His serum revealed IgM anti-HBc for the diagnosis of fulminant hepatitis B. Anti-HDV was negative. His mother had been diagnosed with type B liver cirrhosis, histologically proven by liver biopsy performed 5 y before the delivery, and had moderately high ALT levels. Her serum taken 3½ mo before delivery contained HBsAg with a titer of 2^{11} and HBV DNA at a level of 1–5 pg/50 μL , and was positive for anti-HBe. The baby responded to treatments including exchange transfusion and recovered completely 3 mo after admission.

Serologic testing for HBV markers. HBsAg and anti-HBs were determined by passive hemagglutination with commercial assay kits (MyCell, Institute of Immunology Co., Ltd., Tokyo, Japan). The results were expressed by the highest 2-fold dilution of test sera (2^N) that induced a positive reaction. HBeAg and anti-HBe were determined with solid-phase enzyme immunoassay kits (HBeAg/Ab EIA, Institute of Immunology). IgM anti-HBc was determined by solid-phase enzyme immunoassay after the method described previously (19). Anti-HDV was determined by commercial RIA kits (Abbott Laboratories, North Chicago, IL). HBV DNA was detected by dot blot hybridization (20), and was semiquantitated by comparing the blot with those of controls with known HBV DNA levels.

Amplification of precore region by PCR and determination of nt sequence. HBV DNA was isolated from 100 μL of serum by the method described previously (21) and was dissolved in 20 μL of Tris-HCl buffer (10 mM, pH 8.0) containing 1 mM EDTA. HBV DNA fragments (nt 1653–1972) including the precore region (nt 1814–1900) were amplified, with appropriate oligonucleotide primers (21), by the method of Saiki *et al.* (17). Amplification was performed for 30 cycles in a 100- μL reaction volume with use of a Gene Amp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, CT).

HBV DNA amplified by PCR was digested with *EcoRI* and *HincII* (Takara Biochemicals, Kyoto, Japan), and the resulting fragment of 283 bp, representing nt 1682–1964 and including the precore region (nt 1814–1900), was cloned into M13 mp11 phage vector (Amersham Japan, Tokyo, Japan). The nt sequence of the precore region was then determined by the dideoxy-chain termination method (22).

RESULTS

The results of sequencing the precore region of HBV DNA clones from two mother-baby pairs are given in Table 1 along with serum marker of HBV infection. For family A, sera obtained from mother and her baby on the 16th d of admission were tested. For family B, three sera obtained from mother during 15 mo covering the delivery were tested, along with the serum obtained from her baby on the 7th admission day. Eighteen of 20 (90%) HBV DNA clones from mother in family A revealed defects in the precore region, whereas all 31 clones from her baby were precore-defective. In family B, all 55 HBV DNA clones

from serial sera from mother, as well as all three clones from her baby, disclosed defects in the precore region.

Nucleotide sequences of the precore region, along with deduced amino acid sequences, are depicted for HBV DNA clones from mother-baby pairs in families A and B (Fig. 1). The precore-region sequence of a nondefective HBV DNA clone is indicated at the top; the same sequence, with minor substitutions of nt not affecting the function of precore-region product, was displayed by HBV DNA clones from 14 sera from persistent carriers positive for HBeAg (21, 23). The majority of HBV DNA clones from mother and all clones from baby in family A, as well as all clones from mother and baby in family B, disclosed a point mutation from guanine to adenine at nt 83 in the precore region, converting codon 28 for tryptophan (TGG) to a stop codon (TAG).

In both families, HBV DNA clones from mother were heterogeneous, while those from baby were homogeneous and identical in sequence to one kind of precore-defective clone from mother. Of 20 HBV DNA clones from mother in family A, 15 (75%) had stop codon 28 along with codon 9 for valine, rather than isoleucine, in the precore region (Fig. 1, a). Among the remaining clones, three (15%) had stop codon 28 (Fig. 1, b) and two (10%) were not defective (Fig. 1, c). All 31 clones from baby displayed stop codon 28 (Fig. 1, d) with precore-region sequences identical to those of three clones from his mother (Fig. 1, b).

All 45 HBV DNA clones from mother in family B, propagated from her serum taken 3½ mo before delivery, possessed a G-to-A point mutation at nt 83 in the precore region (Fig. 1, e). Among them, 19 (42%) had an additional point mutation, from adenine to thymine, at nt 33 (Fig. 1, f) that did not change codon 11 for serine (TCA or TCT). Of 10 clones propagated from two sera of the mother, taken after delivery, one had the sequence (Fig. 1, e) and the remaining nine had the sequence (Fig. 1, f). All clones from her baby had stop codon 28 with nt 33 of T (Fig. 1, g), and were identical in sequence to one kind of clone from his mother (Fig. 1, f).

DISCUSSION

Although uncommon, neonatal fulminant hepatitis is a serious disease characterized by elevated levels of serum bilirubin during the earliest months of life and a high mortality. Etiology of childhood fulminant hepatitis is most commonly a complication of viral hepatitis, of which over 50% is associated with HBV infection (24). Fulminant hepatitis B is suspected by the detection of HBsAg or anti-HBs in the serum and diagnosed by high-titered IgM anti-HBc (18). An exaggerated immune response of the host to HBV-related antigens has been proposed as the pathogenesis of fulminant hepatitis B (25,26). Combined infections with HBV and hepatitis delta virus are known to increase the risk of developing fulminant hepatitis (27).

Neonatal fulminant hepatitis B clusters in babies born to certain carrier mothers (9, 10). Remarkably, it tends to develop in babies born to carrier mothers with serum anti-HBe (11–13). These lines of evidence strongly indicate that particularly virulent strains of HBV, possibly associated with serum anti-HBe, would transmit fulminant hepatitis B from mother to baby in the perinatal period.

HBV mutants with precore-region defects, incapable of directing the synthesis and secretion of HBeAg, gradually prevail and finally replace predecessor, nondefective HBV in persistent carriers who seroconvert from HBeAg to anti-HBe (21). To date, at least 25 carriers positive for anti-HBe have been found to possess HBV mutants with precore-region defect, whereas such mutants were not detected in 20 carriers with serum HBeAg; five carriers who were seroconverting from HBeAg to anti-HBe harbored both wild-type HBV and precore-defective mutants (21, 23, 28). Elimination of hepatocytes harboring nondefective HBV with the surface expression of HBeAg, by humoral and cellular immune responses of the host against HBeAg, is postulated as the

Table 1. Defects in precore region of HBV DNA clones from two pairs of mother and baby with neonatal fulminant hepatitis B*

	Date of cloning	HBsAg titer (2 ^N)	HBeAg or anti-HBe	Defects in precore region of HBV DNA†	
				+	-
Family A					
Mother	Mar. 23, 1981	5	Anti-HBe	18	2
Baby	Mar. 23, 1981	§	Anti-HBe	31	0
Family B					
Mother	Dec. 24, 1987	11	Anti-HBe	45	0
	Nov. 10, 1988	9	Anti-HBe	3	0
	Mar. 16, 1989	9	Anti-HBe	7	0
Baby	Apr. 7, 1988	§	Anti-HBe	3	0

* HBV DNA clones were propagated from sera of mother and baby with perinatal fulminant hepatitis B and sequenced for the precore region.

† Point mutation from guanine to adenine at nt 83, converting codon 28 for tryptophan (TGG) to a stop codon (TAG).

§ Anti-HBs was detected.

	10	20	30	40	50	60	70	80	↓
	ATGCAACTTTTTCACCTCTGCCTAATCATCTCATGTTTCATGTCTACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTGGGGC								
	MetGlnLeuPheHisLeuCysLeuIleIleSerCysSerCysProThrValGlnAlaSerLysLeuCysLeuGlyTrpLeuTrpGly								
Family A									
Mother									
(a)	-	-	-	-	-	-	-	-	-
(b)	-	-	-	-	-	-	-	-	-
(c)	-	-	-	-	-	-	-	-	-
Baby									
(d)	-	-	-	-	-	-	-	-	-
Family B									
Mother									
(e)	-	-	-	-	-	-	-	-	-
(f)	-	-	-	-	-	-	-	-	-
Baby									
(g)	-	-	-	-	-	-	-	-	-

Fig. 1. Nucleotide and deduced amino acid sequences of the precore region. The sequences of nondefective clones (21, 23), with the capacity to direct the synthesis and secretion of HBeAg, are indicated at the top. The sequences of HBV DNA clones from sera of mother and baby in families A and B are given below. HBV DNA clones from babies were homogeneous (d and g), all with stop codon 28, whereas those from mothers were heterogeneous (a-c, e, and f) and some of them were without precore-region defects (c). The arrow indicates nt 83 that is either guanine, contributing to codon 28 for tryptophan (TGG) in nondefective clones, or adenine, forming a part of stop codon (TAG) in precore-defective clones.

mechanism for the shift from predecessor, nondefective HBV to mutants with precore-region defects (21).

The precore-region sequences were determined for HBV DNA clones from sera of two babies with neonatal fulminant hepatitis B and their mothers who carried HBV with serum anti-HBe. A recently introduced technique for amplifying desired DNA sequences by PCR (17) was particularly useful in propagating HBV DNA clones from sera of babies without detectable HBsAg and positive for anti-HBs.

A point mutation from guanine to adenine at nt 83 in the precore region, converting codon 28 for tryptophan (TGG) to a stop codon (TAG), was invariably observed in HBV DNA clones from sera of mother and baby in family B, and in 90% of clones from mother and all clones from her baby in family A.

Heterogeneous HBV DNA clones, most with precore-region defects, were propagated from sera of mothers, and homogeneous clones, all with precore-region defects, were obtained from sera of their babies. This indicates that diverse mutations, in addition to the most prevalent G-to-A point mutation at nt 83, would have occurred in the precore region of HBV strains in the carrier mothers who would have had seroconverted from HBeAg to anti-HBe. Only one of the strains with a precore-region defect would have been transmitted to their babies perinatally and induced fulminant hepatitis B.

Anti-HBe detected in the baby in family B, who were infected with precore-defective HBV mutants, without capacity to encode HBeAg, requires an explanation. Anti-HBe in this baby would have been transported from his mother through the placenta or it might have been raised by him against the C-gene product, which is produced by a biosynthetic pathway distinct from HBeAg and assembles to make core particles (15, 16). The C-gene product has antigenic determinants of HBeAg, in addition to those of hepatitis B core antigen (1); a rabbit raised anti-HBe, as well as anti-HBc, when it was immunized with the C-gene product (29).

On the basis of results obtained, HBV mutants defective in the precore region would have been transmitted from mothers to babies, and induced neonatal fulminant hepatitis B. The lack of hepatitis delta virus infection in afflicted babies would stand in support of this view. To establish the causal relationship between precore-defective HBV mutants and fulminant hepatitis B, however, additional mother-baby pairs with perinatal transmission of fulminant hepatitis B would have to be examined, along with adult cases of posttransfusion or sporadic fulminant hepatitis B. It is necessary, also, to accumulate sequence data on HBV DNA clones from HBeAg-positive mothers and anti-HBe-positive mothers whose babies do not develop fulminant hepatitis B.

Neonatal fulminant hepatitis B is reported in babies born to carrier mothers who were HBeAg-positive and to those without detectable HBeAg or anti-HBe in the serum (11, 12). There still is a possibility that precore-defective HBV mutants would have induced fulminant hepatitis B in these babies. In view of a gradual shift from nondefective HBV to precore-defective mutants along seroconversion from HBeAg to anti-HBe (21), these carrier mothers, implicated in the transmission, might have harbored precore-defective mutants, as well as nondefective HBV, even in the presence of HBeAg or without detectable HBeAg/anti-HBe.

In sera from individuals persistently infected with HBV, serum HBeAg signals active replication of HBV with high infectivity, whereas anti-HBe denotes inactive replication with low infectious activity (2). There appear to be exceptions to this general rule, however. Sera from some patients with type B chronic active hepatitis contain high levels of HBV DNA, albeit they are positive for anti-HBe (30, 31). HBV DNA clones propagated from sera of patients with this disease are defective in the precore region and incapable of directing the synthesis and secretion of HBeAg (28, 32, 33). There would be HBV mutants with precore-region defects, therefore, that would have a high replicative activity and, when introduced to susceptible hosts, would be able to induce severe hepatitis.

Apparently, neonatal fulminant hepatitis B is transmitted by only a very minor population of carrier mothers with anti-HBe and precore-defective mutants. This would point to factors on the part of HBV, other than precore-region defects, that are responsible for an enhanced virulence. Of possible relevance to this context is the detection of HBV DNA at 1–5 pg/50 μ L, standing for approximately 10^6 infectious viruses/mL, in sera from both mothers implicated in the transmission of fulminant hepatitis B to their babies, despite the fact that they were positive for anti-HBe. It might be worthwhile to compare HBV DNA levels in sera of anti-HBe-positive carrier mothers who transmit fulminant hepatitis B to their offspring against those from such mothers whose babies do not develop fulminant hepatitis B.

Immunoprophylaxis of perinatal HBV infection, especially in districts of high endemicity, has been primarily indicated to babies born to HBeAg-positive carrier mothers for the purpose of preventing the development of a persistent carrier state. Apparently, babies born to carrier mothers positive for anti-HBe have to be protected also, lest they develop fulminant hepatitis B. The goal will be readily achieved by combined passive and active immunization, the efficacy of which has been proven in protecting babies born to HBeAg-positive carrier mothers (34, 35).

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