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Mutations within the S Gene of Hepatitis B Virus Transmitted from Mothers to Babies Immunized with Hepatitis B Immune Globulin and Vaccine

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ABSTRACT. A variant of hepatitis B virus (HBV) having a specific mutation within the S gene has been found to infect vaccinees. To know whether similar variants were involved in Japan, we analyzed two cases of maternal transmission of HBV in infants immunized with hepatitis B immune globulin and hepatitis B vaccine. DNA clones of HBV S genes were propagated from patients and family members and sequenced. In one family, the DNA clones from the baby patient had a Gly-to-Arg mutation at the 145th codon of the S gene, whereas those from her mother had no such mutations. In the other family, all the DNA clones obtained from the two infected children had the 145th codon intact, but they had a missense mutation at the 126th codon of the S gene, causing an amino acid substitution of Asn for Thr or Ile. This same mutation was observed in 12 of 17 clones of DNA obtained from their mother. In comparison with the wild type HBV-derived hepatitis B surface antigen, the two types of S gene mutations, either at the 145th or the 126th codon, were associated with a significant decrease in the antigenicity of some determinants on the hepatitis B surface antigen, measured by MAb. Amino acid substitution at these sites, therefore, would have induced the escape from conventional vaccines that were S gene products of wild type HBV and also from hepatitis B immune globulin, whose main components were probably also antibodies against the S gene products expressed by wild type HBV. (*Pediatr Res* 32: 264–268, 1992)

Abbreviations

HBV, hepatitis B virus
HBsAg, hepatitis B surface antigen
anti-HBs, antibody to HBsAg
HBeAg, hepatitis B e antigen
anti-HBc, antibody to hepatitis B core antigen
HBIG, hepatitis B immune globulin
A492, absorbance at 492 nm in ELISA
PHA, passive hemagglutination
PCR, polymerase chain reaction

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Prevention of mother-to-child transmission of HBV has been generally successful by immunoprophylaxis with HB vaccines, especially when combined with HBIG. However, a small portion (5–10%) of babies born to HBeAg-positive mothers become infected with the virus despite the preventive procedures (1–3). Initially, infection in vaccinees was associated with whether the vaccinees were responders or nonresponders to the vaccines used. However, Carman *et al.* (4) identified in vaccinated patients a variant of HBV that had a point mutation within the S gene resulting in an amino acid substitution of Arg for Gly at the 145th codon. This mutation, they reported, caused a reduction in the antigenicity of the determinant "a" of HBsAg, which is implicated in virus neutralization. The importance of the "escape mutants" of HBV will depend on their epidemiologic distribution and frequency of infection.

We analyzed two families in Japan where a mother-to-child transmission of HBV occurred despite immunizations with HBIG and vaccine. DNA clones of the HBV S gene from each subject were propagated and sequenced. Several distinct antigenic determinants on HBsAg in the circulation of the subjects were detected using mapped MAb. In this study, we found two types of S gene mutations: one at the 145th codon as reported by Carman *et al.* (4), another at the 126th codon. Both mutations were associated with a significant decrease in the antigenicity of specific determinants on HBsAg, and thus could effect escape from HBIG and wild type vaccines.

MATERIALS AND METHODS

Family 1. A 7-mo-old female baby born to an HBeAg-positive carrier mother developed HBs-antigenemia during the follow-up, although she had initially acquired anti-HBs antibodies passively by HBIG, and then maintained PHA titers of 1:8–1:16 through a protocol of three active immunizations, as depicted in Figure 1. The subject became seropositive for HBeAg and became a persistent carrier of HBV for at least 3 y without any symptoms. Sera from the patient obtained in January 1987 and from her mother in July 1986 were analyzed for the nucleotide sequence of the HBV S gene, and the HBsAg was tested for reactivity with antibodies mapped against known HBsAg determinants.

Family 2. A 9-mo-old female infant presented with a moderate degree of jaundice and hepatomegaly. Laboratory findings suggested that she was suffering from acute hepatitis B, positive for HBsAg and IgM anti-HBc, and with elevated liver enzymes in her serum. The patient, born to an HBeAg-positive carrier mother, had been immunized passively with HBIG twice at 0 and 60 d after birth, and actively immunized with HB vaccine

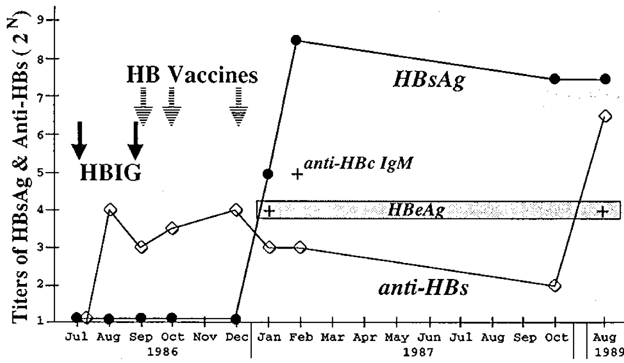

Fig. 1. Family 1 patient profile. The baby was born to an HBeAg-positive mother and was immunized with HBIG and vaccine (*arrows*), but developed persistent HBs-antigenemia. HBsAg and anti-HBs titers were determined by reversed PHA and PHA.

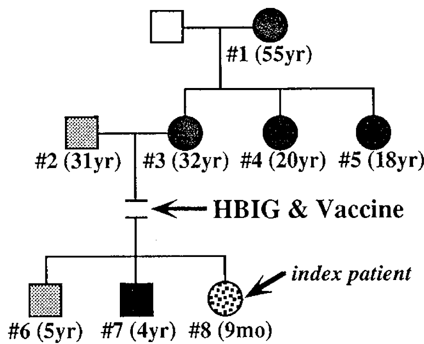

Fig. 2. Family 2 profile. *Squares* are males, *circles* are females. *Black*, HBsAg positive; *gray*, anti-HBs positive; *white*, not determined; and *dots*, acute hepatitis B.

Table 1. *Mother-to-child transmission of HBV variants with S genes mutated at either 126th or 145th codon**

Subjects	No. of clones examined	Codon 126 in S gene			Codon 145 in S gene	
		Thr (ACT)	Ile (ATT)	Asn (AAT)	Gly (GGA)	Arg (AGA)
Family 1						
Mother	4	0	4	0	<u>4</u>	0
Patient	4	0	4	0	0	<u>4</u>
Family 2						
Grandmother	16	<u>14</u>	2	0	16	0
Aunt	18	0	<u>18</u>	0	18	0
Aunt	20	0	<u>20</u>	0	20	0
Mother	17	2	3	<u>12</u>	17	0
Brother	20	0	0	<u>20</u>	20	0
Patient	19	0	0	<u>19</u>	19	0

* The numbers of clones with the highest frequency at the discordant codons are underlined.

three times at 2, 3, and 6 mo after birth. Her hepatitis resolved 2 mo later with a seroconversion to anti-HBs antibodies. Serum obtained during the acute phase was used for HBV DNA sequence analysis.

Clinical and serologic data were available for seven members of the patient's family as shown in Figure 2. One of her brothers (no. 7 in Fig. 2) was found to be an asymptomatic carrier of HBV, noted at 1 y of age. He had also received HBIG and vaccine on the same schedule as the patient. In contrast, another brother (no. 6) was positive for anti-HBs; anti-HBc was negative, suggesting that the anti-HBs detected in this child was a result of immunization with HBIG/vaccine and not a result of a natural seroconversion after cryptic infection. Four other family members (the mother, two aunts, and a grandmother) were healthy carriers of HBV, and the father was positive for anti-HBs antibodies. Sera were obtained in December 1990 or February 1991 from five family members (nos. 1, 3, 4, 5, and 7) and subjected to serologic investigation and analysis of HBV DNA sequence.

Sequence analysis. Serum (100 μL) was mixed with 300 μL of a Tris-HCl buffer (pH 8.0) containing the following ingredients: Tris 10 mM; EDTA 5 mM; SDS 0.5% (wt/vol); and proteinase K 100 μg/mL. The mixture was incubated at 70°C for 3 h. DNA was then extracted with phenol and chloroform, and precipitated with cold ethanol in the presence of 10 μg/mL transfer RNA. The precipitated DNA was dissolved in 20 μL of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA.

A portion of the S gene of HBV DNA was amplified by PCR using previously described primers: S1, 5′-TCGTGTTACAGGCGGGGTTT-3′ (nt 38–57 in the S gene), and S2, 5′-CGAACCACTGAACAAATGGC-′ (nt 531–550 of the complementary strand) (5). This PCR strategy surveys amino acids 14–183 of the 226 amino acid HBsAg, which circumscribes all known significant epitopes in HBsAg. The PCR was cycled 25 times. Each reaction cycle included denaturation at 94°C for 1 min, primer annealing at 55°C for 1.5 min, and primer extension at 72°C for 3 min. The PCR products were digested with the restriction enzymes *Xba*I and *Spe*I (Takara Biochemicals, Kyoto, Japan), and the resulting fragments of 437 bp (nucleotides 93–529 in the S gene) were inserted into the *Xba*I site of M13 mp11 phage vector. Both plus and minus strands were then sequenced by Sanger's method (6).

Monoclonal anti-HBs antibodies. To characterize the HBsAg surface epitopes for mutant and wild type viruses, we used murine monoclonal anti-HBs antibodies, nos. 3207, 824, 3423, and 313, previously mapped by Usuda *et al.* (7), and no. 5124, by Ohnuma *et al.* (8). In addition, we prepared MAb no. 7604 (IgG1/κ), by the following methods. Female BALB/c mice (7 wk of age) received intraperitoneally 200 μg of human plasma-derived HBsAg (subtype adr) as an emulsion with Freund's complete adjuvant (Difco Laboratories, Detroit, MI). After 5 wk, they were boosted by i.v. administration of 40 μg of the HBsAg. Three d later, spleen cells from the mice were fused with NS-1 myeloma cells by a conventional method (9). Clones were selected by an ELISA with the HBsAg/adr solid antigen.

Quantitative measurement of antigenic determinants on HBsAg. The MAb directed to the S gene product of HBV were used for measuring antigenic activities of various determinants on the HBsAg in the circulation of subjects. Sera from the subjects were adjusted to contain HBsAg with similar titer (1:16 by reversed PHA) by dilution with PBS (pH 7.5), and a 50-μL aliquot of each was added to microtiter plate wells that had been coated with 250 ng of a horse polyclonal anti-HBs antibody. After incubation for 1 h at room temperature with shaking, the plate was washed five times with 0.05% Tween20 in 150 mM NaCl. The plate well then received 50 μL of 50 ng/mL of the murine monoclonal anti-HBs, and was incubated for 1 h at room temperature with shaking. After washing five times with the same washing buffer as above, each well of the plate received 50 μL of 12 ng/mL horseradish peroxidase-labeled rabbit polyclonal anti-mouse IgG/M antibody. After the incubation with the tracer antibody at room temperature for 60 min with constant agitation, the plate was washed as above. Each well received 50 μL of 1.33 mg/mL *o*-phenylene diamine (Sigma Chemical Co., St. Louis, MO) and 0.17% (wt/vol) $NaBO_3$ (Wako Pure Chemicals, Osaka, Japan) dissolved in a buffer containing 0.1 M citrate and 0.2 M Na_2HPO_4. After incubation at room temperature for 30 min in the dark, 50 μL of 4 N H_2SO_4 was added to stop the color-developing reaction. Optical densities of the reaction products were then measured at a wavelength of 492 nm (A492). The above methods have been described by Ohnuma *et al.* (8) in greater detail.

Serologic testing for HBV markers. HBsAg (reversed PHA), anti-HBs (PHA), HBeAg (ELISA), and antibody to HBeAg

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       CCAGGAACATCAACTACCAGCACGGG*CCATGCAAGACCTGCACGA*TCCTGCTCAAGGA
Cons   ProGlyThrSerThrThrSerThrGlyProCysLysThrCysThr126 ProAlaGlnGly
       --------------------------A-------------------T-------------
I-M    - - - - - - - - - - - - - - - Ile - - - -
       --------------------------A-------------------T-------------
I-P    - - - - - - - - - - - - - - - Ile - - - -
       --------------------------G-------------------T-------------
II-Ma  - - - - - - - - - - - - - - - Ile - - - -
       --------------------------G-------------------C-------------
II-Mb  - - - - - - - - - - - - - - - Thr - - - -
       --------------------------G-------------------A-------------
II-Mc  - - - - - - - - - - - - - - - Asn - - - -
       --------------------------G-------------------A-------------
II-B   - - - - - - - - - - - - - - - Asn - - - -
       --------------------------G-------------------A-------------
II-P   - - - - - - - - - - - - - - - Asn - - - -

       ACCTCTATGTTTCCCTCTTGTTGCTGTACAAAACCTTCGGAC*GAAACTGCAC*TGTATT
Cons   ThrSerMetPheProSerCysCysCysThrLysProSerAsp145 AsnCysThrCysIle
       ------------------------------------------G----------T------
I-M    - - - - - - - - - - - - - - Gly - - - - -
       ------------------------------------------A----------T------
I-P    - - - - - - - - - - - - - - Arg - - - - -
       ------------------------------------------G----------C------
II-Ma  - - - - - - - - - - - - - - Gly - - - - -
       ------------------------------------------G----------C------
II-Mb  - - - - - - - - - - - - - - Gly - - - - -
       ------------------------------------------G----------C------
II-Mc  - - - - - - - - - - - - - - Gly - - - - -
       ------------------------------------------G----------C------
II-B   - - - - - - - - - - - - - - Gly - - - - -
       ------------------------------------------G----------C------
II-P   - - - - - - - - - - - - - - Gly - - - - -

       CCCATCCCATCATC*TGGGCTTTCGCAAGATTCCTATGGGAGTGGGCCTCAGTCCGTTTC
Cons   ProIleProSerSerTrpAlaPheAlaArgPheLeuTrpGluTrpAlaSerValArgPhe
       --------------C---------------------------------------------
I-M    - - - - - - - - - - - - - - - - - - - -
       --------------C---------------------------------------------
I-P    - - - - - - - - - - - - - - - - - - - -
       --------------T---------------------------------------------
II-Ma  - - - - - - - - - - - - - - - - - - - -
       --------------T---------------------------------------------
II-Mb  - - - - - - - - - - - - - - - - - - - -
       --------------T---------------------------------------------
II-Mc  - - - - - - - - - - - - - - - - - - - -
       --------------T---------------------------------------------
II-B   - - - - - - - - - - - - - - - - - - - -
       --------------T---------------------------------------------
II-P   - - - - - - - - - - - - - - - - - - - -
```

Fig. 3. Nucleotide and deduced amino acid sequences of the S gene of HBV DNA. Nucleotides 331 to 510 (amino acid residues 111 to 170) of the HBV S gene propagated from family 1 (*I-M*, mother; *I-P*, patient) and family 2 (*II-M*, mother; *II-B*, brother; *II-P*, patient). The mother of family 2 had three distinct sequences, *II-Ma*, *II-Mb*, and *II-Mc*, at the frequencies of three of 17, two of 17, and 12 of 17, respectively. Consensus sequences (*Cons*) are on the top line. Missense mutations not seen in wild type HBV are *boxed and shaded.* A *dash* denotes the same nucleotide or amino acid as the consensus.

(ELISA) were measured using commercial products (Institute of Immunology, Co., Ltd., Tokyo, Japan). IgM anti-HBc antibodies were determined by solid-phase enzyme immunoassay by the method described previously (10).

RESULTS

Mutations within S gene of HBV. Results of the sequence analysis of the S gene of HBV DNA in families 1 and 2 are depicted in Table 1 and Figure 3. In family 1, all four clones propagated from the patient had in common a point mutation at the 1st base of codon 145. This was the only difference from the sequence derived from her mother, and the point mutation caused amino acid substitution of Arg for Gly in wild type HBV. In family 2, all the cDNA clones from the patient (19 of 19) and her brother (20 of 20) and most clones from their mother (12 of 17) had a missense mutation at codon 126 in the S gene, leading to a substitution of Asn for Thr or Ile in wild type HBV. The 126th codon in the S gene of all clones from two aunts in family 2 were ATT, coding for Ile, and most clones from the grandmother (14 of 16) were ACT, which codes for Thr.

Altered expression of antigenic determinants on HBsAg. The degree of expression of some antigenic determinants on the HBsAg, as measured by mapped MAb antibodies, was different between mothers and children in both families (Table 2). In family 1, the expressions of no. 3207 (determinant "a") and no. 3423 (determinant "d") epitopes were significantly decreased in the patient, but they were normal in her mother. In family 2, the no. 7604 epitope was poorly expressed on HBsAg from the two infected children compared with those from other family members, including their mother.

DISCUSSION

The present study analyzed the S gene sequence of HBV DNA and the epitope expressions on HBsAg from two families where

Table 2. *Decreased expression of some antigenic determinants on HBsAg from subjects infected with HBV variants**

	HBsAg determinants detected by MAb					
	3207 "a"†	824 "a"	5124 "a"	7604 ‡	3423 "d"	313 "r"
Family 1						
Mother	1.025	1.207	0.591	1.053	0.636	1.765
Patient	0.016	1.408	0.851	1.080	0.010	>2.000
Family 2						
Grandmother	0.630	1.298	0.615	1.061	0.614	1.639
Aunt	0.649	1.008	0.522	1.151	0.520	1.353
Aunt	1.046	1.881	0.672	1.061	0.669	1.873
Mother	0.861	1.268	0.703	0.844	0.709	1.550
Brother	0.861	1.237	0.647	0.170	0.610	1.233
Patient	1.070	1.199	0.705	0.267	0.687	1.588

* ELISA absorbance at 492 nm. Values with significant decreases are underlined.

† Previously mapped epitope specificity for each MAb antibody (References 7, 8).

‡ No. 7604 was raised against HBsAg/adr and is shown by the present study to be linked to codon 126.

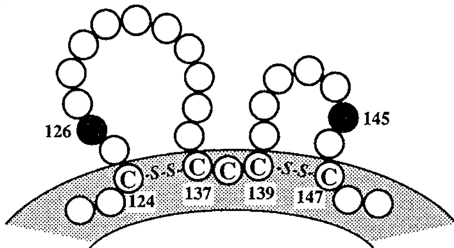

Fig. 4. Common S gene antigenic domain on HBsAg. Amino acid residues 126 and 145 (*black circles*) located at mutually symmetric positions within the structure. Proposed by Carmen *et al.* (4).

HBV was transmitted from mothers to children who were immunized with HBIG and vaccine. In both families, the children were infected with a mutant virus and the mothers had wild type HBV S gene sequences (Table 1). There were two types of missense point mutations within the S gene of the virus from the children: Gly to Arg at codon 145 in family 1 and Thr or Ile to Asn at codon 126 in family 2. Because there was complete identity of the S gene sequences between mothers and children, except for the one mutated codon (Fig. 3), we believe the children were infected with HBV from the mothers, and not from other sources.

It is uncertain when the point mutation at codon 145 may have occurred in family 1. Carman *et al.* (4) previously identified a similar mutant in a vaccinated baby born to a mother harboring a wild type HBV and speculated that the mutant arose *de novo* in the baby and thus called the mutant "vaccine-induced escape mutant." However, in family 2 of the present study it was clearly demonstrated that the point mutation had already occurred before the mother-to-child transmission. The mutant in the mother existed along with wild type virus (12 mutant clones and five wild type; Table 1). The wild type HBV clones in the mother were not found in her two vaccinated children (0 of 19 and 0 of 20 clones; Table 1). This suggests a selective advantage of the mutant over the wild type HBV under immune pressure. HBIG and the vaccine may have succeeded in preventing infection with wild type HBV but failed with the mutant. Unfortunately, we sequenced only four clones from the mother in family 1. The mutant may have been detected if more clones had been examined (Note: After submission of this manuscript, we performed single-track sequencing on an additional 96 clones from the mother and five clones from the child in family 1 only to establish whether the first letter of codon 145 is G of wild type or A of mutant. All 96 clones from the mother had G, whereas the five from the child had A at that position. This suggests that the mutant genotype detected in the infant was not preexisting in the mother at a frequency greater than 1%).

The "common" group-specific antigenic determinant "a" on HBsAg has been proposed by Carman *et al.* (4) as the region of amino acids at 124–147, where the two disulfide bonds stabilize a double-loop structure (Fig. 4). This model is consistent with the finding that a mutant with an amino acid substitution at codon 145 could escape wild type immunity. Our current finding of another "vaccine-escape" variant in which codon 126 was mutated supports the model that both codons are involved in the "a" determinant structure. The two residues at 126 and 145 exist at symmetrical positions in the proposed structure of the "a" domain (Fig. 4). However, the exact sequence recognized by neutralization-effective antibodies is still unknown. Also unknown are the determinants for the antibodies most abundantly induced by active immunization.

In our study, several monoclonal anti-HBs antibodies with known epitope specificities were used to study HBsAg-mutants having amino acid substitutions at either codon 126 or 145. The mutants had decreased antigenic binding activities for some antibodies, as shown in Table 2. The Gly-to-Arg mutation at codon 145 in family 1 was associated with decreased binding activities for one of three "a" epitope antibodies and for a subtype-specific "d" epitope antibody. The Thr or Ile-to-Asn mutation at codon 126 in family 2 was associated with decreased binding of the no. 7604 epitope antibody. The mutant virus was found in vaccinated children; therefore, it must have escaped the protective immunity.

The currently unmapped MAb no. 7604 showed decreased binding with the S gene codon 126 mutant found in the family 2 children. They carried virus with Asn at residue 126, whereas other family members carried virus with Thr or Ile at that position and reacted strongly with the no. 7604 antibody. It is of interest to consider the genotype and the serotype expressions of the S gene in the mother in family 2. She had much more mutant HBV DNA than wild type DNA in the circulation (12 *versus* five clones). The no. 7604 epitope expression of her HBsAg (A492 = 0.844) was comparable to that of other family members with exclusive wild type infections (A492 = 1.053–1.151). The no. 7604 epitope expressed in her children who were infected only with the mutant HBV was much lower (A492 = 0.170–0.267) (Table 2). Coinfections with wild type and mutant viruses present special problems related to preferential synthesis, assembly, secretion, and reinfection. The possibility of phenotype mixing exists, wherein a wild type genome may be encapsulated with mutant envelope or a mutant genome within a wild type envelope. Such mixed phenotypes could have existed in the circulation of the mother in family 2.

An important problem related to HBV surface protein mutants is design of effective vaccines. The current commercial vaccines are composed of plasma-derived or recombinant S gene product with wild type HBV sequence, and infections with mutant strains have been found in vaccinees. Incorporation of HBsAg corresponding to the individual variants would be a possible solution to the problem. Another proposal is to use vaccines that contain the pre-S gene product as a component. The pre-S protein has been found to be a more potent T cell stimulator than the S gene product (11), and a synthetic peptide from the pre-S2 region has shown protective efficacy in chimpanzees (12). These studies suggest that a pre-S or pre-S + S vaccine might circumvent infection by the currently identified S gene escape mutants and possibly also stimulate host immune responsiveness.

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