Naturally Occurring Escape Mutants of Hepatitis B Virus with Various Mutations in the S Gene in Carriers Seropositive for Antibody to Hepatitis B Surface Antigen

KAYOKO YAMAMOTO,1 MINORU HORIKITA,2 FUMIO TSUDA,3 KEICHI ITOH,4 YOSHIHIRO AKAHANE,5 SHIGERU YOTSUMOTO,1 HIROAKI OKAMOTO,1 YUZO MIYAKAWA,1 AND MAKOTO MAYUMI1*

Immunology Division, Jichi Medical School, Tochigi-ken 329-04,1 Second Department of Internal Medicine, University of Tokushima School of Medicine, Tokushima-ken 770,3 Viral Hepatitis Research Foundation of Japan, Tokyo 113,1,3 Japanese Red Cross Blood Center, Yamaguchi-ken 753,4 First Department of Internal Medicine, Yamanashi Medical College, Yamanashi-ken 409-38,5 and Mita Institute, Tokyo 108,6 Japan

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Hepatitis B virus (HBV) DNA was extracted from sera of six carriers with hepatitis B e antigen as well as antibody to hepatitis B surface antigen and sequenced within the pre-S regions and the S gene. HBV DNA clones from five of these carriers had point mutations in the S gene, resulting in conversion from Ile-126 or Thr-126 of the wild-type virus to Ser-126 or Asn-126 in three carriers and conversion from Gly-145 to Arg-145 in three of them; clones with Asn-126 or Arg-145 were found in one carrier. All 12 clones from the other carrier had an insertion of 24 bp encoding an additional eight amino acids between Thr-123 and Cys-124. In addition, all or at least some of the HBV DNA clones from these carriers had in-phase deletions in the S′ terminus of the pre-S2 region. These results indicate that HBV escape mutants with mutations in the S gene affecting the expression of group-specific determinants would survive in some carriers after they seroconvert to antibody against surface antigen. Carriers with HBV escape mutants may transmit HBV either by donation of blood units without detectable surface antigen or through community-acquired infection, which would hardly be prevented by current hepatitis B immunoglobulin or vaccines.

The envelope gene of hepatitis B virus (HBV) codes for three kinds of proteins, which are translated from distinct mRNA (for a review, see reference 30) and collectively called hepatitis B surface antigen (HBsAg). The major HBsAg is coded for by the S gene and made up of 226 amino acids (aa). The middle HBsAg is coded for by the pre-S2 region (55 aa) and the S gene, while the large HBsAg is coded for by the pre-S1 region (119 or 108 aa, depending on subtypes), the pre-S2 region, and the S gene.

Escape mutants of HBV which have point mutations in the S gene resulting in amino acid changes for the loss of the group-specific determinant called a have been reported. They arise in persons infected with HBV after they receive hepatitis B vaccine (3, 5, 7, 19) or in patients with orthotopic liver transplantation on treatment with monoclonal antibody to HBsAg (anti-HBs) (11). HBV escape mutants are reported, also, in a carrier who did not receive hepatitis B immunoglobulin or vaccine (12). HBV mutants with deletions in the pre-S2 region occur in persons persistently infected with HBV and replace wild-type HBV (6, 23). HBV mutants with mutations in the pre-S2 region or the S gene would arise in the natural course of HBV infection, instigated by the selective pressure of immune responses of hosts for the survival of the fittest.

Six individuals with persistent HBV infection who had hepatitis B e antigen (HBeAg) in sera, despite the fact that they tested positive for anti-HBs, were identified. HBV DNA clones were propagated from their sera, and sequences of the pre-S regions and the S gene were determined. Patterns of deletions in the pre-S2 region and point mutations in the S gene, as shown in the analyses below, would be associated with virological, clinical, and epidemiological implications.

MATERIALS AND METHODS

Serum samples. Sera which contained HBeAg and high-titered antibody to hepatitis B core antigen (anti-HBc) in spite of positivity for anti-HBs were selected. They were from an asymptomatic carrier of HBV (case 1), a patient with liver cirrhosis (case 2), a patient with primary hepatocellular carcinoma (case 3), and three patients with chronic hepatitis B (cases 4 to 6). The case 1 carrier was tested at three time points during 11 years, the last serum sample being taken after he had seroconverted to antibody to HBeAg (anti-HBe). Serological profiles of the six carriers are shown in Table 1.

Additionally studied were 14 HBV carriers with conventional serologies. Nine of them had HBsAg and HBeAg, while the remaining five possessed HBsAg and were seropositive for anti-HBe. They all had high-titered anti-HBc.

Serological assays. HBsAg and anti-HBs were determined by enzyme immunoassay (EIA) with commercial kits (AUSZYME II and AUSAB; Abbott Laboratories, Abbott Park, Ill.) with cutoff A492 values of 0.14 and 0.21, respectively. These assays involved the group-specific determinant of HBsAg called a that is maintained by conformation of two loop structures constructed by aa 124 to 147 of the product of the S gene (1, 3). Anti-HBe was determined in sera diluted 1:200 with EIA kits (Corzyme; Abbott). HBeAg and anti-HBe were determined by EIA with commercial kits (HBeAg/Ab EIA; Institute of Immunology Co., Ltd., Tokyo, Japan). Antigenicity of the pre-S2 region product was determined by a sandwich EIA with horse polyclonal anti-HBs immobilized on a solid support and murine monoclonal antibody to pre-S2

* Corresponding author.
antigen labeled with horseradish peroxidase; the antibody (no. 5520) was raised against a synthetic 19-mer peptide representing aa 14 to 32 of the product of the pre-S2 region deduced from an HBV DNA isolate of subtype adr (17). Antibody to the pre-S2 region product (anti-pre-S2) was determined by EIA according to the following procedure. Wells of a plastic microtiter plate (Sumitomo Bakelite Co., Tokyo, Japan) were coated with the synthetic 55-mer peptide, deduced from the pre-S2 region of an HBV DNA isolate of subtype adr, with a sequence of MQWNSTTFHQALLDPRVRLGLYFFA GGSSSVTVNPPVTTAPSSIFSRTGDPAPN, and unsaturated binding sites were quenched with 40% (vol/vol) bovine serum. Test serum (50 μL) was delivered to a well, and bound antibody was detected by Fab' fragments of murine monoclonal antibody specific for human immunoglobulin G (γ) (B22; Institute of Immunology) labeled with horseradish peroxidase. Anti-HBs values of 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, and 10.0 were considered positive in anti-pre-S2 assay, and those exceeding 0.10 were considered positive in anti-pre-S2 assay; the cutoff values represented the mean plus 3 standard deviations of 30 serum samples without markers of HBV infection.

Extraction and quantitation of HBV DNA. HBV DNA was extracted with phenol-chloroform from 100 μl of serum treated with proteinase K-sodium dodecyl sulfate, by the method described previously (20). The extract was serially diluted tenfold with Tris-HCl buffer (10 mM, pH 8.0) supplemented with 1 mM EDTA and 20 μg of glycogen (Boehringer GmbH, Mannheim, Germany) per ml. A sequence of the pre-S2 gene (233 bp) was amplified by PCR with two sets of nested primers (9). The titer of HBV DNA was expressed by the highest dilution of the extract in which HBV DNA was detected by the nested PCR.

Cloning and sequencing of the pre-S regions and the S gene. The pre-S regions and the S gene were amplified on HBV DNA extracted from 25 μl of serum by PCR with AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) and nested primers. The entire sequence of the pre-S regions and the S gene was amplified by two separate PCR procedures, the products of which overlapped within nucleotides (nt) 64 to 137 in the pre-S2 region; nucleotides were numbered from the unique EcoRI site of the HBV genome.

The first round of PCR for the amplification of the pre-S1 region and a part of the pre-S2 region was performed with 5'-TTGCCCTTAGACAAAGGCAT-3' (PS14, nt 2673 to 2692 [plus strand]) and 5'-ACACGAGCAGGGGTCTAGG-3' (PS17, nt 178 to 197 [minus strand]), and the second round was performed with 5'-ACATATACTCTGTGGAAGGCTG-3' (PS13, nt 2750 to 2769 [plus strand]) and 5'-CAGTCCCTCGAGAAAGATTGAC-3' (PS16, nt 118 to 137 [minus strand]). The first round of PCR for the amplification of a part of the pre-S2 region and the S gene was carried out with 5'-GCTGGTGCTGTCTCAGTTC-3' (S006, nt 56 to 75 [plus strand]) and 5'-AATTATATATATATCTTCAAGA-3' (S007, nt 874 to 893 [minus strand]), and the second round was carried out with 5'-TGGTCCCTTCAGTGGACAG-3' (S008, nt 64 to 83 [plus strand]) and 5'-ATATCCCATGAATTTAGGG-3' (S009, nt 866 to 885 [minus strand]). The first round of PCR was performed for 35 cycles, and the second round was performed for 25 cycles; each cycle included denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min.

The product of PCR was treated with T4 DNA polymerase (Takara Biochemicals, Kyoto, Japan) and T4 polynucleotide kinase (New England Biolabs Inc., Beverly, Mass.) and inserted into the M13 phage vector. Nucleotide sequences of amplified HBV sequences were determined for both strands with the Sequenase DNA sequencing kit (7-deaza-dGTP edition version 2.0, United States Biochemical Corp., Cleveland, Ohio) or the AutoRead DNA sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Cloning and sequencing of the pre-C region. Cloning and sequencing of the pre-C region were performed by the method described previously (20).

RESULTS

Table 1 shows serological profiles of the six carriers with HBV DNA and HBeAg as well as high-tiered anti-HBs in sera despite coexisting anti-HBs. Sera from the case 1 carrier were tested at three time points during 11 years, the last serum sample being taken after he seroconverted to anti-HBe; HBeAg and anti-HBs co-occurred in all three serum samples. The antigenic activity for the pre-S2 region product was not detected in sera from any carriers. Antibody to pre-S2 antigen was detected in them all and in high titers in three of them.

HBV DNA clones were propagated from serum samples from the six carriers, and sequences of the pre-S region and the S gene were determined (Table 2). All or at least some of the HBV DNA clones from them possessed deletions in the pre-S2

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age and sex of carrierb</th>
<th>Date of sampling (yr/mo/day)</th>
<th>Dilution of HBV DNA/m³</th>
<th>Surface antigen or antibody (A492)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>31 M</td>
<td>81/03/19</td>
<td>10⁷</td>
<td>&gt;2.00 HBeAg Anti-HBs Pre-S2 antigen Anti-pre-S2</td>
</tr>
<tr>
<td>1b</td>
<td>48/05/10</td>
<td>92/04/30</td>
<td>10⁶</td>
<td>&gt;2.00 &gt;2.00 0.01 0.85</td>
</tr>
<tr>
<td>1c</td>
<td>71 M</td>
<td>85/10/31</td>
<td>10⁴</td>
<td>&gt;2.00 &gt;2.00 0.00 2.00</td>
</tr>
<tr>
<td>2</td>
<td>49 M</td>
<td>88/02/10</td>
<td>10⁴</td>
<td>0.86 0.63 0.01 0.26</td>
</tr>
<tr>
<td>3</td>
<td>55 M</td>
<td>89/06/26</td>
<td>10⁴</td>
<td>1.22 0.61 0.01 0.85</td>
</tr>
<tr>
<td>4</td>
<td>58 F</td>
<td>89/12/8</td>
<td>10³</td>
<td>0.08 &gt;2.00 0.00 2.00</td>
</tr>
<tr>
<td>5</td>
<td>48 M</td>
<td>90/09/28</td>
<td>10⁵</td>
<td>1.30 0.79 0.01 0.80</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>0.13 &gt;2.00 0.01 1.27</td>
</tr>
</tbody>
</table>

a Sera from all carriers were positive for HBeAg (except for the case 1c sample, which was taken after the carrier had seroconverted to anti-HBe) and had high titers of anti-HBe (>70% inhibition when tested at a 1:200 dilution).

b M, male; F, female.

c HBV DNA was extracted from 0.1 ml of serum and titrated in serial tenfold dilutions of the extract.

*HBeAg and anti-HBs were tested for by EIAs with AUSZYME II and AUSAB kits (Abbott) with cutoff values of 0.14 and 0.21, respectively. Pre-S2 antigen and anti-pre-S2 were determined by EIAs (see Materials and Methods) with cutoff values of 0.05 and 0.10, respectively.

d Tested after the case 1 carrier had seroconverted to anti-HBe.
TABLE 2. Mutations in the pre-S regions and the S gene of HBV DNA clones from six HBV carriers with anti-HBs

<table>
<thead>
<tr>
<th>Case no.</th>
<th>No. of clones tested</th>
<th>No. of clones with deletion in:</th>
<th>No. of clones tested</th>
<th>No. of clones with mutations at indicated codon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-S1</td>
<td>Pre-S2</td>
<td>126 (Leu or Thr [AAT or AGT])</td>
</tr>
<tr>
<td>1a</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>6 (Ser [AGT])</td>
</tr>
<tr>
<td>1b</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>6 (Ser [AGT])</td>
</tr>
<tr>
<td>1c</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td>6 (Ser [AGT])</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>9</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>10</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>12</td>
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<td>5</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>12</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

a A 24-bp insertion sequenced AACGCACGGGACCAGCGACC, coding for Asn-Ser-Thr-Gly-Pro-Cys-Thr-Thr, between Thr-123 and Cys-124.

b Two clones with Arg-145 possessed a premature termination codon. One had a C-to-T mutation converting Gln-23 (CAG) to a stop codon (TGA), and the other had a C-to-A mutation converting Ser-143 (TGC) to the stop codon (TGA).

deletion, as shown by HBV DNA clones from all six carriers. Deletions of nucleotides in the pre-S2 region were multiples of three when they occurred within a single span (cases 1 and 3 to 5). When they occurred in two separate spans, both of them were multiples of three (case 2), or they added up to a multiple of three (case 6). Of 27 clones from the case 2 carrier, 9 had an in-phase deletion in the pre-S1 region spanning 18 bp (6 clones), 174 bp (1 clone), or 129 bp (2 clones); one of them

FIG. 1. Deletions of nucleotides in the pre-S1 and pre-S2 regions of HBV DNA clones propagated from sera of six carriers with anti-pre-S2 and anti-HBs. The ranges of pre-S1 and pre-S2 regions with nucleotide numbers are shown above. Deletions are indicated by solid lines with their spans in base pairs, and numbers of nucleotides flanking them are shown. The same deletion was detected in all HBV DNA clones, in numbers indicated in Table 2, from each carrier of cases 1, 3, 4, 5, and 6. Five different patterns of deletions were detected in 10 clones from the case 2 carrier; pattern a was detected in 6 clones and patterns b to e were detected in one clone each.
with a 129-bp deletion co-occurred with deletions in the pre-S2 region.

Mutations in the S gene were much different from those in the pre-S1 or pre-S2 region. Point mutations and insertions of nucleotides were detected, while deletions were not found. Point mutations resulted in changes of codons 126 and 145. Wild-type HBV had either Ile-126 (ATT) or Thr-126 (ACT) (14). A point mutation converting the second base of codon 126 from T or C to G for Ser-126 (AGT) was observed in all 18 HBV DNA clones from the case 1 carrier propagated from three serum samples taken during an 11-year period. Another point mutation converting the second base of codon 126 to A, contributing to Asn-126 (AAT), was detected in clones from the case 5 and case 6 carriers. Essentially all HBV DNA clones from the case 2 and 3 carriers had a G-to-A point mutation at the first base of codon 145 converting Gly-145 (GGG) to Arg-145 (AGG). Ten of eleven clones from the case 5 carrier had mutations, four with Asn-126 and six with Arg-145; two clones with Arg-145 had a mutation converting codon 23 or 143 to a stop codon.

None of 12 HBV DNA clones from the case 4 carrier showed mutations involving codon 126 or 145. All of them possessed an in-phase insertion of 24 bp, however. The insertion encoded eight additional amino acids, Asn-Ser-Thr-Gly-Pro-Cys-Thr-Thr, between Thr-123 and Cys-124 in the S gene product.

The pre-C region was sequenced on more than 10 HBV DNA clones each from the six carriers. The G-to-A point mutation converting Trp-28 to a stop codon (2, 20) was detected in all 10 clones from the serum taken from the case 1 carrier after he seroconverted to anti-HBe and in 3 of 10 clones from the case 4 carrier. This mutation was not detected, however, in any of 20 HBV DNA clones from two serum samples from the case 1 carrier taken while he was positive for HBeAg or in any of 52 clones from the other carriers. None of the sequenced clones had any other mutations aborting the translation of the precursor of HBeAg.

Mutations in the pre-S regions and the S gene were searched for in HBV DNA clones propagated from 14 HBsAg carriers with conventional serologies (Table 3). Nine of them had HBeAg, while the remaining five were positive for anti-HBe. Various deletion mutations were detected in the pre-S1 region of some HBV DNA clones from three carriers with HBeAg and two with anti-HBe. Deletions in the pre-S2 region were detected in some HBV DNA clones from three of the five carriers with anti-HBe; they were not detected in any clones from carriers with HBeAg. None of 42 HBV DNA clones from the 14 carriers had mutations at codon 126 or 145 in the S gene.

**DISCUSSION**

Hosts infected with HBV mount antibody responses of three different categories to viral antigens (8). First, they raise antibodies to the viral nucleocapsid commonly called hepatitis B core antigen (HBcAg). Then, antibody is elicited to HBeAg, which is a secretory form of nucleocapsid protein produced by a distinct biosynthetic pathway (10, 31) and associated with active replication of HBV (15). Last, hosts raise anti-HBs for the termination of HBV infection. Although anti-HBe does not interfere with viral replication, occurring in high titers in persistently infected hosts, anti-HBe and anti-HBs responses have crucial significance in the life cycle of HBV. They would be responsible for mutants with various mutations to evade immune surveillance occurring in persistently infected hosts.

Best known are HBV mutants with mutations in the pre-C region for aborting the secretion of HBeAg. The G-to-A point mutation at nt 1896, converting codon 28 of the pre-C region
from TGG for Trp to TAG for a stop codon, is most frequently observed (2, 20). These mutations prevail as the host seroconverts to anti-HBe, because hepatocytes infected with wild-type HBV and surface expression of HBeAg are selectively eliminated.

Although not frequent, mutations with mutations in the S gene affecting the expression of group-specific determinants of HBsAg are reported. The potent B-cell epitope of HBsAg is borne by aa 124 to 147 of the S gene product, which, it is proposed, make two loop structures maintained by a disulfide bond between Cys-124 and Cys-137 as well as between Cys-139 and Cys-147 (1, 3). Point mutations converting Gly-145 to Arg-145, or Ile (Thr)-126 to Asn-126, abolish the expression of group-specific HBsAg determinants; mutations with these mutations can escape the vaccination or therapeutically administered monoclonal anti-HBs and prevail in circulation (3, 5, 7, 11, 19).

HBV mutants with mutations in the envelope gene were searched for in sera from six carriers who had HBeAg in serum despite the fact that they were positive for anti-HBs. Various mutations affecting the expression of the pre-S2 region product and the S gene product were detected. Their analyses, taken along with similar mutations in previous reports, would help outline the pattern of mutations in the HBV genome for evolution in persistently infected hosts.

The G-to-A point mutation at nt 587 converting Gly-145 to Arg-145 was detected in three carriers. On the basis of this fact taken together with five previous reports describing this mutation in eight carriers (3, 5, 7, 11, 19), nt 587 seems to be the hottest spot for an escape mutation to occur. Also notable were conversions of codon 126 in the S gene, which encodes either Ile or Thr in the wild-type HBV regulating allelic subtypic determinants of HBsAg called i and t (14). Ser-126 was found in all 18 HBV DNA clones from the case 1 carrier, while Asn-126 was detected in all 10 clones from the case 6 carrier as well as in 4 of 11 clones from the case 5 carrier. Ser-126 has been reported in HBV DNA clones from a 66-year-old Japanese man with chronic hepatitis who was positive for HBeAg and anti-HBs (12), and Asn-126 has been reported in a baby who was born to an HBeAg-positive carrier mother and who received combined passive and active immunization (19). Since the S gene sequence of these clones, except for codon 145 or codon 126, was not different from that of wild-type HBV, these changes would be responsible for the loss of group-specific determinants of HBsAg in these five carriers. A change not documented previously was an insertion of eight amino acids between Thr-123 and Cys-124 in all 12 clones from one of the carriers. Such a mutation is reasonably presumed to affect the expression of a group-specific determinant maintained by aa 124 to 147 of the S gene product.

Escape mutants with mutations in the S gene would pose a substantial risk to the community, because current hepatitis B immunoglobulin and vaccines are not effective in preventing infection with them. Blood units containing such mutants are missed by routine screening for HBsAg and can transmit HBV infection to recipients. Since these mutations can replicate actively with potent anti-HBc responses in hosts, the exclusion of blood units with high-titered anti-HBc (9) is expected to be effective for preventing their transmission by transfusions.

Deletions in the pre-S2 region were observed in all or at least some HBV DNA clones from six carriers studied. All of them occurred in the S’ terminus of the pre-S2 region, like those described in HBV DNA clones from carriers infected with escape mutants (12) or without them (6, 23). All deletions in the pre-S2 region were in phase; the expression of large or middle-sized HBsAg species or the P gene product would not be affected, albeit the product would be a little shorter than the authentic products. The pre-S2 region is doubly coded, in a different reading frame, for the DNA polymerase reverse transcriptase (30). It falls on the spacer-tether region of the P gene product, which is not essential for enzyme activity (22).

The deletion in the pre-S2 region, therefore, would be permissible for the replication of mutants. This view goes along with the recent report by Fernholz et al. (4), who have demonstrated that the expression of the pre-S2 protein is not essential for HBV replication, viral morphogenesis, secretion, or in vitro infectivity. Deletions in the pre-S2 region in HBV DNA clones from the six carriers fell on one of the three transcriptional initiation sites for the S mRNA (24). There remains a possibility, therefore, that pre-S2 deletions might lead to a decreased expression of HBsAg at the transcription level.

Anti-pre-S2 was detected in sera from all six carriers, and this would have been responsible for the selection of mutants with pre-S2 deletions. Anti-pre-S2 develops early in persons with acute HBV infection (13) and is detectable in some carriers with anti-HBe (18). Anti-pre-S2 seroconversion seems to be linked, in some way, to anti-HBe seroconversion; HBsAg particles co-occurring with HBsAg in sera of carriers possess the pre-S2 region product in much higher quantities than those coexisting with anti-HBe (28).

Deletions in the pre-S2 region were searched for in HBV DNA clones from 14 HBV carriers with conventional serologies, including nine with HBeAg and five with anti-HBe. Pre-S2 deletions were detected in some HBV DNA clones from three carriers with anti-HBe but not in any of 31 clones from nine carriers with HBeAg. Pre-S2 deletions in HBV DNA clones from the six carriers infected with escape mutants, therefore, would be unique in that they occurred despite coexisting HBeAg. It is not known whether pre-S2 deletions have any significance for the development of escape mutants. A similar pre-S2 deletion has been reported in an escape mutant (12). It would be worthwhile to sequence the pre-S2 region in the other escape mutants to substantiate this association further and to evaluate any causal relationship between them.

HBV evolves rapidly for a DNA virus (16), because its replication involves the reverse transcription of an RNA intermediate (27), a process prone to errors due to the lack of proofreading enzymes (26). Because of seroconversion to anti-HBc and anti-pre-S2 in hosts occurring earlier than anti-HBs responses, mutants with mutations in the pre-C and pre-S2 regions would commonly occur. Since, in general, the replication of HBV is virtually shut off after the host seroconverts to anti-HBe (15), chances are slim for escape mutants with mutations in the S gene to appear in circulation. In rare carriers such as those described here and reported previously (12), however, anti-HBs seroconversion may occur before anti-HBe seroconversion, for some reason or other, and escape mutants would arise in them naturally.

Given the evidence for versatility of the HBV genome in the evasion of various immune pressures of the host, it is not very hard to imagine mutations in the C gene to deprive core particles of immunogenicity for HBeAg. This view would be supported by the failure of chimeric HBV core particles assembled by hybrid core proteins with an insertion of 27 aa between positions 75 and 83 to bind with anti-HBe (25).

Indeed, HBV might eventuate in a cyborg mutant that does not stimulate anti-HBe, anti-pre-S2, anti-HBs, or even anti-HBe responses in the host, accounting for the mystery of HBV DNA sequences in sera and hepatocytes of patients without any serological markers of HBV infection (21, 29).
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REFERENCES