Mutations in the envelope gene of hepatitis B virus variants co-occurring with antibody to surface antigen in sera from patients with chronic hepatitis B

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Three clones of hepatitis B virus (HBV) DNA were propagated from sera of each of five patients with chronic hepatitis B who possessed hepatitis B surface antigen (HBsAg) and antibody to HBsAg in their serum. The clones were sequenced within the envelope gene (the preS1, preS2 regions and the S gene). Clones from four patients had various missense mutations involving codons 124–147 of the S-gene which encode amino acids in the loop structures that form the conformational, common antigenic determinant of HBsAg. Clones from three patients had Asn-130 (Giy in the wild-type), which generated a potential N-glycosylation site, Asn-Thr-Ser, spanning amino acids 130–132 of the S-gene product. In addition, clones from one patient had Arg-145 (Gly in the wild-type), which has been reported in escape mutants of HBV. One of the three clones from another patient had Ser-126 in place of Ile or Thr in wild-type HBV, but the remaining two had no mutations known to affect expression of the common determinant of HBsAg. The remaining patient possessed HBsAg of subtype adr and anti-HBs specific for the w determinant. Clones from this patient did not reveal any mutations which are known to affect the common antigenic determinant of HBsAg.

Introduction

Hepatitis B surface antigen (HBsAg) can co-occur with the corresponding antibody (anti-HBs) in the circulation of some individuals who carry hepatitis B virus (HBV). HBV mutants encoding HBsAg without the conformational, common antigenic determinant designated ‘a’ arise typically in persons who receive hepatitis B vaccine, with or without combined immunoglobulins, and who seroconvert to anti-HBs (Carman et al., 1990; Hino et al., 1995; Karthigesu et al., 1994; Okamoto et al., 1992), and in patients who are injected with human monoclonal anti-HBs (McMahon et al., 1992). Since such mutants can evade the host immune responses, they are called escape mutants. HBV mutants encoding HBsAg without the ‘a’ determinant can occur naturally even without such artificial immune pressure (Yamamoto et al., 1994).

The common antigenic determinant ‘a’ is considered to be borne by one or both of the two loops conformed by amino acids (aa) 124–147 in the S-gene product (Brown et al., 1984; Guerrero et al., 1988; Howard et al., 1988). Several mutations in the S gene resulting in changes to aa in these loops have been identified in HBV escape mutants, including changes of Gly-145 to Arg- or Lys-145 (Carman et al., 1990; Fujii et al., 1992; Harrison et al., 1991; Hino et al., 1995; McMahon et al., 1992; Okamoto et al., 1992; Yamamoto et al., 1994), Lys-141 to Glu- or Ile-141 (Karthigesu et al., 1994; McMahon et al., 1992) and Asp-144 to Ala-144 (Harrison et al., 1994). Amino acid conversion at position 126, from Ile or Thr in the wild-type to Asn or Ser has been reported also (Okamoto et al., 1992; Yamamoto et al., 1994). The full range of variability for escape mutants of HBV has yet to be seen, however.

For the purpose of identifying other varieties of HBV escape mutant, DNA clones were propagated from sera of patients with chronic hepatitis B who possessed both HBsAg and anti-HBs, and nucleotide sequences of the envelope gene (preS1 and preS2 regions and S gene; Tiollais et al., 1985) were determined. The results obtained have revealed novel HBV escape mutants, and will, it is hoped, give further insight into mechanisms that result in co-occurrence of HBsAg and anti-HBs in persistent infection with HBV.
Methods

Serological assays. HBsAg and anti-HBs were determined by radioimmunoassay (AUSRII-125 and AUSAB; Abbott Laboratories) and the antibody was quantified with Anti-HBs (Human) AUSAB Quantification Panel (Abbott Laboratories). Antibody to the preS2 region product was determined by ELISA with a synthetic 55-mer peptide mimicking its sequence (Yamamoto et al., 1994). Subtypes of HBsAg were determined by ELISA with commercial kits (HBsAg SUBTYPE EIA; Institute of Immunology Co., Tokyo, Japan). Hepatitis B e antigen (HBeAg) and corresponding antibody (anti-HBe) were determined by ELISA with commercial kits (HBeAg/Ab EIA; Institute of Immunology Co.). Antibody to hepatitis B core (anti-HBc) was determined by haemagglutination inhibition and the results were expressed as the highest twofold dilution (2^n) of sera that inhibited haemagglutination, as described by Iizuka et al. (1992).

Antigenic determinants of envelope protein. Antigenic determinants of HBsAg and those of the preS1 and preS2 sequences, as well as subtypic determinants borne by HBsAg, were determined by a sandwich ELISA with murine monoclonal antibody to various epitopes of the envelope protein and horse polyclonal anti-HBs. Wells of a plastic microtitre plate (Sumitomo Bakelite Co., Tokyo, Japan) were coated with each monoclonal antibody, and unsaturated binding sites were quenched with 40% (v/v) bovine serum. Subviral particles in the test serum (50 µl) were captured on a well, to which was added horse polyclonal anti-HBs. Wells of a plastic microtitre plate (Sumitomo Bakelite Co., Tokyo, Japan) were coated with a synthetic oligopeptide representing aa 95-107 in the preS1 region product (Takai et al., 1987). Monoclonals #3417 and #3423 (Usuda et al., 1991) were raised against the conformational, common determinant of HBsAg and high titres of HBsAg subtype adr. All five patients had high serum titres of anti-HBC indicating ongoing HBV infection. Three of them (Cases 1, 4 and 5) were positive for HBeAg, while the remaining two (Cases 2 and 3) had anti-HBe.

Results

Patients with HBsAg and anti-HBs in serum

In the 4 year period from September 1990 to October 1994, 73 patients with hepatitis B were enrolled at the First Department of Internal Medicine of Yamanashi Medical University. Anti-HBs was detected in 15 (21%) patients, and five with high serum titres of HBV DNA were selected for molecular biological analyses of the HBV mutants infecting them.

Clinical features and serological profiles of these five patients are shown in Table I. Case 1 had liver cirrhosis and his serum contained too low a titre of HBsAg to allow subtyping by ELISA. The remaining four cases had chronic active hepatitis and high titres of HBsAg subtype adr. All five patients had high serum titres of anti-HBC indicating ongoing HBV infection. Three of them (Cases 1, 4 and 5) were positive for HBeAg, while the remaining two (Cases 2 and 3) had anti-HBe.

Deletions in the preS2 region of HBV DNA clones from patients

Three clones were propagated from the serum of each patient and sequenced within the envelope gene (preS1 and preS2 regions and S gene). Fig. 1 shows the consensus sequence of deduced amino acids for the HBV strain infecting each of the five patients in comparison with wild-type HBV subtype adr. They all possessed Lys-122 and Arg-160, which is indicative of subtype adr (Okamoto et al., 1987). A few amino acid substitutions were observed in the preS1 region product, but there were no deletions or insertions of amino acids in this region.

There were in-phase deletions in the 5'-terminal part of the preS2 region in HBV DNA clones from four of the five patients, resulting in the loss of 11–17 aa. All three clones from Cases 2, 3 and 4 had deletions, while only one of the three clones from Case 1 did. All three clones from Cases 2 and 4 had identical deletions of 45 bp (nt 8–52) and 33 bp (nt 20–53), respectively, while two of the three clones from Case 3 had a 51 bp deletion spanning nt 4–54 and the other (not shown in Fig. 1) had a 33 bp deletion spanning nt 24–56.

One of the three clones from Case 1 had a deletion of 42 bp (nt 3214–3215/1–40) and an additional deletion of 6 bp (nt 48–53) 8 bp downstream of it (not shown in Fig. 1). It

HBV DNA extracted from 25 µl of serum by PCR with nested primers; they overlapped and included the entire envelope gene. Nucleotide sequences were obtained from the unique EcoRI site of the HBV genome as described previously (Okamoto et al., 1988). The PCR product was treated with T4 DNA polymerase (TaKaRa Biochemicals, Kyoto, Japan) and T4 polynucleotide kinase (New England Biolabs) 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) or the AutoRead DNA sequencing kit (Pharmacia LKB).
Table 1. Clinical and virological features of the five patients with HBsAg and anti-HBs

| Case no. | Age/sex | Diagnosis* | ALT (IU/l)† | HBV DNA (10^3 copies/ml) | HBsAg (COI)‡ | Anti-HBs (mIU/ml) | HBeAg (A492)§ | Anti-HBe (% inhibition) || Anti-HBc (2N) ‖ |
|----------|---------|------------|-------------|--------------------------|--------------|------------------|---------------|-------------------|-----------------|
| 1        | 78/M    | LC         | 24          | 6                        | 2.0          | 57               | > 3.00        | 0                | 0              | 8               |
| 2        | 26/M    | CAH        | 844         | 6                        | 7.78         | 250              | 0.01          | 100              | 10             |
| 3        | 38/M    | CAH        | 245         | 5                        | 20.1         | 185              | 0.01          | 97               | 10             |
| 4        | 46/M    | CAH        | 133         | 6                        | 7.91         | 54               | > 3.00        | 0                | 10             |
| 5        | 23/F    | CAH        | 545         | 7                        | 5.7          | 162              | 2.80          | 0                | 10             |

* LC, liver cirrhosis; CAH, chronic active hepatitis.
† Alanine aminotransferase (normal values ≤ 35 IU/l).
‡ Cut-off index in ELISA. Values > 1.0 are considered positive.
§ Absorbance at 492 nm in ELISA. Values > 0.07 are considered positive.
‖ Percentage inhibition in ELISA for HBeAg. Values > 70% are considered positive.

Fig. 1. Amino acid sequence of the envelope protein of HBV mutants co-occurring with anti-HBs. The sequence of wild-type HBV, subtype adr, shown at the top, was deduced from the consensus sequence of this subtype retrieved from the GenBank/EMBL/DBJ DNA databases (V00867, X01597, M12906, D00630 and X14193). The consensus sequences of three HBV DNA clones propagated from each of five patients with HBsAg and anti-HBs are shown below. Starting positions of the preS1, preS2 and S-gene products are indicated by vertical bars with arrows. Numbers in parentheses are amino acid positions in the S-gene product which are known to affect the antigenicity of the common determinant 'a' (126, 130 and 145) and those specifying subtypic determinants 'd' or 'y' (122) and 'w' or 'r' (160) (Okamoto et al., 1987). All clones had Lys-122 and Arg-160, indicative of subtype adr.

coded for a preS2 region product shorter than that of the wild-type by 16 aa (aa 4-17, aa 21 and 22). The deletion in clones from Cases 2, 3 and 4 terminated at the identical position, aa 22 in the preS2 region product, as did one of the two deletions in a clone from Case 1 (not shown in Fig. 1).

None of the three clones from Case 5 had deletions in the preS2 region. However, they had three amino acid changes unique to them – Ala-32, Thr-45 and Ile-49 – within a stretch of 55 aa representing the preS2 region product.

Point mutations resulting in amino acid changes in the S-gene product

Point mutations resulting in amino acid changes that would affect the antigenicity of the common determinant 'a' were
Table 2. Mutations in the preS region and S gene in HBV DNA clones from the five patients with HBsAg and anti-HBs

Three HBV DNA clones were propagated from sera of each of the five cases of chronic hepatitis B, and sequenced within the envelope gene. Clones with mutations in the preS1, preS2 region or the S gene are shown.

<table>
<thead>
<tr>
<th>PreS region Clones with deletions in:</th>
<th>126 Ile (ATT) or Thr (ACT)</th>
<th>130 Gly (G GC)</th>
<th>145 Gly (G GA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case no.</td>
<td>PreS1</td>
<td>PreS2</td>
<td>0/3</td>
</tr>
<tr>
<td>1</td>
<td>0/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>2</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>3</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>4</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>5</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

Antigenic determinants of subviral particles as detected by monoclonal antibodies

Subviral particles in sera from Cases 2, 4 and 5 were subjected to analyses for antigenic determinants by ELISA with murine monoclonal antibodies (Table 3). They all exhibited a high reactivity with the monoclonal antibody (#T0606) that was directed to the product of the preS1 region. Low reactivity with monoclonals to the preS2 region product was exhibited by subviral particles of Case 2 in ELISA with #5158, which recognizes the amino-terminal part of the preS2 region product, and by those of Case 4 in ELISA with #5158 and also with #4408, which recognizes a central part of this region. Subviral particles from all three cases revealed high binding activity with #5156 which recognizes the carboxyl-terminal part of the preS2 region product. Subviral particles from Case 5 reacted with all monoclonals with an activity comparable with that of wild-type particles. None of the three unique amino acid changes in them, therefore, affected the antigenicity of the preS2 region moiety of subviral particles.

In tests to detect the common determinant ‘a’ with various monoclonals, subviral particles from Case 4 had low activity with #7604/a. Subviral particles from all the three patients tested bound with monoclonals #3423/d and #313/r, in agreement with their subtype being adr.

Table 3. Antigenic determinants detectable by monoclonal antibodies on subviral particles co-occurring with anti-HBs

Subviral particles of subtype adr co-occurring with anti-HBs in three cases were tested by ELISA for binding with monoclonal antibodies directed to the common as well as subtypic determinants of HBsAg and with those to preS1 or preS2 region product. Absorbance values at 492 nm are shown. To equalize the number of subviral particles, sera were diluted so as to have an HBsAg ELISA value of 2.0.

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Wild-type*</th>
<th>Case 2</th>
<th>Case 4</th>
<th>Case 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-preS1 #T0606</td>
<td>2.49</td>
<td>2.18</td>
<td>2.32</td>
<td>2.01</td>
</tr>
<tr>
<td>Anti-preS2 #5158</td>
<td>2.23</td>
<td>0.34</td>
<td>0.09</td>
<td>1.87</td>
</tr>
<tr>
<td>#4408</td>
<td>2.31</td>
<td>1.57</td>
<td>0.08</td>
<td>2.29</td>
</tr>
<tr>
<td>#5520</td>
<td>2.78</td>
<td>1.98</td>
<td>0.29</td>
<td>2.50</td>
</tr>
<tr>
<td>#5156</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
</tr>
<tr>
<td>Anti-HBs #3207/a</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
</tr>
<tr>
<td>#824/a</td>
<td>1.82</td>
<td>1.67</td>
<td>1.66</td>
<td>1.92</td>
</tr>
<tr>
<td>#5124/a</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
<td>&gt; 3.00</td>
</tr>
<tr>
<td>#7604/a</td>
<td>1.50</td>
<td>1.26</td>
<td>0.11</td>
<td>1.47</td>
</tr>
<tr>
<td>#3423/d</td>
<td>1.20</td>
<td>1.09</td>
<td>0.98</td>
<td>1.18</td>
</tr>
<tr>
<td>#313/r</td>
<td>2.46</td>
<td>2.20</td>
<td>2.68</td>
<td>2.40</td>
</tr>
</tbody>
</table>

* Subviral particles in serum from a symptom-free carrier with HBeAg whose HBV DNA clone (pNDR260) has been sequenced in its entirety (Okamoto et al., 1988).

Specificity of anti-envelope antibodies

Antibody to the preS2 region product was detected in sera from Cases 1, 2, 3 and 4, but not in serum from Case 5.
Readings of $A_{492}$ in ELISA were 0·50 for Case 3 and between 0·13 and 0·15 for Cases 1, 2 and 4. Negative controls as well as the serum from Case 5 had $A_{492}$ values of $\leq 0·02$.

Sera from the five patients were tested for ability to agglutinate detector cells coated with small HBsAg particles of subtype adw or those of subtype adw. Serum from Case 5 agglutinated cells coated with HBsAg/adw with a haemagglutination titre of 2$^6$, but did not haemagglutinate cells coated with HBsAg/adw at all. Hence, this serum contained antibodies directed to the $w$ determinant. Sera from Cases 1–3 agglutinated cells coated with HBsAg/adw or HBsAg/adr with comparable titres ranging from 2$^6$ to 2$^7$. Antibodies in serum from Case 4 were present at too low a titre (< 2$^3$) to be evaluated for subtypic specificities.

**Discussion**

Co-occurrence of HBsAg and anti-HBs appears to be rather common among patients with chronic hepatitis B, being detected in 15 (21%) of the 73 patients who were enrolled in our hospital over a 4 year period. Some of them would have been seroconverting from HBsAg to anti-HBs with HBsAg particles in their circulation complexed with antibodies. The simultaneous detection of HBsAg and anti-HBs, especially when they both occur in high titres, may, however, represent HBV variants that do not express the common determinant ‘a’ of HBsAg. In a previous report (Yamamoto et al., 1994), we described point mutations resulting in amino acid changes in the loop structure that is implicated in expression of the ‘a’ determinant (Brown et al., 1984; Guerrero et al., 1988; Howard et al., 1988); these mutations occurred naturally in six carriers with anti-HBs and HBeAg. Hence, HBV escape mutants may not only occur in vaccinees and recipients of anti-HBs antibodies, but also can arise naturally and thrive as the dominant virus population in persistent HBV infection.

This view is reinforced by the results of molecular biological analyses performed on the present series of five patients with chronic hepatitis B who were positive for both HBsAg and anti-HBs, and had HBV DNA in high titres in serum. One patient was infected with an HBV variant with a point mutation in the $S$ gene resulting in the change Gly-145 to Arg. This mutation has been reported in escape mutants induced by vaccines or anti-HBs immune globulins by many groups of investigators (Carman et al., 1990; Fuji et al., 1992; Harrison et al., 1991; Hino et al., 1995; McMahon et al., 1992; Okamoto et al., 1992). A point mutation resulting in the change of aa 126 to Ser was found in one of the three clones from another patient. Wild-type HBV has either Ile-126 or Thr-126, for allelic subtypes $i$ and $t$ (Ohnuma et al., 1993), and mutations encoding Asn-126 or Ser-126 have been reported in HBV DNA clones from HBsAg carriers who have anti-HBs in high titres (Okamoto et al., 1992; Yamamoto et al., 1994). Such mutations, therefore, might affect expression on HBsAg particles of the ‘a’ determinant as a result of the microconformation maintained by the disulphide bond between Cys-121 and Cys-124 (Ohnuma et al., 1990), a possibility which is supported by observations on double mutants generated by site-directed mutagenesis of both cysteine residues (Mangold & Streeck, 1993; Antoni et al., 1994). This has to be evaluated, however, by expressing HBsAg particles with Asn-126 or Ser-126 and determining the extent of binding with anti-HBs.

Two G to A point mutations at the first and second positions of codon 130 for Gly (GGC) in wild-type HBV, converting it to a codon for Asn (AAC), had not been documented previously. Such mutations were observed in all three clones from each of three cases, including a clone that had Arg-145 also. The mutation created a new potential N-linked glycosylation site, Asn-Thr-Ser, corresponding to a 130–132 in the $S$-gene product. Inasmuch as the change from Thr/Ile-126 to Asn-126 is implicated in the loss of the ‘a’ determinant (Okamoto et al., 1992; Yamamoto et al., 1994), positioned at the base of the first loop of the HBsAg protein (Brown et al., 1984; Guerrero et al., 1988; Howard et al., 1988), the conversion from Gly-130 to Asn at its centre may well result in an escape mutant.

In addition, it is tempting to speculate that binding of a carbohydrate moiety to Asn-130 would affect expression of the ‘a’ determinant, although it is not clear to what extent glycosylation occurs. The $S$-gene product has an N-linked glycosylation site at Asn-146 and binding of a carbohydrate moiety increases the molecular mass of the small surface protein from 24 to 27 kDa (Peterson et al., 1982; Tiollais et al., 1985). Association of a carbohydrate moiety with the small surface protein may or may not affect expression of the ‘a’ determinant on HBsAg particles since the constituent proteins that assemble to form HBsAg particles are not fully glycosylated (Peterson, 1981).

The serum from Case 5 contained anti-HBs specific for the $w$ subtypic determinant. It might be that Case 5 was originally infected with HBV of subtype adw, with codon 160 encoding Lys in the $S$-gene product (Okamoto et al., 1987), and developed monospecific antibody to the $w$ determinant. An HBV mutant with an A to G point mutation could have occurred in this case, resulting in codon 160 encoding Arg and a subtypic change from adw to adw; such a mutant may have survived its predecessor owing to immune pressure exerted by antibodies to the $w$ determinant. Indeed, this may be another mechanism to account for escape mutants. The co-occurrence of HBsAg particles of a certain subtype and anti-HBs with a specificity for subtypic determinants not borne by the particles has been documented in some HBV carriers (Le Bouvier et al., 1976; Sasaki et al., 1976).

One of the three clones from Case 2 had a mutation for Ser-126, while the other two clones possessed Thr-126, as does the wild-type. Hence, the mutation scarcely accounts for the escape. Perhaps there is some mechanism for co-occurrence of
HBsAg and anti-HBs which is unfathomable in view of present knowledge on the immunochemistry of HBsAg. Alternatively, HBsAg particles in this case might circulate complexed with antibodies, despite the fact that their sera tested strongly positive for both HBsAg and anti-HBs.

Deletions of nucleotides were not observed in the preS1 region of any of 15 HBV DNA clones from the five patients. This may reflect its pivotal role, indispensable for virus infection, as the possible HBV receptor binding site (Neurath et al., 1986; Ishikawa & Ganem, 1995).

Various deletions were observed in HBV DNA clones from four of the patients. All of them were in phase, and when two separate deletions occurred they added up to a multiple of 3, as reported previously (Yamamoto et al., 1994). Antibodies directed to the preS2 region product were detected in sera from all patients infected with HBV variants with deletions in the preS2 region. Hence, such mutants may have been selected by immune pressure with anti-preS2 antibodies. This view was supported by the diminished binding of monoclonal antibodies to preS2 determinants exhibited by subviral particles in sera from two of the patients. Unlike the preS1 region product, the product of the preS2 region may be dispensable for HBV. This observation reinforces the proposal of Femholz et al. (1993) that HBV mutants devoid of preS2 protein can occur in vivo as a dominant or exclusive virus population and that expression of the preS2 protein is not essential for HBV replication, virion morphogenesis, secretion or in vitro infectivity.

A number of clinical, epidemiological and preventive implications can be conceived for HBV escape mutants. They can evade detection by HBsAg tests, especially those based on monoclonal antibodies in current use. Blood for transfusion that is contaminated with such mutants would transmit HBV infection to recipients because it escapes the screening for HBsAg. However, such blood can be identified by testing for anti-HBc and excluding that with high titres (Iizuka et al., 1984). Affinity of antibody responses in man to hepatitis B vaccine determined with synthetic peptides. Lancet ii, 184–187.


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