A novel hepatitis B virus variant in the sera of immunized children

Vasandra Devi Karthigesu, Lisa M. C. Allison, Marijke Fortuin, Maimuna Mendy,
Hilton C. Whittle and Colin R. Howard

Department of Pathology and Infectious Diseases, Royal Veterinary College, Royal College Street, London NW1 0TU, U.K. and Medical Research Council Laboratories, P.O. Box 273, Fajara, The Gambia

A novel hepatitis B virus (HBV) variant was detected in the sera of two children in The Gambia, West Africa. The children had been immunized with plasma-derived vaccine and had developed antibody titres of 1448 international units x 10^-3 (mIU)/ml and 133 mIU/ml respectively against the hepatitis B surface antigen (HBsAg). Despite the protective levels of antibodies, HBV DNA was subsequently detected in both children. The complete surface (S) protein gene sequence demonstrated that this HBV isolate was closely related to the ayw4 subtype. However, five nucleotide changes were identified and two of these were unique to the Gambian isolate. One of these changes was within the region of the S gene coding for the immunodominant a determinant of the S protein. A unique nucleotide change from adenosine to guanosine at nucleotide 421 was found, resulting in an amino acid substitution at residue 141 from lysine to glutamic acid. Previous studies have shown that amino acids 141 to 146 are critical for binding to the protective anti-HBsAg antibodies. The presence of a variant HBV in these children suggests the emergence of a novel strain of HBV which can evade immune recognition. This has potential implications for HBV diagnosis and prophylaxis.

Immunization against hepatitis B virus (HBV) infection has been effective in preventing the establishment of chronic HBV infections (Fortuin et al., 1993). HBV vaccines contain the 22 nm subviral hepatitis B surface antigen (HBsAg) particle, which consists mainly of the 226-amino acid S protein, the major polypeptide of the viral surface protein. Protective antibodies induced by vaccines are directed against the common group a determinant shared by all known subtypes of HBV, which is located in a cysteine-rich region between amino acids 124 and 150 of the S protein. High affinity antibodies against a cyclical synthetic peptide representing a beta-turn of the amino acid sequence from 139 to 147 (Stirk et al., 1992) have been found in sera of recovered patients and vaccinated individuals (Brown et al., 1984a, b). Immunodominant B and T cell epitopes have been demonstrated in this region of the S protein (Howard et al., 1986; Steward et al., 1986).

In all known serotypes of HBV, the a determinant site between amino acids 139 and 147 is highly conserved. However, some subtype variation between threonine and serine has been observed at residue 143 and less frequently at residue 140, where threonine is substituted by serine in HBV strains of the ayw4 and adw4 subtypes (Norder et al., 1992a). There is evidence that other changes within the a determinant may be selected as a result of exposure of the virus to anti-HBsAg (anti-HBs) antibodies. An amino acid substitution at residue 145 from glycine to arginine has been reported in several individuals who developed HBV infections despite receiving anti-HBs immunoglobulin and vaccine (Carmen et al., 1990; Harrison et al., 1991), or monoclonal antibodies (MAbs) (McMahon et al., 1992). Studies of vaccination efficacy in two villages in The Gambia have revealed breakthrough HBV infections characterized by the presence of anti-hepatitis B core (anti-HBc) antibodies (Whittle et al., 1991). Post-vaccination serum samples of 57 of these children were screened for HBV DNA by molecular hybridization. All samples contained protective levels of anti-HBs antibodies [greater than 10 International Units x 10^-3 (mIU)/ml] and 31 were also positive for anti-HBc antibodies. One child who had anti-HBc antibodies was found to be HBV DNA-positive by DNA hybridization analysis. In addition, PCR amplification from serum DNA extracts of a further 16 of the children with anti-HBc antibodies revealed five who were positive for HBV DNA. We report the S gene sequence, and a novel substitution within the loop of amino acid sequence 139 to 147 of the a determinant, of an HBV variant isolated from two of these children.

The two children, who had non-carrier mothers and were from separate villages in The Gambia, had been

The sequence data reported in this paper have been submitted to the GenBank database and assigned the accession number L24071.

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immunized with the plasma-derived HBV vaccine (Merck, Sharp & Dohme). The clinical history and serological markers of HBV detected in the children are shown in Table 1. No clinical evidence of liver disease was reported in either child.

DNA was recovered from serum samples by proteinase K digestion, extracted with phenol and chloroform, and precipitated with absolute ethanol. Samples, equivalent to 27 μl of sera, were screened for HBV by dot-blot DNA hybridization using cloned HBV DNA, radiolabelled with [α-32P]dCTP, as a probe (Sambrook et al., 1989). The limit of detection for this assay was estimated to be 0.25 pg/μl of HBV DNA in serum.

PCR amplification (Saiki et al., 1988) of HBV DNA was carried out with the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus). To prevent cross-contamination, DNA from each serum sample together with an HBV-negative control serum was individually extracted and PCR-amplified. Oligonucleotide primers HBSQ6A and HBL5 (Table 2) were used to amplify a 782 nucleotide DNA fragment, which included the complete S gene, from HBV DNA derived from child M74. HBSQ3A and HBL5 were used to amplify DNA extracts obtained from child M06 resulting in a 616 bp fragment encoding amino acids 42 to 226 of the S gene. Thirty cycles of amplification (1 min denaturation at 94 °C, 2 min annealing at 45/50 °C and 3 min extension at 72 °C) were carried out. The PCR products from both children were subjected to a second round of amplification, purified using the Magic DNA Clean-up System (Promega) and blunt end-ligated into a SmaI-cut pGEM-3Z vector (Promega).

Sequencing of both strands of DNA was carried out by the dideoxynucleotide chain termination method (Sanger et al., 1977) using the Taqtrack Sequencing System, Deaza (Promega). The a determinant encoding region for amino acids 139 to 147 of the S protein from each HBV isolate was sequenced from a minimum of 12 clones derived from two PCR products using oligonucleotide primers HBSQ4A and HBSQ5B (Table 2).

The complete HBV S gene, isolated from child M74, was sequenced using primers HBSQ4A, HBSQ4B, HBSQ5A, HBSQ5B, PGP and PGN (Table 2). Both strands of the DNA of four clones derived from two PCR products were sequenced. Thermocycle sequencing (fmol DNA Sequencing System, Promega) was used to sequence clones using primers PGP and PGN (Table 2), and also to sequence PCR products directly using primers HBSQ6A and HBSQ4B to overcome compressions caused by the occurrence of G + C-rich tracts at both the 5' and 3' ends of the S gene.

Protective anti-HBs antibody levels were detected in both children at 1.5 and 3.5 years of age (Table 1). Although there was no serological evidence of HBV infection in child M74 at 1.5 years, the presence of contamination, DNA from each serum sample together with an HBV-negative control serum was individually extracted and PCR-amplified. Oligonucleotide primers HBSQ6A and HBL5 (Table 2) were used to amplify a 782 nucleotide DNA fragment, which included the complete S gene, from HBV DNA derived from child M74. HBSQ3A and HBL5 were used to amplify DNA extracts obtained from child M06 resulting in a 616 bp fragment encoding amino acids 42 to 226 of the S gene. Thirty cycles of amplification (1 min denaturation at 94 °C, 2 min annealing at 45/50 °C and 3 min extension at 72 °C) were carried out. The PCR products from both children were subjected to a second round of amplification, purified using the Magic DNA Clean-up System (Promega) and blunt end-ligated into a SmaI-cut pGEM-3Z vector (Promega).

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**Table 1. Clinical history and serological markers of HBV in the two children, M74 and M06**

<table>
<thead>
<tr>
<th>Child</th>
<th>M74</th>
<th>M06</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Age at vaccination (months)</td>
<td>1.5, 2.5, 4.5, 8.5</td>
<td>1, 2, 3.5, 8</td>
</tr>
<tr>
<td>HBV serology at 1-5 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HBs (mIU/ml)*</td>
<td>1342</td>
<td>133</td>
</tr>
<tr>
<td>Anti-HBe*</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>HbsAg</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>HBV serology at 3-5 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HBs (mIU/ml)*</td>
<td>1349</td>
<td>47</td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>HbsAg</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>HbeAg</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

* Tested by radioimmunoassay (Sorin Biomedica).
† Not done.
‡ Dot-blot hybridization positive.
§ PCR positive.
|| Tested by RPHA (Wellcome Diagnostics).

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**Table 2. Synthetic oligonucleotide primers used for PCR amplification and DNA sequencing**

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
<th>Position</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSQ6A*</td>
<td>Forward</td>
<td>1394–1413</td>
<td>CATATCGTCAATCTTTCGCA</td>
</tr>
<tr>
<td>HBSQ3A*</td>
<td>Forward</td>
<td>1560–1579</td>
<td>CTAGGGGGGACACCCGTGTG</td>
</tr>
<tr>
<td>HBSQ4A*</td>
<td>Forward</td>
<td>1755–1774</td>
<td>TGTCCTCTAAATCCAGATC</td>
</tr>
<tr>
<td>HBSQ4B*</td>
<td>Reverse</td>
<td>1774–1755</td>
<td>GATCCTGGAATTAGGACA</td>
</tr>
<tr>
<td>HBSQ5B*</td>
<td>Reverse</td>
<td>1977–1958</td>
<td>GAAACAAATGGGCTAGTAAA</td>
</tr>
<tr>
<td>HBL5*</td>
<td>Reverse</td>
<td>2175–2156</td>
<td>GGGGACGTCCCAATGACATGCCCATGA</td>
</tr>
<tr>
<td>PGP†</td>
<td>Forward</td>
<td>2657–2676</td>
<td>AAGGGCTTAAGTGGGTAAA</td>
</tr>
<tr>
<td>PGN†</td>
<td>Reverse</td>
<td>94–113</td>
<td>CAGCTATGACCATGATTACG</td>
</tr>
</tbody>
</table>

* HBV-specific sequences numbered from the ATG of the HBV core gene (Vaudin et al., 1988).
† Plasmid pGEM-3Z sequences (Promega) numbered from the initiation codon of the T7 promoter.
A unique change, A → G, involving nucleotide 421 numbered from the ATG of the S gene resulted in an amino acid substitution of residue 141 from lysine to glutamic acid. (ii) Changes described in (i) were detected in 12 clones derived from child M06. Clone 6/21 had an additional change resulting in a TAA stop codon at amino acid 140. Clone 6/03 had classical HBV sequence for this region.

1 Glutamic acid; 2, stop codon; 3, threonine. (b) Autoradiographs of the DNA sequences described above showing the nucleotide changes in variant strains, nucleotide 421, A → G, (i and iii), and in the variant clone 6/21, nucleotide 419, C → A (iii). Sequence (ii) is that of the classical isolate.

circulating anti-HBc antibodies and the detection of HBV DNA by PCR 2 years later suggested that the child had become infected in the interim period.

Anti-HBc antibodies were present in child M06 at 1-5 years of age in the absence of HBV DNA (Table 1), but were no longer detectable 2 years later. These could have represented maternal antibodies which may persist for 1 to 2 years (Chotard et al., 1992). HBV DNA estimated at 740 pg/ml, equivalent to 2.5 × 10⁸/ml of virus particles in the serum, was detected by dot-blot hybridization assay and PCR at 3.5 years of age, indicating that this child had also become infected despite the presence of protective anti-HBs antibodies.

Anti-HBs antibody titres of 10 mIU/ml or greater are considered protective against infection with HBV (Hadler et al., 1986) and are normally present following recovery from infection or vaccination. Both children had therefore developed protective levels of anti-HBs antibodies, but had subsequently become infected with HBV as indicated by circulating DNA, which was relatively high in the case of child M06. The absence of anti-HBeAg (anti-HBe) antibodies also suggested that the HBV infection was in a viraemic phase in both children. In addition, child M74 had developed an anti-HBc antibody response to infection.

All 12 clones from child M74 were found to show a characteristic sequence between amino acids 139 and 147 of the a determinant site (Fig. 1). Comparison with the sequence of a classical, ayw4 subtype HBV (Norder et al., 1992a) demonstrated a change at nucleotide 421, numbered from the ATG of the S gene, from adenosine to guanosine resulting in an amino acid substitution at residue 141 from lysine to glutamic acid.

This a determinant site of HBV DNA isolated from child M06 was sequenced using eight clones from one PCR product and six clones from an additional PCR. Twelve of these clones contained the nucleotide 421 change observed in the HBV isolate from child M74 (Fig.
adenosine resulting in a TAA stop codon at amino acid 140. The translation product is deduced to be a truncated S polypeptide consisting of 139 amino acids instead of the normal 226. The remaining clone (6/03) had a threonine at residue 140. The translation product is deduced to be a truncated S protein of 139 amino acids instead of the normal 226.

Amino acids 139 to 147 of the S protein have been shown to be critical in the immune response to HBV (Howard et al., 1986; Steward et al., 1986) and substitutions from glycine to arginine at amino acid 145 have been related to the ability of HBV isolates to avoid protective anti-HBs immunoglobulins or MAbs (Carman et al., 1990; Harrison et al., 1991; McMahon et al., 1992). Results obtained in the present study suggest that in The Gambia an additional HBV variant is circulating which is capable of avoiding protective levels of anti-HBs antibodies. This variant is characterized by a unique amino acid change within the 139 to 147 amino acid loop of the a determinant.

Neither of the children investigated here showed any evidence of either HBsAg or HBeAg circulating in association with HBV DNA. It is possible that sampling was carried out during the 'window' period in convalescence when levels of HBsAg and HBeAg had declined to below detectable levels, particularly using the relatively insensitive reverse passive haemagglutination method (RPHA) for HBsAg. However, the detection of HBV DNA, in the absence of anti-HBe antibodies, suggests a persistence of the viraemic phase. It is likely that the antigens were present as immune complexes and were therefore not detectable. Previous studies in the villages of Keneba and Manduar have shown that 14% to 16% of children, between the ages of 0·5 to 4 years, who were chronic carriers of HBsAg had no serologically detectable levels of HBeAg (Whittle et al., 1990). However, the absence of HBsAg and HBeAg could reflect variations in either or both of these antigens. Studies using amino acid replacement assays have shown that for HBsAg, replacing residues 141 to 146 markedly reduced or abolished reactivity of synthetic peptides with rabbit anti-HBs antibodies. In particular, amino acid replacement of lysine at 141 by glutamic acid resulted in a complete loss of reactivity of the peptide with anti-HBs antibodies as measured by radioimmunoassay (Neurath et al., 1990) and ELISA (Steward et al., 1993). Lysine, a basic residue with a long side chain and glutamic acid, an acidic residue with a shorter side chain are molecules of similar mass (lysine 128·18 and glutamic acid 129·12), but different volume (glutamic acid 18·9% less than lysine). This substitution is therefore likely to affect anti-HBs recognition of the variant HBV in vivo and of HBsAg in serological tests.

The S gene sequence of HBV DNA from child M74 was examined to characterize the sub-determinant sites identified by Okamoto et al. (1987). The presence of an arginine at 122 was indicative of the $y$ subtype and lysine at residue 160 specified the $w$ subtype. This HBV isolate therefore appeared to be of the $yw$ subtype. The sequence was compared to the $ayw4 (P4)$ sequence (Norder et al., 1992a) to which it appeared to be particularly closely related, with nucleotide and amino acid variations of 0·7% and 0·9% respectively (Fig. 2). The $ayw4$ sequence was originally identified in HBV isolates from the West African countries of Senegal, which surrounds The Gambia, and Nigeria (Norder et al., 1992b).

The HBV polymerase gene open reading frame, which overlaps the S gene, is in the +1 reading frame in relation to the latter. The two unique nucleotide substitutions at positions 421 and 625 of the S gene (Fig. 2) resulted in amino acid substitutions at codon 484 of the polymerase from lysine to arginine and at codon 552 from leucine to proline respectively. These changes are within a region of the putative DNA polymerase/reverse transcriptase segment of the gene which is poorly conserved among mammalian strains of HBV, and in which there is a 54-amino acid deletion in the duck HBV polymerase (Radziewl et al., 1990). It is likely that these amino acid substitutions did not adversely affect viral polymerase function as the virus appears to be replication-competent.

Variant HBV has been detected in The Gambia in vaccinated children with protective levels of anti-HBs antibodies. It remains to be determined whether the children were originally infected with a classical HBV strain which underwent mutation and immune selection resulting in the variant strain becoming the predominant species. HBV has a relatively high mutation rate attributed to the virus-encoded reverse transcriptase which is utilized in replication. The rate has been estimated at $2 \times 10^{-4}$ substitutions per site per year for the woodchuck hepadnavirus (Girones & Miller, 1989). A variant strain, once established, could spread quite readily among the children in The Gambia where the main mode of transmission is horizontal, from sibling to sibling (Whittle et al., 1990). It is also possible that these children were initially infected with the variant strain that had arisen in the population or, as in the case of the child M06, with a mixed population of the virus. It is extremely unlikely that this initial infection was a result of the variant strain being present as a contaminant in the HBV vaccine the children received because the vaccine is formalin-treated during manufacture. In addition, the serological profiles of the children did not show any evidence of HBV infection in either child at about 10 months following vaccination which is outside the usual incubation period of between 6 weeks and 6
Fig. 2. The S gene and deduced amino acid sequences of the variant isolate is compared with the sequences of HBV ayw4 (4) subtype (Norder et al., 1992a). Unique amino acid and nucleotide variations are underlined. Variations of the amino acid sequence of the variant strain are indicated in parentheses. Amino acids 139 to 147, and the corresponding nucleotides, are boxed. (4) Stop codon. In addition to the change from threonine to serine at codon 140, both strains shared two additional substitutions (nucleotide 6, G to A, amino acid 2 conserved; and nucleotide 8, A to G, resulting in asparagine being replaced by serine at amino acid 3). Besides the change at nucleotide 421, an additional unique nucleotide change was detected in the Gambian variant virus (base 625, T to C; amino acid 209 conserved). The nucleotide sequence of the variant HBV differed from the ayw4 subtype at three other sites (nucleotide 135, T to A, amino acid 45 conserved; nucleotide 176, G to A, with serine being substituted by asparagine at codon 59; and nucleotide 642, G to T, with amino acid 214 conserved).

months. Furthermore, the DNA sequence of the S gene of the variant strain is very closely related (99.3%) to the ayw4 wild-type virus found in other West African countries suggesting that it had originated from strains circulating in the region. Longitudinal studies involving the analysis of HBV DNA isolates from vaccinated and unvaccinated children are currently being carried out and should serve to resolve these questions. In any event, current evidence suggests that mutations introduced into the HBV genome enable the virus to escape immune
recognition. This may have to be taken into consideration in the future design of HBV vaccines and diagnostic assays.

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References


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