Hepatitis B Surface Antigen Variant with Multiple Mutations in the a Determinant in an Agammaglobulinemic Patient

Alexandra Alexopoulou,1 Gerasimos Baltayiannis,2 Saffie Jammeh,2 Jenny Waters,2 Spyros P. Dourakis,1 and Peter Karayiannis2*

Academic Department of Medicine, Hippokration General Hospital, Athens, Greece,1 and Department of Medicine, Imperial College London, Faculty of Medicine, St. Mary’s Campus, London, United Kingdom2

Received 4 December 2003/Returned for modification 26 January 2004/Accepted 17 February 2004

A patient with agammaglobulinemia developed acute hepatitis that progressed to chronic liver disease with high levels of hepatitis B virus (HBV) DNA in the absence of detectable HBsAg. Sequencing of the a determinant region of HBsAg revealed multiple amino acid substitutions that, unusually, also included a substitution at position 122 that defines subtype specificity. All of these mutations had a profound effect on the antigenicity of this region, which led to the complete failure of variant detection by commercially available routine diagnostic assays or laboratory-based monoclonal antibody assays.

CASE REPORT

A 50-year-old agammaglobulinemic Greek male patient with a past medical history of multiple respiratory and skin infections presented with acute hepatitis. He had been receiving treatment for polycythemia rubra vera with hydroxyurea during the 2 years prior to admission. In addition, prior to presentation he had been receiving treatment with nimesulide and 32 mg of methylprednisolone daily for 6 and 5 months, respectively, for non-specific arthritis. The dose of the latter was tapered down during the last month of treatment, and prior to its withdrawal, the patient presented with acute hepatitis with alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, and serum bilirubin levels of 360 IU/liter, 107 IU/liter, and 1.0 mg/dl, respectively. Total immunoglobulin levels were very low at 55 mg/dl (IgG, 33 mg/dl; IgA, 7 mg/dl; IgM, 11 mg/dl). CD4+/H11001 and CD8+/H11001 ratios of 1 were recorded in peripheral blood. The B-lymphocyte number was reduced. Gamma globulin (Sandoglobulin; Novartis) was first infused at a dose of 400 mg/kg of body weight 1 week after admission, and infusions were repeated every 3 weeks thereafter. Steady state was not achieved, as indicated by the low levels of immunoglobulin detected prior to each infusion. Family contacts were negative for markers of past or present hepatitis B virus (HBV) infection, and HBV DNA was undetectable in their sera.

HBV DNA levels of 1.1 × 10⁷ copies/ml were recorded on presentation and 6 months later (samples 1 and 2, respectively), even though the patient was HBsAg negative (HBV Monitor; Roche Diagnostic Systems Inc., Branchburg, N.J.). At 6 months, liver aminotransferase levels were still elevated (AST level, 94 IU/liter; ALT level, 121 IU/liter) and the HBV serological profile was the same as that at presentation. Treatment with lamivudine was initiated at this point, with a gradual decrease in the viral load to 10⁶ copies/ml during the 5th month after the start of treatment. This was accompanied by normalization of ALT levels.

Amplification and sequencing. HBV DNA was extracted from sample 1 (acute-phase serum), and 5 μl was used to amplify the surface gene with primers S1 and S4, as described elsewhere (17, 27). Amplicons were purified with a QIAEX II gel extraction kit (Qiagen Ltd., Crawley, United Kingdom) and then cloned into the TA vector pGEM-T easy (Promega, Southampton, United Kingdom). Transformation of Escherichia coli was followed by the selection of up to 20 colonies for preparation of plasmid DNA. Plasmids containing inserts were sequenced with a BigDye Terminator Ready Reaction kit and an ABI Prism 377 automatic sequencer (Applied Biosystems, Warrington, United Kingdom).

The nucleotide and amino acid sequences were edited, aligned, and compared with each other and with published sequences by using Dnasis and Prosis software, respectively (Hitachi, Yokohama, Japan). The amino acid sequences obtained are shown in Fig. 1. Between the cysteine residues at positions 124 and 147, there were 5 amino acid substitutions in all. These were T for M at position 124, H for Y at position 134, Y for C at position 139, G for D at position 144 (32), and...
the well-known R-for-G change at position 145. The M residue at position 125 is present in other subtypes and genotypes of viruses with normal HBsAg reactivities. However, the effect of this substitution on HBsAg antigenicity in the context of the other changes seen here remains unknown. The Y-to-H substitution at position 134 is quite novel. Previously described changes at this position in liver transplant recipients treated with immunoglobulin involved I or Y to F and F to T, depending on the subtype (11, 26). The cysteine (C) residues are highly conserved among all subtypes. In vitro studies have shown that antigenicity is lost when any one of the C residues at position 124, 137, 139, 147, or 149 is replaced with serine, suggesting that these amino acids play a critical role in the conformational structure of the a determinant. On the contrary, the C residue at position 138 has little or no effect on antigenicity (1, 4, 8). All clones from our patient had a replacement of cysteine with Y at position 139, and an additional change of C149 to Y was detected in one other clone. Replacement of C124 and C137 with Y has previously been reported in liver transplant recipients following monoclonal antibody treatment (20).

There were, in addition, two other amino acid substitutions between cysteine residues 121 and 124. One replaced the subtype-determining amino acid at position 122 with I, and the other replaced the adjacent amino acid at position 123 with N. Mutations of amino acids in this region have been described only once before (12). Moreover, all clones sequenced had A instead of T at position 118 and N instead of S at position 174. Other changes seen here remain unknown. The Y-to-H substitution on HBsAg antigenicity in the context of the other changes described by Gunther et al. (13) and directly sequenced. Phylogenetic tree analysis and tree construction performed with the MEGA (version 2.1) program firmly placed the patient isolate within the ayw cluster (Fig. 2).

**Phylogeny.** Comparison of the sequence of the isolate from our patient with those of strains of various genotypes indicated that the isolate from our patient was of genotype D. Despite the absence of K or R at position 122, which are determinants for subtype d and y specificities, respectively, the isolate from our patient was determined to have originated from subtype ayw3. This was based on the presence of T46, G159, and A168, all of which are yw subtype determinants, and y allelic determinant T68 in the absence of R122. The presence of T127 determines the w3 specificity (22–24). That the isolate was of the ayw subtype was further substantiated by nucleotide sequence comparisons, which showed more than 97% sequence identity with other ayw isolates and 88 and 89% identities with the adr and adw subtypes, respectively. Moreover, the entire genome was amplified with the primers and under the conditions described by Gunther et al. (13) and directly sequenced.

**Monoclonal antibody binding studies.** An attempt was made to further characterize the epitopes of the variant HBsAg by using a panel of monoclonal antibodies, one of which had a bias against ayw subtypes (19). This antibody (antibody 1021) and one other (antibody 1044) were kindly provided by Morag Ferguson, National Institute of Biological Standards and Control, South Mimms, United Kingdom, and, together with our Royal Free Hepatitis B surface series of monoclonal antibodies, were used to coat polystyrene beads in bicarbonate buffer overnight (29, 30). The beads were then used in enzyme-linked immunosorbent assays (ELISAs) with an antibody from the Murex kit used as the detection antibody. The results obtained with the acute-phase serum sample (sample 1) and that obtained at 6 months (sample 2), together with serum samples each containing subtypes adr, adw, and ayw, are given in Table 1. Whereas wild-type HBsAgs were detected by all monoclonal antibodies and with the Murex kit, neither of the two patient serum samples showed any binding whatsoever, even though the viral load in serum sample 2 was 10 times higher than that for the control serum sample containing subtype ayw. In view of this, it was difficult to determine serologically the ayw specificity of the present isolate. In our hands, antibody 1021 detected HBV in all positive control sera equally well, even when the sera were diluted 1/5,000 (data not shown).

**Other genomic regions.** The effects of the mutations in the a-determinant region on the overlapping reverse transcriptase (RT) domain of the polymerase gene were also investigated. Nucleotide substitutions at codons 118, 122, 123, 134, and 145 of the surface antigen gene (S) open reading frame (ORF) led to amino acid changes at RT positions 126 (R to H), 130 (H to P), 131 (K to D), 142 (A to V), and 153 (Q to R), respectively. All these changes were in the intervening region (fingers subdomain), between domains A and B of the RT. All other changes were silent, including that at position 174 of the S ORF (RT position 182), the only one that occurred within one of the functional RT domains (domain B). Mutations in the RT region of the polymerase gene have been associated with resistance to nucleoside analogues (2). However, none of the mutations in the S ORF of the present isolate affected any of the

<table>
<thead>
<tr>
<th></th>
<th>120</th>
<th>130</th>
<th>140</th>
<th>150</th>
<th>160</th>
<th>170</th>
<th>180</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AYW</strong></td>
<td>PGSSTSTG</td>
<td>CRTMTIAGQ</td>
<td>TSMYPSCCCT</td>
<td>KPSDGNCCTC</td>
<td>FIPSSWAFPG</td>
<td>FLWEWAAGRF</td>
<td>SWLSSLVFVF</td>
<td></td>
</tr>
<tr>
<td><strong>ADW</strong></td>
<td>-T-</td>
<td>-K-</td>
<td>-T-</td>
<td>-K-</td>
<td>-T-</td>
<td>-K-</td>
<td>-T-</td>
<td></td>
</tr>
<tr>
<td><strong>ADR</strong></td>
<td>-T-</td>
<td>-K-</td>
<td>-T-</td>
<td>-K-</td>
<td>-T-</td>
<td>-K-</td>
<td>-T-</td>
<td></td>
</tr>
<tr>
<td>Clone 4</td>
<td>-Y-</td>
<td>-Y-</td>
<td>-GR-</td>
<td>-Y-</td>
<td>-GR-</td>
<td>-Y-</td>
<td>-Y-</td>
<td>9</td>
</tr>
</tbody>
</table>

FIG. 1. Distribution of the amino acid substitutions detected in the variant. The amino acid sequences from codon positions 110 to 180 of the surface antigen gene were compared with three reference sequences, those of subtypes ayw (GenBank accession no. HPBAYW), adw (GenBank accession no. M54923), and adr (GenBank accession no. D00630). The percentages of the major and minor clones detected are also noted. Boldface letters represent the a-determinant region.
well-recognized functional domains of the RT. The high viral loads seen in the patient's serum in the absence of wild-type virus suggest that they had no adverse effect on polymerase function. Two of the mutations seen in the a determinant in our patient (T123N, G145R), which fall within the fingers subdomain of RT, have recently been shown to replicate with equal efficiency as the wild-type sequence and, in addition, to restore the replication capacity of lamivudine-resistant variants (28).

Amplification of the entire precore-core region with primers PC and 3C (17), followed by cloning and sequencing, showed that the virus carried the G1896A stop codon mutation, but the core promoter sequences were those of wild-type strains (1762A, 1764G).

**Discussion.** Antibodies against the group-specific a determinant (codon positions 124 to 147), a complex antigenic structure with multiple immunogenic epitopes, normally neutralize virus and confer cross-protective immunity to all HBV subtypes. Historically, the secondary structure of this epitope is presented as a double loop, formed through disulfide bridges between cysteine residues 124 and 137 and residues 139 and 147 (3). Nucleotide substitutions that lead to amino acid changes within this region may result in reduced binding or failure to detect serum HBsAg in diagnostic assays with polyclonal and/or monoclonal antibodies (29). Amino acid changes have been described at positions 126, 129, 131, and 133 of the first loop of the a determinant and positions 141, 142, 144, and 145 of the second loop (14, 16, 18, 31). By far the most common mutation that has been reported is G145R (6), which occurs either alone or in combination with others. This variant displays reduced levels of binding to anti-HBs antibodies, and it is usually selected under immune pressure after administration of the HBV vaccine, with or without concurrent hepatitis B immunoglobulin (HBIG), or following treatment with polyclonal or monoclonal HBIG (20). Naturally occurring surface gene variants have also been reported around the world in persons who have not been immunized (9, 10, 15, 31).

This is the first time that a surface antigen gene variant has been described in an agammaglobulinemic patient with a deficient antibody response. The patient had never been vaccinated or received immunoglobulins prior to the development of acute HBV infection. The most plausible explanation for the acute episode of hepatitis B in our patient is reactivation of HBV infection after tapering and withdrawal of corticosteroid therapy. It is very likely that our patient was a carrier of the

**TABLE 1.** Ratios of optical densities for samples containing known HBV subtypes and the two patient sera to the optical densities for negative controls obtained by monoclonal antibody-based ELISAs and with the Murex kit

<table>
<thead>
<tr>
<th>Sample</th>
<th>MAb&lt;sup&gt;a&lt;/sup&gt; 1044</th>
<th>MAb&lt;sup&gt;a&lt;/sup&gt; 1021</th>
<th>MAb&lt;sup&gt;a&lt;/sup&gt; RFHBs1</th>
<th>MAb&lt;sup&gt;a&lt;/sup&gt; RFHBs2</th>
<th>MAb&lt;sup&gt;a&lt;/sup&gt; RFHBs7</th>
<th>MAb&lt;sup&gt;a&lt;/sup&gt; RFHBs18</th>
<th>Murex kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>adr serum</td>
<td>4.58</td>
<td>5.42</td>
<td>3.0</td>
<td>5.33</td>
<td>1.5</td>
<td>2.91</td>
<td>6.67</td>
</tr>
<tr>
<td>adw serum</td>
<td>4.72</td>
<td>5.33</td>
<td>3.08</td>
<td>7.94</td>
<td>2.98</td>
<td>6.4</td>
<td>6.64</td>
</tr>
<tr>
<td>ayw serum</td>
<td>4.7</td>
<td>5.47</td>
<td>3.07</td>
<td>1.4</td>
<td>1.79</td>
<td>5.89</td>
<td>6.71</td>
</tr>
<tr>
<td>Sample 1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>1.0</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> All samples were tested at a 1/10 dilution. Samples 1 and 2 were from the acute phase and 6 months after the acute phase, respectively.

<sup>b</sup> MAb, monoclonal antibody.

<sup>c</sup> ND, not determined.
variant with latent virus replication (occult HBV) infection, as indicated by the absence of any evidence of fibrosis in liver sections. This is further supported by the detection of the precore stop codon mutation, normally seen in virus isolates from patients in the anti-HBe-positive phase of chronic infection (5). The delay in the detection of the HBV carrier state was primarily due to our inability to detect HBsAg by conventional diagnostic methods. As has been reported previously (6, 27, 29), the loss of certain conformational epitopes within the a determinant as a result of important amino acid substitutions would modify its antigenicity and render it undetectable. In a patient with a primary defect in immunoglobulin production, the variant could represent the original infecting virus. However, the presence of anti-HBs in the serum of the patient would indicate that he was capable of producing at least some antibody (15), but whether this antibody selected the variant previously and kept it under control until steroid withdrawal or whether it was produced after the acute episode cannot be established in the absence of sera from before steroid withdrawal. The level of anti-HBs produced was relatively low compared with the high viral load and therefore may have been insufficient to clear the virus. The humoral immune system defect in our patient prevented him from producing IgM-class antibodies, and this may account for the absence of such antibodies during the reaction period. Carman et al. (7) reported on a similar case in a patient who received corticosteroids for lymphoma and who developed fulminant hepatitis B due to an envelope protein mutant that escaped detection by a monoclonal HBsAg assay.

One other possibility is that the patient may have been immune to HBV because of the presence of anti-HBs and anti-HBe antibodies, and he may have newly contracted the G145R variant. At that time, the existing neutralizing antibody may not have been able to prevent infection by the variant virus. However, this explanation can be discounted, as the patient was not at risk of contracting HBV infection. He had a monogamous sexual relationship, he denied any extramarital sexual contacts, and he did not belong to any of the high-risk groups. He lived on a sparsely populated Aegean island.

In conclusion, a surface gene variant has been described in a patient with agammaglobulinemia. The variant had an unprecedented number of amino acid substitutions in the a-determinant region. In spite of this, the variant appeared to be replication competent, achieving high levels of viremia.

Nucleotide sequence accession number. The full nucleotide sequence of the variant HBV isolate has been submitted to GenBank under accession number AY341335.

REFERENCES


