A quasi-monoclonal anti-HBs response can lead to immune escape of ‘wild-type’ hepatitis B virus

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Hepatitis B virus (HBV) infections can be prevented or controlled by the host humoral immune response (anti-HBs) directed against the major surface antigen (HBsAg), elicited either naturally or by vaccination. A chronic HBV carrier was found to have high levels of both virus and anti-HBs. Full-length HBV genomes were amplified from the patient’s serum, sequenced and cloned. The genome was ‘wild-type’ HBV of genotype C and serotype adr. The sequence has remained stable, with no signs of emergence of an immune-escape mutant population. To study what was recognized by the patient’s serum, viral particles were 35S-labelled and then immunoprecipitated by using the patient’s serum or control sera. The patient’s serum immunoprecipitated the adr HBsAg encoded by his HBV genome poorly, but efficiently recognized HBsAg of serotype ayr. When his HBV genome was modified by a point mutation to express HBsAg of serotype ayr, the patient’s serum could recognize the antigen, as well as the control anti-HBs-positive serum. The patient appeared to have made a quasi-monoclonal humoral response to the y epitope. By switching to the d epitope, which requires only a point mutation, the virus could replicate, despite the high levels of anti-HBs. This study underlines the subtleties of virus–host interactions. Implications for HBV vaccination are discussed.

INTRODUCTION

Unlike viruses that replicate via an error-prone RNA-intermediate pathway, such as hepatitis C or human immunodeficiency viruses, hepatitis B virus (HBV) infections can be controlled effectively by the host humoral immune response, either natural or elicited by vaccination. In vaccinated individuals, the response is neutralizing and immune response, either natural or elicited by vaccination. A chronic HBV carrier was found to have high levels of both virus and anti-HBs. Full-length HBV genomes were amplified from the patient’s serum, sequenced and cloned. The genome was ‘wild-type’ HBV of genotype C and serotype adr. The sequence has remained stable, with no signs of emergence of an immune-escape mutant population. To study what was recognized by the patient’s serum, viral particles were 35S-labelled and then immunoprecipitated by using the patient’s serum or control sera. The patient’s serum immunoprecipitated the adr HBsAg encoded by his HBV genome poorly, but efficiently recognized HBsAg of serotype ayr. When his HBV genome was modified by a point mutation to express HBsAg of serotype ayr, the patient’s serum could recognize the antigen, as well as the control anti-HBs-positive serum. The patient appeared to have made a quasi-monoclonal humoral response to the y epitope. By switching to the d epitope, which requires only a point mutation, the virus could replicate, despite the high levels of anti-HBs. This study underlines the subtleties of virus–host interactions. Implications for HBV vaccination are discussed.

The GenBank/EMBL/DDBJ accession number for the sequence determined in this work is AJ749098.

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surveillance by anti-HBs antibodies. The corollary is that, at least initially, these mutant antigens are recognized poorly or not at all by the commercial HBsAg detection assays (Coleman et al., 1999; Ireland et al., 2000). The fact that these mutations have not become fixed in the general HBV population during the course of evolution indicates that the mutants are less fit than the ‘wild-type’ viruses, as a result of either the mutations also affecting the overlapping P gene or the altered structure of HBsAg reducing infectivity. However, the existence of these mutants creates a situation in which there is circulating HBV, despite the presence of significant levels of anti-HBs. We describe here a similar situation, but one in which the circulating virus is apparently ‘wild-type’, and put forward a mechanism of immune escape that appears to be the most probable reason for the existence of this situation.

METHODS

Patient. The patient was code-named Z1. He was born in Vietnam in 1998, was adopted and arrived in France at the age of 6 months. The foster-parents had signed an informed-consent letter. Obtained during regular consultations. The foster-parents had signed in 1998, was adopted and arrived in France at the age of 6 months. The existence of this situation.

Table 1. Virological and biochemical follow-up of patient Z1

<table>
<thead>
<tr>
<th>Date</th>
<th>α-HBs (IU l⁻¹)</th>
<th>HBV DNA (×10⁶ equivalents ml⁻¹)</th>
<th>HBsAg</th>
<th>α-HBc</th>
<th>HBeAg</th>
<th>α-HBe</th>
<th>HIV1/2</th>
<th>γGT (IU l⁻¹)</th>
<th>ASAT (IU l⁻¹)</th>
<th>ALAT (IU l⁻¹)</th>
<th>αFP (µg ml⁻¹)</th>
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<td>9</td>
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<td>7</td>
<td>33</td>
<td>36</td>
<td>34</td>
<td>1-40</td>
</tr>
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*-Bayer Versant Quantiplex HBV-DNA.
†Tested for IgM anti-HBc.
‡Bayer Versant HBV 3.0.
Immunoprecipitation. Clarified medium (300 μl) was diluted with an equal volume of 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 2 % BSA (TNE-BSA) and 5 μl antiserum was added. Three antisera were used: the August 2002 serum of Z1, an anti-HBs-positive serum (AK) and an anti-HBs-negative serum (AK-PI). Sera AK and AK-PI came from one of the authors, who had been initially vaccinated and boosted with Genhevac B, which contains an HBsAg of serotype ayw, and was reboosted 7 years later with Engerix B, which contains an HBsAg of serotype adr. The anti-HBs titre of serum AK was 1000 IU l<sup>-1</sup>. AK-PI was the pre-immune serum collected before the first HBV vaccination. The mixtures were incubated on a rotating wheel for 90 min at 4 °C. A 20 % (v/v) suspension of Protein A-Sepharose CL-4B (Amersham Biosciences) in TNE-BSA (100 μl) was then added to each tube and incubation was continued for a further 90 min. The tubes were centrifuged briefly, the supernatants were removed and the beads were washed four times with 1 ml TNE-BSA. In order to keep the viral particles in their native form, detergents were avoided. The beads were transferred to clean tubes by using water, centrifuged and, after removal of the supernatants, the beads were dried under vacuum and resuspended in 30 μl sample loading buffer [Tris/HCl (pH 6.8), 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 0.001 % bromophenol blue]. After boiling for 5 min, 15 μl aliquots were analysed by SDS-PAGE (12 % gel; acrylamide : bis-acrylamide, 30 : 0.8). After electrophoresis, the gels were fixed, impregnated with En3Hance fluor (Perkin Elmer), dried and exposed to autoradiography film at −70 °C for 1–3 days. Films were scanned and the results were mounted in PowerPoint.

Mutagenesis. The cloned Z1 genome was mutagenized by using a QuikChange Mutagenesis kit (Stratagene) and the oligonucleotide 5′-GGACCAGTCGACGATCCGCTTGCTC-3′ (nt 509–541) and its complement. By using degenerate nucleotides, all three of the desired mutants (Z1-122R, Z1-126I and Z1-122R126I) could be obtained in one mutagenesis reaction. In addition, the nucleotide changes conformed to the sequence of the isolate with GenBank accession no. AF223955 and did not affect the overlapping polymerase gene. In addition, changing nt 519 from A to G introduced a TfiI site. These were used for initial screening of the clones obtained after mutagenesis. One clone of each type was selected and the HBsAg-coding regions were sequenced to make sure that only the desired mutations had been introduced. The three constructions, along with the original Z1 genome, were transfected into HuH7 cells, which were then 35S-labelled. Viral particles were immunoprecipitated as described above.

RESULTS

Code-named Z1, the patient was born in 1998 in a rural province of central Vietnam. He was adopted by French foster-parents and arrived in France at the age of 6 months. He was certainly not vaccinated against HBV in France and was probably not vaccinated in Vietnam, a neonatal vaccination programme having been initiated in only a part of the country in 1997 and on a national scale only in 2003 (www.who.int/vaccines-surveillance/WHO UNICEF_Coverage_Review/pdf/viet_nam.pdf). During the adoption process he was found to be HBsAg-positive (Table 1). He was and has remained asymptomatic. During a routine check-up in April 2002, he was found to have not only a moderately high level of viraemia ( > 5 × 10<sup>7</sup> copies ml<sup>-1</sup>), but also a high titre of anti-HBs antibodies ( > 600 IU l<sup>-1</sup>). By August 2002, the viraemia had increased tenfold and has remained high since then, as have his anti-HBs titres, although these have decreased slowly over time (Table 1). Suspecting the presence of an immune-escape mutant, full-length HBV genomes from Z1 were amplified and sequenced. The genome was found to be genotype C (Fig. 1) and, more particularly, a C-1858 variant that is found in Southeast Asia, including Vietnam (Alestig et al., 2001). To our surprise, the sequence was apparently ‘wild-type’, with the notable exception of an 18 nt deletion involving the ATG codon normally used for initiation of the preS1 protein. However, many HBV genomes have deletions in this region and several have a deletion identical to that of the Z1 genome, including two C-1858 variants (Fig. 1). A viable preS1 protein can be initiated at an in-phase ATG codon 13 codons downstream from the normal initiation codon, and HBV genotype D naturally has such a truncated preS1 protein. The genome of Z1 encoded an HBsAg of serotype adr, with residues 122 and 160 being Lys and Arg, respectively (Table 2; Fig. 2). When compared with the consensus HBsAg sequence of C-1858 variants, the HBsAg of Z1

![Fig. 1. Phylogenetic tree of nucleotide sequences of complete HBV genomes representing the eight known HBV genotypes (A–H), showing that the HBV genome of Z1 belongs to the C-1858 variant group of genotype C. The geographical origins of the genotype C isolates are shown and the C-1858 variant cluster is indicated. Large arrowheads point to two C-1858 variant genomes that have an 18 bp deletion identical to that found in the HBV genome of Z1. Sequences are identified by their GenBank accession numbers.](http://vir.sgmjournals.org)
Table 2. Molecular basis for HBsAg serotypes

The conformational α determinant that is common to all of the serotypes is defined by residues 124–147. Genotypes that can contain the serotype are shown in parentheses. Small capitals indicate genotypes that contain the serotype infrequently.

<table>
<thead>
<tr>
<th>Serotype</th>
<th>HBsAg residues involved</th>
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<tbody>
<tr>
<td>ayw1 (A, B)</td>
<td>122R + 160K + 127P + (134F and/or 159A)</td>
</tr>
<tr>
<td>ayw2 (D)</td>
<td>122R + 160K + 127P</td>
</tr>
<tr>
<td>ayw3 (D, C)</td>
<td>122R + 160K + 127T</td>
</tr>
<tr>
<td>ayw4 (E, D, F)</td>
<td>122R + 160K + 127L</td>
</tr>
<tr>
<td>ayr (C)</td>
<td>122R + 160R</td>
</tr>
<tr>
<td>adw2 (A, B, G, C)</td>
<td>122K + 160K + 127P</td>
</tr>
<tr>
<td>adw3 (α)</td>
<td>122K + 160K + 127T</td>
</tr>
<tr>
<td>adw4&lt;− (F, H)</td>
<td>122K + 160K + 127L + 178Q</td>
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<td>adr&lt;+ (C)</td>
<td>122K + 160R + 177V + 178P</td>
</tr>
<tr>
<td>adr&lt;− (C)</td>
<td>122K + 160R + 177A</td>
</tr>
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</table>

differed at only two positions, residues 5 and 126. A Ser residue at position 5 is rare in human HBsAg, but has been found in strains from Vietnam (Hannoun et al., 2000). A Thr at position 126 is very common in HBV isolates, including those of genotype C.

Full-length genomes were also amplified from the three subsequent samples. The HBsAg-coding region of the December 2002 sample and the complete genomes of the two other samples were sequenced. All were identical to the sequence determined from the August 2002 sample. To see whether a minor population of immune-escape mutants was emerging, 29 clones in total, 11 from the August 2002 sample and 18 from the June 2003 sample, were sequenced in the HBsAg-coding region. Of these, only six showed substitutions compared with the HBsAg sequence obtained from direct sequencing (not shown). No substitution involved residues known to be implicated in HBV immune escape. Also, substitutions were all due to point mutations and no two clones showed an identical substitution. Therefore, it was not possible to discriminate between a real microheterogeneity of the genome and errors introduced during PCR. However, there were no indications of an emerging immune-escape mutant population.

The first hypothesis to explain the paradox between circulating ‘wild-type’ HBV and high levels of anti-HBs is that the Z1 genome overexpresses HBsAg, thereby swamping the anti-HBs humoral immune response. However, when the Z1 genome and a control ‘wild-type’ genome (15803) isolated from a typical chronic HBV carrier were transfected into HuH7 cells, there were no significant differences in the levels of HBV DNA or RNA synthesized, including HBsAg mRNA, or in HBsAg expression and secretion (not shown).

To see what the serum of Z1 was capable of recognizing, viral particles were 35S-labelled after transfection of full-length genomes into HuH7 cells and immunoprecipitated. Three genomes were used: Z1, 15803 encoding HBsAg of serotype ayw, and CS, a ‘wild-type’ genome that, like Z1, encodes an HBsAg of serotype adr, but which was isolated from a typical chronic HBV carrier. Three sera were used: Z1 serum, an anti-HBs-positive serum (AK) and an anti-HBs-negative serum (AK-PI). Sera AK and Z1 recognized the serotype ayw HBsAg equally well (Fig. 3). Conversely, whilst serum AK efficiently recognized HBsAg expressed by genomes Z1 and CS, these antigens were recognized poorly by Z1 serum.

This experiment indicated that Z1 serum did not strongly recognize the normally immunodominant α determinant that is common to the three antigens studied and did not allow discrimination between the possible implication of the d/y or the r/w subtype determinants. However, genotype C HBsAgs, especially those from Vietnam, rarely possess the
they encode are shown. MT, Mock-transfected cells. The genomes transfected and the serotypes of the HBsAg that vary serotype. Viral particles were 35 S-labelled after transfection of full-length HBV genomes in HuH7 human hepatocyte cells. Three sera were used for immunoprecipitation: an anti-HBs-negative serum (AK-Pi), an anti-HBs-positive serum from a vaccinee (AK) and the August 2002 serum of patient Z1. The non-glycosylated form of HBsAg and the monoglycosylated form (HBsAg-CHO) are indicated. The mobilities of molecular mass markers (kDa) are shown on the right. The figure shows that Z1 serum recognized the HBV (serotype adw) currently circulating in his bloodstream only very weakly, but showed strong recognition of viral particles carrying HBsAg of serotype ayr. The genomes transfected and the serotypes of the HBsAg that they encode are shown. MT, Mock-transfected cells.

**Fig. 3.** Immunoprecipitation of 35 S-labelled HBsAg particles of varying serotype. Viral particles were 35 S-labelled after transfection of full-length HBV genomes into HuH7 human hepatocyte cells. Three sera were used for immunoprecipitation: an anti-HBs-negative serum (AK-Pi), an anti-HBs-positive serum from a vaccinee (AK) and the August 2002 serum of patient Z1. The non-glycosylated form of HBsAg and the monoglycosylated form (HBsAg-CHO) are indicated. The mobilities of molecular mass markers (kDa) are shown on the right. The figure shows that Z1 serum recognized the HBV (serotype adw) currently circulating in his bloodstream only very weakly, but showed strong recognition of viral particles carrying HBsAg of serotype ayr. The genomes transfected and the serotypes of the HBsAg that they encode are shown. MT, Mock-transfected cells.

**Fig. 4.** Immunoprecipitation of 35 S-labelled viral particles expressed by modified Z1 genomes. The original Z1 genome and three mutant constructions were transfected. The figure shows that, when HBsAg was converted from serotype adr to serotype ayr (construction Z1-122R), there was strong detection of the viral particles by Z1 serum. For other details, see legend to Fig. 3.

The serum of Z1 was unable to efficiently recognize the serotype adr HBV that is currently circulating in his bloodstream. However, when the viral genome was modified by a point mutation so that it expressed HBsAg of serotype ayr, recognition was completely restored. Z1 appears to have made a quasi-monoclonal response to the y epitope, which has allowed the adr strain to replicate freely. We cannot rule out the possibility that Z1 was initially infected with two HBV populations, one probably of serotype ayr and another of serotype adr. The patient could have mounted an anti-y response, so that the ayr virus disappeared and the adr virus became the only viral population in the patient. However, mixed infections are rare, and one would have to postulate the co-existence of two exceptional events: a mixed infection and a monoclonal response to one epitope. A simpler explanation is that the patient was initially infected with an ayr strain and, under the pressure of the anti-y response, a variant expressing the d epitope, which would require only a point mutation, was selected and escaped immune surveillance. Although the notion of quasispecies is not usually invoked in the case of HBV, because of the error-prone reverse-transcription step of the HBV replication cycle, point mutations are not uncommon. This is shown by the diversity of HBV genomes (Norder et al., 2004), the emergence of vaccine-escape mutants (Basuni & Carman, 2004), most of which involve single-base substitutions, and the fact that such mutations can be found in the non-vaccinated population (Oon & Chen, 1998). There is a precedent for point mutations changing subtype determinants (Okamoto et al., 1987): that study described patients expressing HBsAgs of either subtypes adr and ayr, or adr and adw. Two HBV genomes were found in each patient that differed in their S genes by only the point mutations that affect the subtype specificities and, in the complete genomes, there were, at most, ten differences between the two genomes. The authors concluded that point mutations within one original genome were responsible for the mixed HBsAg subtypes, which could co-exist as the patients, unlike Z1, had not mounted an anti-HBs response against one of the subtype epitopes. Incidentally, our study showed that an anti-HBs response directed against a subtype epitope can be neutralizing, as Z1 has successfully eliminated the viral population that originally expressed the y epitope.

How frequent are such cases? In the 1970s, there were several reports of co-existence of HBsAg and anti-HBs, with discrepancies between the serotype of circulating HBsAgs and the serotypes recognized by the sera (Koziol et al., 1976; Le Bouvier et al., 1976; Sasaki et al., 1976). In one study, out of 140 consecutive HBsAg-positive blood donors, three...
(2-1 %) also had heterotypic anti-HBs (Le Bouvier et al., 1976), a prevalence that is low, but not negligible. However, the limited analytical tools available at that time meant that the studies were descriptive, although the conclusions that were drawn were remarkably prescient. Here, we provide evidence for the molecular and immunological basis of the phenomenon. More recently, leaving aside immune-escape mutant cases, there have also been reports of co-existence of HBV and anti-HBs, but involving low levels of viraemia that can only be detected by PCR (Bahn et al., 1997) or borderline anti-HBs titres (Zaaijer et al., 2002). Only one reported case (Kohno et al., 1996) seems similar to that of Z1, the HBsAg also being ‘wild-type’ for serotype adr. In a haemagglutination test, the patient’s serum did not recognize HBsAg of serotype adr, but did recognize HBsAg of serotype adw. This, therefore, seems to be a case of epitope switching, involving not the d/y determinant but the r/w determinant. Viraemia (10⁴ copies ml⁻¹) and anti-HBs titres (162 IU l⁻¹) were both lower than in patient Z1, but the patient was older (23 years) and this may reflect virus-host adaptation.

What are the implications for HBV vaccination? This study illustrates the intricacies of the virus-host relationship. Z1 had mounted an anti-HBs response far stronger than that considered to be protective, but the humoral (and presumably also the T-cell) response was insufficiently broad. This left open a loophole that permitted a ‘wild-type’, and therefore fit, virus to replicate freely. If naturally infected individuals who are confronted with a full range of potential neutralization epitopes, not only on HBsAg but also on the minor envelope proteins, can sometimes mount a quasi-monoclonal response to one epitope, this may also be true for some vaccinated individuals. It is possible that some cases of vaccine escape, especially those involving mutations outside the a determinant, may be due to a humoral response that is limited in scope. Only close study of breakthrough patients in mass-vaccination programmes can answer this point. If this turns out to be a problem, then it may be necessary to increase the variety of the epitopes present in vaccines. This could involve using mixtures of HBsAgs, for example adw/ayr or adr/awy, as well as the introduction of the minor envelope proteins.

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REFERENCES


