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 prevents establishment of the infection. In acute infections, the humoral response controls virus replication and eventually, probably with the aid of appropriate T-cell responses, clears the infection. In both cases, the response is directed mainly against the major surface antigen (HBsAg) and especially against the major hydrophilic region [comprising residues approx. 103–173 of HBsAg (Persson & Argos, 1994), although other models exist (Chen et al., 1996)], which is exposed on the surface of viral particles. The major antigenic determinant in this region is the a determinant (residues 124–147), but other important determinants exist, such as the d/y determinants, which are defined by the nature of the residue at position 122 of HBsAg (Arg or Lys, respectively) and the r/w determinants at position 160 (again Arg or Lys) (Kidd-Ljunggren et al., 2002; Norder et al., 1992, 2004). In adults with acute HBV infection, over 90% develop anti-HBs antibodies and clear the virus permanently (Robinson, 1999). HBsAg is also the major component of HBV vaccines, sometimes in association with a minor envelope protein, the middle surface antigen (preS2 protein). Anti-HBs titres of >10 IU l$^{-1}$ are generally considered to be protective (Jack et al., 1999). Neonatal vaccination in areas highly endemic for HBV infection has proved to be highly effective (Hino et al., 2001; Kao & Chen, 2002; Poovorawan et al., 2000; Viviani et al., 1999), but breakthrough infections can occur, in most cases presumably due to non-response or poor response to vaccination. The effectiveness of the anti-HBs response is due, at least in part, to the limited variability of HBsAg that the virus can tolerate (Mizokami et al., 1997; Ogura et al., 1999; Torresi, 2002; Zollner et al., 2001), the reading frame for HBsAg being overlapped completely by a region of the P gene that encodes vital functional domains of the viral polymerase. However, with the advent of mass vaccination, so-called vaccine-escape mutants have emerged (Basuni & Carman, 2004; Chen & Oon, 1999; Zuckerman & Zuckerman, 1999, 2003). These mutants can in fact be detected, albeit very infrequently, in the non-vaccinated population (Oon & Chen, 1998). Most of the escape mutants described so far contain substitutions within or in proximity to the a determinant (Oon et al., 1999). It is thought that these substitutions alter the structure of the a determinant in such a way that the virus remains viable, but is able to avoid
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 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 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 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 patient was code-named Z1. He was born in Vietnam in 1998, was adopted and arrived in France at the age of 6 months.

**PCR amplification, sequencing and cloning.** Nucleic acids were extracted from 200 µl serum by using a Nucleospin Blood kit (Machery-Nagel). Full-length HBV genomes were amplified by using a one-step PCR method (Günther et al., 1995) and Platinum Taq High Fidelity DNA polymerase (Invitrogen). After purification with Wizard PCR Preps (Promega), the PCR fragment was sequenced directly by using HBV-specific primers and a BigDye terminator Ready Reaction kit (Applied Biosystems). Sequencing reactions were analysed on an Applied Biosystems Prism 377 automated-sequencing machine. For the August 2002 sample, each nucleotide was checked at least twice and, except for the two extremities, which could be sequenced in only one direction by direct sequencing, the genome was sequenced fully on both strands. The genomic sequence has been deposited in GenBank under accession no. AJ748098. For cloning, the PCR fragment was digested with restriction enzymes HindIII and SacI, whose restriction sites were contained within the two primers used for PCR amplification. The genome was then ligated into pGEM-3Zf(+) (Promega), which had been digested with the same enzymes. The region encoding HBsAg was amplified from the June 2003 sample by using the primers Pol1 (5’-CTGGTGCTCCAGTTTC-3’, nt 56–75) and Pol4 (5’-TACCCAAAGACAAAGAAAATTTG-3’, nt 826–830) and AmpliTaq DNA polymerase (Perkin Elmer). After purification, the PCR fragment was cloned directly into pGEM-T (Promega).

**Cell transfection and measurement of HBV DNA, mRNA and HBsAg-expression levels.** HuH7 cells, a human hepatocyte cell line, were grown and transfected as described previously (Jeantet et al., 2002). Two HBV genomes were used: the cloned Z1 genome and a control ‘wild-type’ genome, 15803 (GenBank accession no. AJ344117), of genotype D isolated from a typical chronic HBV carrier and amplified by the same one-step PCR used for Z1. Five days after transfection, total extra- and intracellular nucleic acids were extracted and HBV DNA and RNA levels were measured by hybridization as described previously (Jeantet et al., 2002). Extra- and intracellular expression of HBsAg was measured by using the Monolisa AHBs Plus Detection Assay (Bio-Rad).

**35S-Labelling.** HuH7 cells were grown and transfected as described above except that the cells were grown in Dulbecco’s modified Eagle’s medium (DMEM). Three HBV genomes were transfected: Z1, which encoded an HBsAg of serotype adr; 15803, which encoded an HBsAg of serotype ayw; and CS, which also encoded an HBsAg of serotype adr, but which was isolated from a typical chronic HBV carrier. The plasmid containing the CS genome, which had also been amplified by the one-step PCR method, was a generous gift of Dr Camille Sureau, INSERM U76, INTS, Paris, France, who also supplied the unpublished sequence of the genome. Three days after transfection, the cells were starved for 2 h by using DMEM without L-Cys and L-Met or 10% dialysed fetal bovine serum. The medium was then removed and replaced with 1 ml per well of the same medium containing 200 µCi (7-4 MBq) [35S]-Cys and [35S]-Met ml⁻¹. The cells were labelled for 24 h and the medium was then collected and clarified by centrifugation at 800 g for 5 min.

**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>HBcAg</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>642</td>
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<td>+</td>
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<td>598</td>
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<td>Aug 2002</td>
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<td>Dec 2002</td>
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<td>Jun 2003</td>
<td>362</td>
<td>435–0*</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Aug 2004</td>
<td>247</td>
<td>1456–0†</td>
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<td>+</td>
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<td>+</td>
<td>−</td>
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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 antiseras 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⁻¹. 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 QuickChange Mutagenesis kit (Stratagene) and the oligonucleotide 5′-GGACCATGCARGACCTGAGATTTCTGCTGATCAGAA-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 new PpuMI restriction site and changing nt 531 from C to T introduced an I site. These were used for initial screening of their GenBank accession numbers. The genome was found to be genotype C (Fig. 1) and, more particularly, a C-1858 variant that is found in South-East 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 remained high since then, as have his anti-HBs titres, although these have decreased slowly over time (Table 1).

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/WHOUNICEF_Coverage_Review/pdf/viet_nam.pdf). During the adoption check-up in April 2002, he was found to have not only a moderately high level of viraemia (>5 x 10⁷ copies ml⁻¹), but also a high titre of anti-HBs antibodies (>600 IU l⁻¹). By August 2002, the viraemia had increased tenfold and has

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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.
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 (o)</td>
<td>122K+160K+127T</td>
</tr>
<tr>
<td>adw4q− (F, H)</td>
<td>122K+160K+127L+178Q</td>
</tr>
<tr>
<td>adrq+ (C)</td>
<td>122K+160R+177V+178P</td>
</tr>
<tr>
<td>adrq− (C)</td>
<td>122K+160R+177A</td>
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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, 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-P1). 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
w epitope (Table 2). Whilst most C-1858 variants code for HBsAg of serotype adr, one (GenBank accession no. AF223955) encodes an HBsAg of serotype ayr. The HBV genome of Z1 was therefore modified to convert residue 122 of HBsAg to Arg (Z1-122R, serotype ayr). As Z1 serum seemed to recognize HBsAg expressed by the CS genome somewhat better than that expressed by its own genome (Fig. 3), a Z1-126I construction was also made, as was a double mutant (Z1-122R126I). Conversion of residue 122 from Lys to Arg resulted in recognition of the modified HBsAg by Z1 serum as well as, if not better than, that of the control anti-HBs positive serum (Fig. 4). Changing residue 126 from Thr to Ile seemed to have little effect.

**DISCUSSION**

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 (Zaaier 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 (10^6 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/ayw, as well as the introduction of the minor envelope proteins.

ACKNOWLEDGEMENTS

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REFERENCES


