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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 ^{35}S-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 *ayw*. 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

The GenBank/EMBL/DDBJ accession number for the sequence determined in this work is AJ748098.

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 relevant biochemical and virological parameters for the period 1999–2004 are shown in Table 1. All sera used in this study were obtained during regular consultations. The foster-parents had signed an informed-consent letter.

PCR amplification, sequencing and cloning. Nucleic acids were extracted from 200 μl serum by using a Nucleospin Blood kit (Macherey-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 *Hin*dIII and *Sac*I, 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′-CCTG-CTGGTGGCTCCAGTTC-3′, nt 56–75) and Por4 (5′-TACCC-AAAGACAAAAGAAAATTGG-3′, nt 826–803) 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 AgHBs Plus Detection Assay (Bio-Rad).

^{35}S-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) [^{35}S]L-Cys and [^{35}S]L-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

+, Positive result; −, negative result; γGT, γ-glutamyl transferase; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; αFP, α-fetoprotein.

Date	α-HBs (IU l^{-1})	HBV DNA ($\times 10^6$ equivalents ml^{-1})	HBsAg	α-HBc	HBeAg	α-HBe	HIV1/2	γGT (IU l^{-1})	ASAT (IU l^{-1})	ALAT (IU l^{-1})	αFP (μg ml^{-1})
Jan 1999			+		+		−				
Mar 1999			+	+	+	−			33	41	
Sep 2000			+	+	+					18	
Mar 2002	642		+	+	+	−	−		27	25	1·01
Apr 2002	598	55·9*	+	−†	+	−			44	32	
Aug 2002	513	558·4*	+	−†	+	−					
Dec 2002	376	229·5*	+		+	−		9	40	39	1·10
Jun 2003	362	435·0*	+	+	+	−		7	33	36	1·19
Aug 2004	247	1456·0‡	+	+	+	−			34	34	1·40

*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 *adw*. 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 En^3Hance 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′-GGACCATGCARGACCTGCACGAYTCCTGCTCAA-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 *Ppu*MI restriction site and changing nt 531 from C to T introduced a new *Tfi*I 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 ^{35}S-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/WHOUNICEF_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 ($>5\times10^7$ 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 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 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

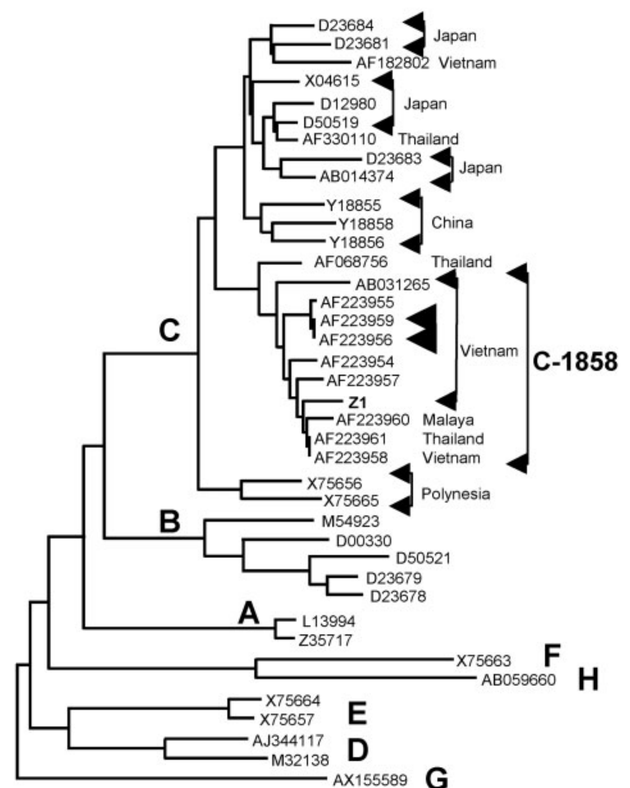

Fig. 1. Phylogenetic tree of nucleotide sequences of complete HBV genomes representing the eight known HBV genotypes (A–H), showing that the HBV genome of Z1 belongs to the C-1858 variant group of genotype C. The geographical origins of the genotype C isolates are shown and the C-1858 variant cluster 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 *a* 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.

Serotype	HBsAg residues involved
ayw1 (A, B)	122R + 160K + 127P + (134F and/or 159A)
ayw2 (D)	122R + 160K + 127P
ayw3 (D, C)	122R + 160K + 127T
ayw4 (E, D, F)	122R + 160K + 127L
ayr (C)	122R + 160R
adw2 (A, B, G, C)	122K + 160K + 127P
adw3 (D)	122K + 160K + 127T
adw4q− (F, H)	122K + 160K + 127L + 178Q
adrq^{+} (C)	122K + 160R + 177V + 178P
adrq^{-} (C)	122K + 160R + 177A

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 ^{35}S-labelled after transfection of full-length genomes into HuH7 cells and immunoprecipitated. Three genomes were used: Z1, 15803 encoding HBsAg of serotype

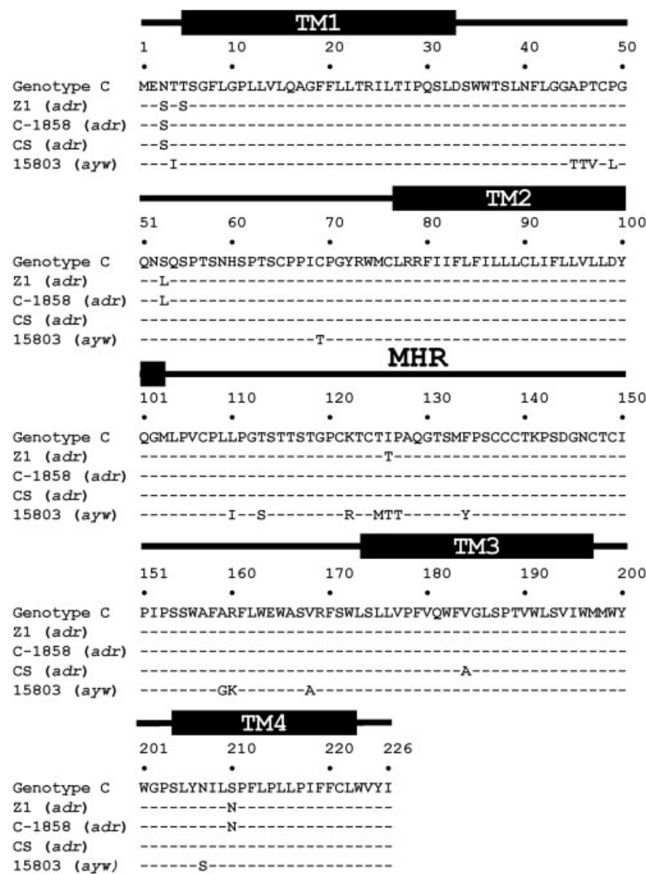

Fig. 2. Alignment of the amino acid sequences (single-letter code) of the HBsAgs encoded by the genomes used in this study (Z1, 15803 and CS) with consensus genotype C and C-1858 variant HBsAg sequences. The consensus genotype C sequence was derived from alignment of 103 genotype C HBsAg sequences found in databases and the consensus C-1858 sequence was derived from eight described sequences (Alestig *et al.*, 2001). A possible topological model (Persson & Argos, 1994) for HBsAg is shown schematically above the sequences. MHR, Major hydrophilic region; TM, transmembrane region.

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 *a* 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

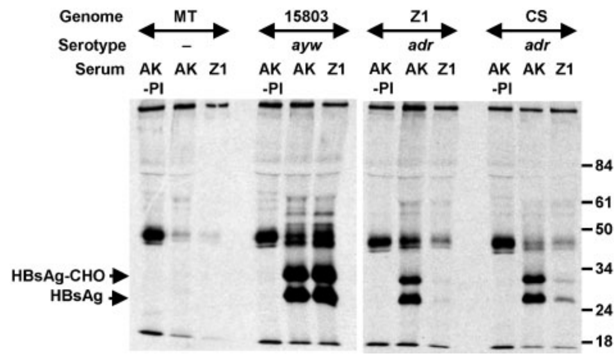

Fig. 3. Immunoprecipitation of ^{35}S-labelled HBsAg particles of varying serotype. Viral particles were ^{35}S-labelled after transfection of full-length HBV genomes into HuH7 human hepatocyte cells. Three sera were used for immunoprecipitation: an anti-HBs-negative serum (AK-PI), an anti-HBs-positive 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 *adr*) currently circulating in his bloodstream only very weakly, but showed strong recognition of viral particles carrying HBsAg of serotype *ayw*. The genomes transfected and the serotypes of the HBsAg that they encode are shown. MT, Mock-transfected cells.

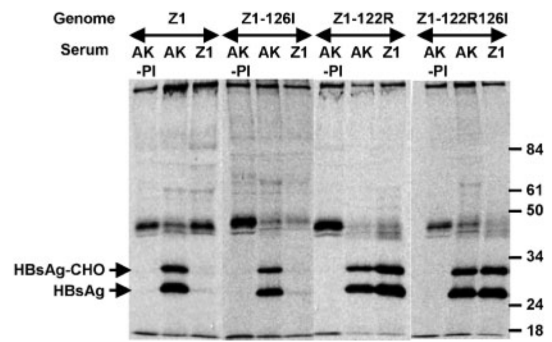

Fig. 4. Immunoprecipitation of ^{35}S-labelled viral particles expressed by modified Z1 genomes. The original Z1 genome and three mutant constructions were transfected. The figure shows that, when HBsAg was converted from serotype *adr* to serotype *ayr* (construction Z1-122R), there was strong detection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

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 (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 (10^7 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.

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REFERENCES

Alestig, E., Hannoun, C., Horal, P. & Lindh, M. (2001). Phylogenetic origin of hepatitis B virus strains with precore C-1858 variant. *J Clin Microbiol* **39**, 3200–3203.

Bahn, A., Gerner, P., Martiné, U., Bortolotti, F. & Wirth, S. (1997). Detection of different viral strains of hepatitis B virus in chronically infected children after seroconversion from HBsAg to anti-HBs indicating viral persistence. *J Hepatol* **27**, 973–978.

Basuni, A. A. & Carman, W. F. (2004). HBV vaccine-escape variants. *Methods Mol Med* **95**, 115–124.

Chen, W. N. & Oon, C. J. (1999). Human hepatitis B virus mutants: significance of molecular changes. *FEBS Lett* **453**, 237–242.

Chen, Y.-C. J., Delbrook, K., Dealwis, C., Mimms, L., Mushahwar, I. K. & Mandecki, W. (1996). Discontinuous epitopes of hepatitis B surface antigen derived from a filamentous phage peptide library. *Proc Natl Acad Sci U S A* **93**, 1997–2001.

Coleman, P. F., Chen, Y.-C. J. & Mushahwar, I. K. (1999). Immunoassay detection of hepatitis B surface antigen mutants. *J Med Virol* **59**, 19–24.

Günther, S., Li, B.-C., Miska, S., Krüger, D. H., Meisel, H. & Will, H. (1995). A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* **69**, 5437–5444.

Hannoun, C., Horal, P. & Lindh, M. (2000). Long-term mutation rates in the hepatitis B virus genome. *J Gen Virol* **81**, 75–83.

Hino, K., Katoh, Y., Vardas, E., Sim, J., Okita, K. & Carman, W. F. (2001). The effect of introduction of universal childhood hepatitis B immunization in South Africa on the prevalence of serologically negative hepatitis B virus infection and the selection of immune escape variants. *Vaccine* **19**, 3912–3918.

Ireland, J. H., O'Donnell, B., Basuni, A. A., Kean, J. D., Wallace, L. A., Lau, G. K. & Carman, W. F. (2000). Reactivity of 13 *in vitro* expressed hepatitis B surface antigen variants in 7 commercial diagnostic assays. *Hepatology* **31**, 1176–1182.

Jack, A. D., Hall, A. J., Maine, N., Mendy, M. & Whittle, H. C. (1999). What level of hepatitis B antibody is protective? *J Infect Dis* **179**, 489–492.

Jeantet, D., Chemin, I., Mandrand, B., Zoulim, F., Trepo, C. & Kay, A. (2002). Characterization of two hepatitis B virus populations isolated from a hepatitis B surface antigen-negative patient. *Hepatology* **35**, 1215–1224.

Kao, J.-H. & Chen, D.-S. (2002). Global control of hepatitis B virus infection. *Lancet Infect Dis* **2**, 395–403.

Kidd-Ljunggren, K., Miyakawa, Y. & Kidd, A. H. (2002). Genetic variability in hepatitis B viruses. *J Gen Virol* **83**, 1267–1280.

Kohno, H., Inoue, T., Tsuda, F., Okamoto, H. & Akahane, Y. (1996). 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. *J Gen Virol* **77**, 1825–1831.

Koziol, D. E., Alter, H. J., Kirchner, J. P. & Holland, P. V. (1976). The development of HBsAg-positive hepatitis despite the previous existence of antibody to HBsAg. *J Immunol* **117**, 2260–2262.

Le Bouvier, G. L., Capper, R. A., Williams, A. E., Pelletier, M. & Katz, A. J. (1976). Concurrently circulating hepatitis B surface antigen and heterotypic anti-HBs antibody. *J Immunol* **117**, 2262–2264.

Mizokami, M., Orito, E., Ohba, K., Ikeo, K., Lau, J. Y. N. & Gojobori, T. (1997). Constrained evolution with respect to gene overlap of hepatitis B virus. *J Mol Evol* **44** (Suppl. 1), S83–S90.

Norder, H., Courouce, A.-M. & Magnius, L. O. (1992). Molecular basis of hepatitis B virus serotype variations within the four major subtypes. *J Gen Virol* **73**, 3141–3145.

Norder, H., Couroucé, A.-M., Coursaget, P., Echevarria, J. M., Lee, S.-D., Mushahwar, I. K., Robertson, B. H., Locarnini, S. & Magnius, L. O. (2004). Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HB_sAg subtypes. *Intervirology* **47**, 289–309.

Ogura, Y., Kurosaki, M., Asahina, Y., Enomoto, N., Marumo, F. & Sato, C. (1999). Prevalence and significance of naturally occurring mutations in the surface and polymerase genes of hepatitis B virus. *J Infect Dis* **180**, 1444–1451.

Okamoto, H., Imai, M., Tsuda, F., Tanaka, T., Miyakawa, Y. & Mayumi, M. (1987). Point mutation in the *S* gene of hepatitis B virus for a d/y or w/r subtypic change in two blood donors carrying a surface antigen of compound subtype adyr or adwr. *J Virol* **61**, 3030–3034.

Oon, C.-J. & Chen, W. N. (1998). Current aspects of hepatitis B surface antigen mutants in Singapore. *J Viral Hepat* **5** (Suppl. 2), 17–23.

Oon, C.-J., Chen, W. N., Koh, S. & Lim, G. K. (1999). Identification of hepatitis B surface antigen variants with alterations outside the "a" determinant in immunized Singapore infants. *J Infect Dis* **179**, 259–263.

Persson, B. & Argos, P. (1994). Prediction of transmembrane segments in proteins utilising multiple sequence alignments. *J Mol Biol* **237**, 182–192.

Poovorawan, Y., Theamboonlers, A., Vimolket, T. & 7 other authors (2000). Impact of hepatitis B immunisation as part of the EPI. *Vaccine* **19**, 943–949.

Robinson, W. S. (1999). Hepadnaviruses (*Hepadnaviridae*): hepatitis B virus: general features. In *Encyclopedia of Virology*, 2nd edn, pp. 640–645. Edited by A. Granoff & R. G. Webster. San Diego: Academic Press.

Sasaki, T., Ohkubo, Y., Yamashita, Y., Imai, M. & Miyakawa, Y. (1976). Co-occurrence of hepatitis B surface antigen of a particular subtype and antibody to a heterologous subtypic specificity in the same serum. *J Immunol* **117**, 2258–2259.

Torresi, J. (2002). The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *J Clin Virol* **25**, 97–106.

Viviani, S., Jack, A., Hall, A. J., Maine, N., Mendy, M., Montesano, R. & Whittle, H. C. (1999). Hepatitis B vaccination in infancy in The Gambia: protection against carriage at 9 years of age. *Vaccine* **17**, 2946–2950.

Zaaijer, H. L., Lelie, P. N., Vandenbroucke-Grauls, C. M. J. E. & Koot, M. (2002). Concurrence of hepatitis B surface antibodies and surface antigen: implications for postvaccination control of health care workers. *J Viral Hepat* **9**, 146–148.

Zöllner, B., Petersen, J., Schröter, M., Laufs, R., Schoder, V. & Feucht, H.-H. (2001). 20-fold increase in risk of lamivudine resistance in hepatitis B virus subtype adw. *Lancet* **357**, 934–935.

Zuckerman, A. J. & Zuckerman, J. N. (1999). Molecular epidemiology of hepatitis B virus mutants. *J Med Virol* **58**, 193–195.

Zuckerman, J. N. & Zuckerman, A. J. (2003). Mutations of the surface protein of hepatitis B virus. *Antiviral Res* **60**, 75–78.