Original article

Antiviral resistance mutations potentiate hepatitis B virus immune evasion through disruption of its surface antigen *a* determinant

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Background: The hepatitis B virus (HBV) *pol* gene overlaps the *S* gene encoding surface antigen (HBsAg). It has been reported previously that drug-induced changes in HBsAg alter its binding to sera from humans immunized against HBV. We investigate here the changes to specific epitopes in the *a* determinant (the major target of neutralizing antibody) caused by a number of drug-resistant mutations.

Methods: Recombinant HBsAgs, produced by transfection of Chinese hamster ovary cells with *S* gene plasmids into which lamivudine, adefovir and entecavir resistance and common antibody-escape mutations had been introduced, were probed with monoclonal antibodies to epitopes in the first and second loops of the *a* determinant.

Results: The mutations rtF166L/sF158Y (lamivudine-associated, compensatory) and rtI169T/sF161L (entecavir-associated, primary) acting alone, and the mutations rtV173L/sE164D (lamivudine-associated, compensatory) and rtSilent/sD144E (antibody escape-associated) each when combined with rtM204V/sI195M (lamivudine-associated, primary) led to decreases in antibody reactivity to epitopes in the first or second loop, or in both loops. The rtM204V/ sI195M+rtV173L/sE164D mutations yielded an epitope–antibody profile similar to the rtR153Q/sG145R vaccine escape mutant. The rtM204V/sI195M mutation combined with the rtF166L/sF158Y or rtR153Q/sG145R mutation restored reactivity to second-loop epitopes previously abrogated by single mutations.

Conclusions: Mutations associated with resistance to nucleos(t)ide analogue therapy, singly or in combination with each other or antibody escape-associated mutations, alter HBsAg immunoreactivity through concomitant amino acid substitutions at codons within and downstream of the *a* determinant. The findings have implications for understanding the native structure of HBsAg, optimizing treatment of chronic hepatitis B and evaluating the success of immunization programmes.

Introduction

It is estimated that two billion people worldwide have been infected with the hepatitis B virus (HBV) and 400 million of these remain persistently infected [1]. More than one quarter of them will die of hepatocellular carcinoma (HCC) [2] and worldwide deaths from HBV-associated liver cancer probably exceed one million per year [3]. The prevalence of persistent infection will not be reduced imminently by implementation of the World Health Organization (WHO) global immunization strategy, which is primarily aimed at interrupting mother-to-child and infant-to-infant transmission. The disease burden of the persistently infected host and their infectivity may be mitigated by antiviral therapy. In a recent study, disease progression was associated with the plasma HBV DNA load [4], indicating that the suppression of HBV replication is likely to give clinical benefit. Currently, four antiviral nucleoside/nucleotide analogues targeting the viral polymerase are licensed

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for the treatment of chronic HBV infection: lamivudine (a cytidine analogue), adefovir (which has an acyclic adenosine ring), entecavir (a guanosine analogue) and telbivudine (a thymidine analogue). Frequently used as monotherapy, all are associated with the evolution of viral resistance mutations in the HBV polymerase (*pol*) gene. For lamivudine, the prevalence of resistance rises rapidly, reaching 14% after 1 year [5] and 70% after 5 years [6] of monotherapy. Resistance may develop less rapidly with adefovir (5.9% over 144 weeks) [7] and entecavir (5.8% over 1 year in lamivudine-treated patients), although there is evidence that previous lamivudine therapy facilitates entecavir resistance [8–10]. Drug resistance is associated with the development of primary resistance mutations in the reverse transcriptase (rt) coding domain of *pol* which facilitate DNA synthesis in the presence of the drug, although at a cost of reduced fitness. Continued therapy leads to the accumulation of compensatory mutations that restore viral fitness. With lamivudine, the primary amino acid substitutions, rtM204V/I, arise in the tyrosine-methionine-aspartate-aspartate (YMDD) motif of *pol*, and the compensatory changes are rtL180M [11] and rtV173L [12]. Adefovir resistance is characterized by rtN236T and/or rtA181V/T [13]. Entecavir resistance also arises in a stepwise manner with the rtS202I change occurring sequentially in viruses already bearing lamivudine resistance mutations [14].

HBV is a small virus and the genes that encode the polymerase enzyme and hepatitis B surface antigen (HBsAg) overlap such that the gene encoding HBsAg (*S*) is embedded within that of the *pol* gene [15]. The surface protein open reading frame (ORF) encodes three co-carboxyl-terminal proteins, the shortest of which is HBsAg (S) the principal component of the external envelope of the 42 nm virion. HBsAg carries the major target of neutralizing antibody, the *a* determinant. Although it is well known that resistance mutations also induce nonsynonymous changes in *S* [16–20], only one study has sought to determine how these might affect HBsAg protein conformation and its antigenicity. Torresi and colleagues [21] showed that the common lamivudine resistance mutations rtM204V/sI195M, rtM204I/sW196S, rtM204I/sW196L and rtM204V/ sI195M+ rtV173L/sE164D all led to reductions in the reactivity of HBsAg with vaccine-induced antibody. In addition, the obverse has been shown in that changes in the *S* gene introduce changes in *pol* that correspond with lamivudine resistance compensatory mutations [22].

In order to investigate more comprehensively the effect of mutations in *pol* on the antigenicity of HBsAg, we carried out a series of site-directed mutagenesis experiments, inserting *pol* resistance mutations into a common genotype A HBV backbone expressed in Chinese hamster ovary (CHO) cells. The effects of these mutations, singly or in conjunction, were determined using three monoclonal antibodies that bind distinct and defined epitopes within the *a* determinant.

Methods

HBV amplification and cloning

Parental HBV DNA was derived from plasmids bearing the *S* gene originally isolated from serum of a patient known to carry genotype A HBV with HBsAg of serological subtype *adw2*. The *S* gene was PCR-amplified with sense primer 5′-GGGTCCCATATTTCTTGGGTACC-3′ and antisense primer 5′-GTGAAAAGGGGGCAGCAAAGC-3′. Each reaction used 5 µl 10× *Pfu* buffer (Stratagene, La Jolla, CA, USA), 5 µl of 2 mM dNTPs (Promega, Madison, WI, USA), 2.5 µl sense and antisense primers (each primer at 10 pmol/µl), 1 unit (1 µl) *Pfu* Turbo (Stratagene), 33 µl nuclease-free water and 1 µl plasmid bearing the parental HBV genome (1×10^{-4} µg/ml). Thermocyling conditions involved initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by a final extension of 72°C for 5 min. Amplicons were purified using the Wizard PCR preps DNA purification kit (Promega) and cloned into the phagemid transfection vector pBK-CMV (Stratagene) via *Kpn*I and *Xho*I digestion of both amplicon and vector. After digestion, cut pBK-CMV was treated with shrimp alkaline phosphatase and ligated with T4 DNA ligase.

The nucleotide sequence of the *S* cassette within the vector pBK-CMV, hereafter referred to as pBK-CMV[smallS], was confirmed by sequencing using the vector T3 and T7 promoter sites as sequencing primers. The products were analyzed in a Beckman CEQ2000 automated capillary array sequencer (Beckman-Coulter, Fullerton, CA, USA) and raw chromatograph data assembled using the SeqMan sequence analysis programme. Clustal W alignments were made in the MegAlign program from the LASARGENE sequence analysis package (DNAstar, Madison, WI, USA). Predicted amino acid sequences for the *S* and *pol* ORFs were generated in the EMBOSS suite of programs accessed from the website of the Health Protection Agency Bioinformatics Unit (http://www.hpa.org.uk/cfi/bioinformatics/tools/tools.htm) using the programme PlotORF, which generates amino acid translations from all theoretically possible reading frames.

Mutagenesis of the *S* cassette within the pBK-CMV[smallS] was carried out using the Quikchange II mutagenesis kit (Stratagene). The various mutations and the cognate amino acid generated therefrom are listed in Table 1. The mutated plasmids were sequenced as before using primers in the

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T7 and T3 promoter sites. Plasmids carrying the desired sequences were grown in overnight culture and DNA extracted with the EndoFree maxiprep kit (Qiagen, Hilden, Germany), whereupon the sequence was checked again prior to storage. Multiple mutations were engineered by introducing the first mutation and then subjecting the mutated plasmid to subsequent rounds of mutagenesis as required.

Cell culture and transfections

CHO cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 4.5 g/l glucose, L-glutamine, sodium pyruvate and 10% heat-inactivated fetal calf serum. Cells were seeded at a concentration of 5×10^4 viable cells/ml into six-well plates in 2 ml growth medium with no antibiotics. Cells were cultured to 50–80% confluence. Cells were then transiently transfected with 4 µg wild-type plasmid vector DNA with 24 µl FuGene 6 (Roche, Basel, Switzerland) per well. Each transfection cycle utilized a six-well plate in which four wells were used for transfection with the pBK-CMV[smallS] vector and two transfected with the empty vector as a negative control. Transfection cycles were repeated three times for all wild type and mutant vectors. Following transfection, cells were then grown under standard culture conditions and 200 µl growth medium added to each well every other day. Five days post-transfection, the supernatants from the wells were pooled separately, clarified by low-speed centrifugation and stored at -20°C.

Monoclonal antibodies for solid-phase capture of HBsAg

Purified murine monoclonal antibodies (mAbs) against three distinct conserved epitopes on HBsAg were used as solid-phase capture reagents. mAb P2D3 recognizes a first loop, linear epitope between surface residues s121–129 that requires a threonine-threonine-proline (TTP) motif [23]. The mAbs H3F5 and D2H5 had been raised against a mixture of *ad/ay* HBsAg and selected to recognize all 10 members of the Paris panel of serological subtypes [24]. They are related in cross-competition assays but recognize distinct conformational epitopes within the second loop of the *a* determinant [24], which have been mapped to s131–142 for H3F5 and s142–147 for D2H5 (J Duncan, personal communication).

HBsAg capture ELISAs

The mAbs P2D3 and H3F5 were supplied precoated onto eight-well strips (in a 96-well plate format) at concentrations of 1 µg/ml. Purified D2H5 immunoglobulin G (IgG) was coated onto the solid phase of sterilized 96-well plates at a concentration of 1 µg/ml in carbonate buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.0) at 4°C overnight and then washed with phosphate-buffered saline (PBS). Plates were then blocked by incubation overnight at 4°C with 3% bovine serum albumin in PBS and washed with PBS. The presence of HBsAg in the supernatant dilution series of transfected cell supernatants for each transfection cycle pool was detected in triplicate for each capture mAb. A 100 µl aliquot of the clarified supernatants or dilutions thereof

Table 1. HBV *pol* and *S* gene mutants studied

	Polymerase					Surface					
		Wild type		Mutant			Wild type		Mutant		
Mutation type	Codon	Nucleotides	Amino acid*	Nucleotides	Amino acid*	Codon	Nucleotides	Amino acid*	Nucleotides	Amino acid*	Reference
Lamivudine	204	ATG	M	GTG	V	195	ATA	I	ATG	M	[31]
primary	204	ATG	M	ATC	I	194	TGG	W	TCG	S	[31]
	204	ATG	M	ATT	I	194	TGG	W	TTG	L	[31]
Lamivudine	180	CTG	L	ATG	M	171	TCC	S	TCA	S	[32]
compensatory	173	GTG	V	TTG	L	164	GAG	E	GAT	D	[12]
	166	TTT	F	TTA	L	158	TTC	F	TAT	Y	[21]
Adefovir	181	GCT	A	ACT	T	172	TGG	W	TGA	*†	[13]
compensatory	181	GCT	A	GTT	V	173	CTC	L	TTC	F	[13]
Entecavir	202	AGT	S	ATT	I	194	GTT	V	TTT	F	[8]
primary	184	ACT	T	AGT	S	176	CTA	L	GTA	V	[8]
	169	ATT	I	ACT	T	161	TTC	F	CTC	L	[8]
Vaccine	128	ACC	T	AAC	N	120	CCA	P	ACA	T	[21]
escape	153	CGG	R	CAG	Q	145	GGA	G	AGA	R	[33]
	153	CGG	R	AGG	R	144	GAC	D	GAA	E	[21]
	153	CGG	R	AAG	K	144	GAC	D	GAA	E	
						and 145	GGA	G	AGA	R	[21]

*Amino acid given in single-letter code. †Stop codon.

was incubated overnight at room temperature in coated wells. A 50 μl aliquot of polyclonal immune-affinity-purified goat anti-HBsAg–horseradish-peroxidase conjugate (Abbott–Murex) was then added to the sample in the well for incubation for 4 h at 37°C. Bound conjugate was revealed by the addition of 3,3′5,5-tetramethylbenzidine and hydrogen peroxide, thereafter sulfuric acid. Optical density (OD) was measured spectrophotometrically at 450 nm absorbance with a 620 nm reference wavelength. All samples and dilutions were assayed in duplicate. A quantified, patient-derived HBsAg standard at concentrations of 3 ng/ml and 0.3 ng/ml HBsAg in normal human plasma and a negative control consisting of maintenance medium were used in 100 μl volumes as controls solely to validate the sensitivity of each assay run. For each transfection cycle pool, a series of one tenthfold dilutions of supernatants from neat were assayed with each of the three mAbs.

ELISA data analysis

The binding ratio (BR) was calculated by dividing the sample OD by the mean of mock-transfection OD. The SD for all BRs from each supernatant dilution were calculated for the wild type and each mutant. Dilution curves derived from mean BRs and SDs from each assay were plotted and the area under the curve (AUC) was measured across each dilution series (see Figure 1 for example of AUC data). The SD for each AUC measurement were determined. For each wild type and mutant antigen, the AUC values separately obtained after capture by P2D3, H3F5 and D2H5 are summated to provide a measure of the total epitope density. The contribution that each mAb made towards this density is expressed as a percentage proportion and all three percentages are presented to give a measure of epitope balance. As all assays were conducted across the detection endpoint, changes in the efficiency of cell export exhibited by the different recombinants would not be expected to affect the comparative findings which are in effect internally controlled.

Results

As all transfected HBV *S* genes in this study carried the TTP motif between residues 125–127 of HBsAg [25], the P2D3 ELISA was considered the reporter assay for HBsAg expression. Data from transfections whose supernatants did not react to P2D3 or did so at a low level were excluded from further analysis; thus, data from the rtA181T/sW172*, rtS202I/sV194F+rtT184S/sL176V and rtR153K/sD144E+sG145R mutants were excluded from further analysis. Secretion efficiency of mutant HBsAg was not directly studied, but by characterizing all mAb interactions relative to P2D3 binding (and therefore supernatant HBsAg expression) it was possible to account for epitope balance regardless of export efficiency.

Transfection of the wild type *S* gene construct produced HBsAg with an epitope density (the summation of AUC values for all three mAbs) of 199.3 and an epitope balance (derived on the basis of the relative contribution of P2D3, H3F5 and D2H5) of 43%, 27% and 30%, respectively (Figure 1A). The SDs of the proportional representation of the AUC for the wild type construct across the dilution series ranged from ±1.8 to ±1.9%, thus indicating the robustness of using AUC values as the basis of expressing epitope density and balance.

The epitope balance results for all HBsAgs bearing single substitutions could be divided into two groups: the non-effectors and sole effectors (Table 2). The antigens that showed a wild-type-like epitope balance are classified as non-effectors. In contrast, those constructs bearing a single mutation, which altered the wild-type-like balance of the HBsAg, are classified as sole effectors. Four sole effector constructs gave a greatly altered epitope balance. Two were are associated with drug-associated mutations. The first was the lamivudine-associated compensatory substitution, rtF166L/sF158Y, which abrogated all the epitopes recognized by H3F5 and D2H5. The second was the entecavir-associated mutation, rtI169T/sF161L, which abrogated only the D2H5 epitope. The other two mutations represent antibody-escape variants. The prototype mutant, rtR153Q/G145R, spared the H3F5 epitope to a slight degree but abrogated binding to D2H5. The rtT128N/sP120T mutant abrogated the H3F5 epitope.

Mutations which, in combination (Table 3), did not alter the epitope balance from that of the parental single changes are termed non-modulators. However, some constructs bearing two substitutions produced epitope profiles that were quite different from either parent. Thus, when the rtM204V/sI195M mutation was combined with any one of four others, altered epitope balances resulted. These phenomena are best exemplified by the rtV173L/sE164D+rtM204V/sI195M mutation pair (Figure 1B). Although each of the rtV173l/sE164D and rtM204V/sI195M substitutions yielded a wild-type-like epitope balance (Table 2), their combination resulted in D2H5 epitope loss (Table 3). An almost identical display of modulation was seen in the interaction between the rtSilent/sD144E and rtM204V/sI195M substitutions. Conversely, there were also instances in which a reverse order of synergy was observed leading to rescue of epitopes. For example, the substitution rtR153Q/sG145R led to complete loss of the D2H5 epitope and partial loss of the H3F5 epitope, but the addition of rtM204V/sI195M to the rtR153Q/sG145R backbone partially restored the

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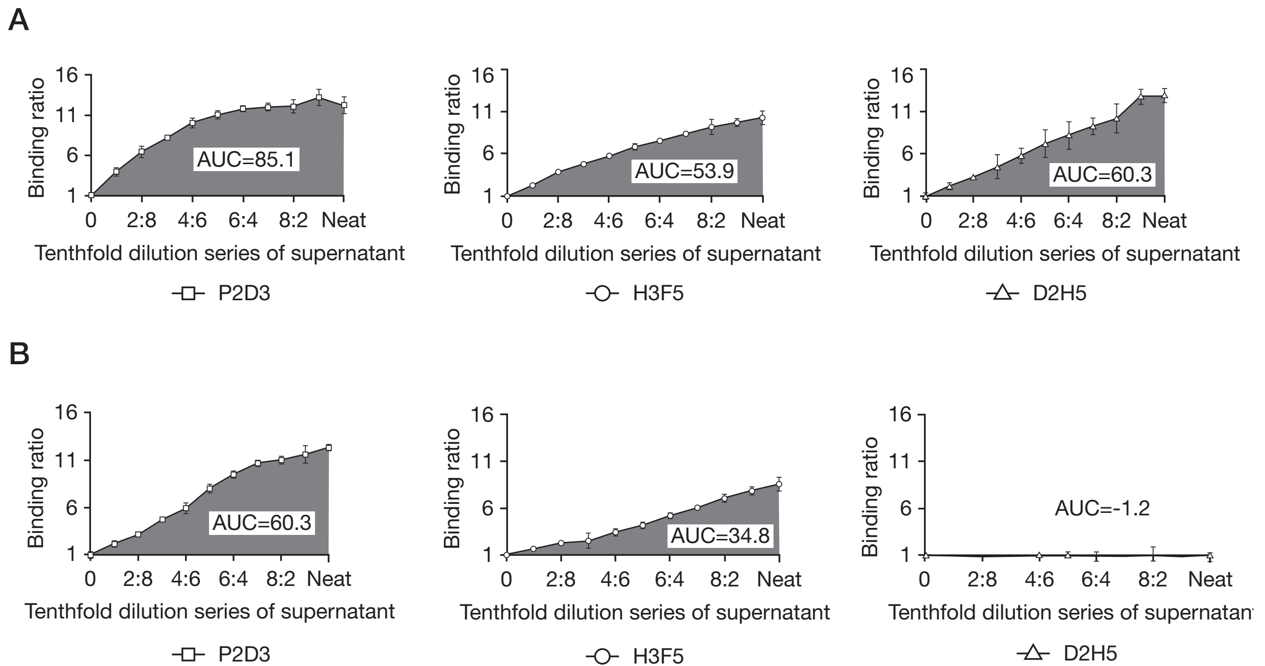
Figure 1. mAb ELISA results of wild type and mutant HBsAg



Results of dilutions in individual mouse monoclonal antibodies (mAb) ELISAs are presented for **(A)** wild type hepatitis B surface antigen (HBsAg) and **(B)** the combined substitutions rtV173L/sE164D+rtM204V/sI195M. Calculated area under curve (AUC) is presented on each plot. Total epitope density is calculated by combining all three AUCs for P2D3, H3F5 and D2H5 in each plot; thus, for wild type the result was 85.1+53.9+60.3=199.3. When each mAb contribution was taken as a percentage of epitope density, this gave the percentage epitope balance. Hence, for wild-type HBsAg, the epitope balance was 43%, 27% and 30% for P2D3, H3F5 and D2H5, respectively. For substitutions rtV173L/sE164D+rtM204V/sI195M in HBsAg, the epitope balance was 64%, 46% and 0% for P2D3, H3F5 and D2H5, respectively. This approach allowed mutant HBsAg ELISA results to be compared regardless of variations in supernatant HBsAg titre.

H2F5 epitope. Combining rtF166L/sF158Y with rtM204V/sI195M resulted in restoration of both the H3F5 and D2H5 epitopes.

Discussion

The availability of mAbs against defined and discrete epitopes carried in the first and second loops of the *a* determinant of HBsAg enabled mapping of the epitope expression of recombinant HBsAg and, in turn, an investigation into the effect that drug-associated mutations in HBV *pol* may have on the overlapping *S* gene. Although it has previously been reported that lamivudine-associated mutations reduce the binding avidity of antibodies to HBsAg, those experiments used sera pooled from vaccinees [21], as a consequence of which topological details of the antibody–antigen interaction could not be evaluated. Although differing from traditional measurements of antibody–antigen affinity (*K*), our novel approach of measuring AUCs derived from epitope-specific, solid-phase capture ELISAs has permitted estimates of the relative contribution of each epitope to the overall antigenicity. The relative precision of this approach facilitated the investigation of base changes in the *S* gene and the resulting alteration of the epitope expression on HBsAg. These findings would predict that further determination of *K* for some of the antibody–antigen interactions will show reduced affinity for the reaction between the mAbs and the altered epitope. This approach is likely to be more fruitful in defining the sensitivity of this comparative binding approach and putting a measure on the significance between similar but obviously different patterns of reactivity

The primary mutations in *pol* that are associated with resistance to lamivudine, which arise frequently during monotherapy, affect the YMDD motif (changing the methionine to either isoleucine or valine), and also cause amino acid substitutions in HBsAg (Table 1). The changes resulting from the *pol* substitutions include sI195M, sW196S and sW196L, which are not found among antibody-escape variants and none on their own influenced the balance of the three epitopes investigated in this study; thus, their interaction with the *a* determinant is wild-type-like. Similarly, the lamivudine-associated compensatory substitution, rtV173L/sE164D, and the primary entecavir-associated substitutions, rtA181V/sL173F and s202I/sV194F, in

spite of causing amino acid substitutions in HBsAg, alone did not affect epitope reactivity. The lamivudine-associated compensatory mutation rtL180M/sSilent was not expected to affect the expression of HBsAg epitopes and experimental data were consistent with that expectation.

Antibody-escape variants also were investigated in this study. The mutant rt/Silent sD144E, which may be found in conjunction with sG145R following antibody escape, did not elicit any altered epitope expression. However, the two immune-escape variants, rtR153Q/sG145R and rtT128N/sP120T, which may also be considered lamivudine-associated [22], each exerted a profound effect on antibody-binding to the second loop epitopes. Interestingly, the rtT128N/sP120T substitution appears to affect only the H3F5 epitope. Another lamivudine-compensatory mutation, rtF166L/sF158Y, and the entecavir primary resistance mutation, rtI169T/sF161L, induced significant alterations, abrogating, respectively, the H3F5 and D2H5 epitopes. Whilst none of these mutations involve amino acid residues within the mAb-binding sites, both were within 15 residues away from these sites. Given the paucity of definitive structural and protein folding

Table 2. Epitope balance profiles of wild type and single-mutant transfectant HBsAg

			mAb epitope balance, %		
Polymerase	Surface	P2D3 AUC reporter	P2D3 s121–129	H3F5 s131–142	D2H5 s142–147
Non-effectors					
Wild type	Wild type	85.1	43	27	30
M204V	I195M	95.0	40	26	34
M204I	W196S	94.9	34	30	36
M204I	W196L	83.1	40	26	34
L180M	Silent	85.0	40	25	35
V173L	E164D	77.0	46	24	30
S202I	V194F	90.7	40	30	30
A181V	L173F	79.3	36	26	38
T184S	L176V	85.0	42	32	26
Silent	D144E	85.7	44	28	28
Sole effectors					
F166L	F158Y	72.1	91	3	6
I169T	F161L	74.0	56	40	4
R153Q	G145R	68.5	82	18	0
T128N	P120T	95.5	50	0	50

Non-effectors demonstrated wild-type-like phenotype whilst sole effectors demonstrated altered phenotype. AUC, area under curve; HBsAg, hepatitis B surface antigen; mAb, monoclonal antibody.

Table 3. Epitope density and balance profiles of double-mutant transfectant HBsAg

			mAb epitopes (alone) balance, %				mAb epitopes (with *rtM204V/sI195M*) balance, %		
Polymerase	Surface	P2D3 AUC reporter	P2D3 s121–129	H3F5 s131–142	D2H5 s142–147	P2D3 AUC reporter	P2D3 s121–129	H3F5 s131–142	D2H5 s142–147
Non-modulators									
Wild type	Wild type	85.1	43	27	30	95.0	40	26	34
L180M	Silent	85.0	40	25	35	84.2	40	26	34
T128N	P120T	95.5	50	0	50	76.5	60	0	40
Modulators									
V173L	E164D	77.0	46	24	30	63.0	64	36	0
F166L	F158Y	72.1	91	3	6	40.8	57	24	18
Silent	D144E	85.0	44	28	28	92.2	61	39	0
R153Q	G145R	68.5	82	18	0	67.5	65	35	0

Non-modulators demonstrated a phenotype that did not differ from their single mutations, whilst modulators demonstrated a phenotype unlike either component mutation. AUC, area under the curve; HBsAg, hepatitis B surface antigen; mAb, murine monoclonal antibodies.

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data for HBsAg, it is not profitable to speculate further on the mechanism by which these changes affect HBsAg immunogenicity.

Changes in the reverse transcriptase YMDD motif are a common outcome of long-term nucleoside/nucleotide therapy not only for HBV but also for HIV. They persist in the face of combined therapy and, in the case of HIV, even after treatment is terminated. In the case of the rtM204V/sI195M substitution in HBV, the isoleucine-to-methionine substitution in HBsAg is considered to be structurally conservative. Methionine is a neutral, non-polar amino acid that is marginally less hydrophobic than isoleucine. Although it also has a sulfur-containing side chain, it is not prone to forming structural interactions because it is not highly nucleophilic. On its own, this mutation does not influence epitope expression (Table 2). We observed that the rtM204V/sI195M substitution and the rtV173L/sE164D substitution, both non-effectors alone, when combined led to the loss of the D2H5 epitope 3 (Table 3). It is surprising that the M204V codon change affecting HBsAg at residue sI195M should have such an influence, because it is a relatively conservative change and is distant from the D2H5 epitope. The isoleucine substitution at codon 195 of HBsAg also has an effect on the same epitope in the presence of the sD144E substitution. It also influences the expression of the H3F5 epitope in conjunction with the rtF166L/sF158Y and rtR153Q/sG145R mutations, where it enhances the reactivity of H3F5 rather than abrogate it. These findings suggest considerable plasticity in HBsAg and the potential for drug-induced changes in antigenicity. It has been reported previously that lamivudine resistance associated substitutions in the YMDD motif in isolation and in combination can lead to reduced HBsAg binding to pooled anti-HBs from vaccine recipients [21]. This observation implies that changes in the predicted downstream HBsAg epitope in residues s187–196 [25] are responsible for the changed phenotype. Our data confirm the effect of such substitutions upon *a* determinant epitopes. Changes at the YMDD motif in isolation do not appear to affect the *a* determinant but, in conjunction with various other substitutions can act synergistically to modulate it at a distance. Specifically, in the case of the combined substitution rtV173L/sE164D+rtM204V/sI195M it is thought that the reduced binding of antibody to HBsAg reported previously [21] may be primarily mediated through the distortion of second loop epitopes. Although the aim of this study was to describe the interaction of defined mAbs with HBsAg bearing antiviral resistance mutations, in the future it may also be useful to consider how the observed changes would affect the interaction of patient-derived polyclonal anti-HBs with such HBsAg. In practice polyclonal antisera are found to react with G145R-bearing HBsAg through the presence of antibody to conserved epitopes, as was the case here with hyperimmune equine anti-HBs (data not shown), but to what extent such reactions protect *in vivo* is not known.

Our data imply that conformational interactions of amino acids located in the first and second loops of the *a* determinant may have a more complex role than previously thought in determining the immunoreactivity of HBsAg. Moreover, our data suggest a greater importance for downstream regions on HBsAg epitope conformation. In a longitudinal study of sequence variance in the carrier whose serum does not contain HBeAg (to be reported elsewhere) this C-terminal region of HBsAg has been found to be under strong selection pressure, indicating that codon changes in this region may result in antigenic modulation. Current models of HBsAg structure, one model based on phage display [25] and another on bioinformatic analysis [26], generally agree on a two-loop structure for the *a* determinant. However, there is disagreement as to the structural nature of the regions downstream of the *a* determinant, particularly towards the C terminus. The existence of the s187–196 epitope described by Chen and colleagues [25] is further confounding. Moreover, the discovery of a monoclonal antibody that is able to bind to s178–186 [27] was surprising, as the earlier models predicted the region between s178–186 to be buried within the lipid bilayer of HBsAg.

From a diagnostics perspective, the drug-driven changes in HBsAg will not substantially affect the performance of diagnostic assays for HBsAg, as ELISAs are increasingly based on multiple monoclonal and polyclonal components or on mAbs that bind to known HBsAg variants [28]. Manufacturers have generally developed assays capable of detecting HBsAg in spite of second loop substitutions; nonetheless, this does mean that the presence and proliferation of these variants will not be recognized without specific studies. From a public health perspective, however, our observations may have profound consequences for HBV immunization campaigns, particularly where HIV coinfection occurs. In Africa, the high prevalence of chronic HBV carriage in adulthood [29], combined with a high prevalence of HIV infection, already provides a population in which immune escape is likely. Widespread use of Combivir, a combined lamivudine and zidovudine pharmaceutical, is in effect monotherapy for HBV infection. In this situation, acquisition of primary and secondary lamivudine mutations should be commonplace. Recently, the emergence of such mutations was documented in HIV–HBV coinfected patients [30]. The substitutions that occur in HBsAg consequent to drug selection in the HBV *pol* do so in codons previously not subjected to such changes and may serve as an alternative and additional means

of selection of antibody-escape mutants over and above that seen in the face of immunosuppression. This may have the uncomfortable outcome of subverting global immunization efforts. It may now be imperative that any drug intervention against HBV must have as one of its aims the prevention of drug resistance.

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Disclosure statement

SI and RST receive personal and institutional royalties on sales of diagnostic kits using mAb P2D3. The other authors declare no conflicts of interest.

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