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Genomic variability associated with the presence of occult hepatitis B virus in HIV co-infected individuals

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SUMMARY

Occult hepatitis B virus (O-HBV) infection is characterized by the presence of HBV DNA without detectable hepatitis B surface antigen (HBV DNA+/HBsAg−) in the serum. Although O-HBV is more prevalent during HBV/HIV co-infection, analysis of HBV mutations in co-infected patients is limited. In this preliminary study, HBV PreSurface (PreS) and surface (S) regions were amplified from 33 HIV-positive patient serum samples − 27 chronic HBV (C-HBV) and six O-HBV infections. HBV genotype was determined by phylogenetic analysis, while quasispecies diversity was quantified for the PreS, S and overlapping polymerase regions. C-HBV infections harboured genotypes A, D and G, compared to A, E, G and one mixed A/G infection for O-HBV. Interestingly, nonsynonymous-synonymous mutation values indicated positive immune selection in three regions for O-HBV vs one for CHBV. Sequence analysis further identified new O-HBV mutations, in addition to several previously reported mutations within the HBsAg antigenic determinant. Several of these O-HBV mutations likely contribute to the lack of detectable HBsAg in O-HBV infection by interfering with detection in serologic assays, altering antigen secretion and/or decreasing replicative fitness.

Keywords

diversity; HBV polymerase; HBV/HIV co-infection; hepatitis B surface antigen; occult hepatitis B virus

INTRODUCTION

There are 350 million chronic carriers of the hepatitis B virus (C-HBV) worldwide. C-HBV infection is characterized by detectable hepatitis B surface antigen (HBsAg) in the serum [1]. Occult HBV infection (O-HBV), in contrast, is defined as low level HBV replication without detectable circulating HBsAg [2]. Antibodies against HBV core protein (anti-HBc) had been considered the sole serological marker of O-HBV infection [3]; however,

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serologically negative individuals have been described with HBV DNA as the only detectable marker of infection [4].

Occult HBV infection is transmissible through blood transfusion from human to human [5] and human to chimpanzee [6]. In addition, C-HBV infection developed after a liver transplant from an O-HBV-infected donor [7]. Retrospective studies have also identified O-HBV infection in 16–68% of tumours in patients with hepatocellular carcinoma (HCC) [8,9]. Similarly, O-HBV infection was associated with development of cirrhosis and HCC [10]. Nonetheless, the clinical consequences of prolonged O-HBV infection remain unclear.

HIV co-infection is common because of shared blood-borne transmission routes. While advances in antiretroviral therapy (ART) have prolonged AIDS-free survival in HBV/ HIV co-infected patients, liver disease has emerged as a leading cause of morbidity and mortality [11]. In HIV-positive cohorts, the prevalence of O-HBV infection is highly variable: Nunez *et al*. [12] did not identify any patients with detectable HBV DNA among 85 HIV+/HBsAg −/anti-HBc+ injection drug users (IDUs); while Hofer *et al*. [13] detected serum HBV DNA in 51 of 57 patients (89.5%) who were solely anti-HBc+. O-HBV infections have also been identified in hepatitis C virus (HCV)-positive IDUs [14] and liver biopsies from patients with normal liver biochemistry and without prior liver disease [15].

It is unclear why HBsAg is undetectable during O-HBV infection, although several hypotheses exist. One possibility is that HBsAg is not produced or is expressed at levels below detectable limits of current diagnostic assays. Alternatively, HBsAg could be produced but not secreted from infected hepatocytes. Importantly, altered expression of HBsAg likely results from HBV mutation(s) [16]. Mutations in either the surface open reading frame (ORF), containing the PreS and surface (S) regions, or their corresponding spacer and reverse transcriptase (RT) regions of the overlapping polymerase ORF could directly affect HBsAg production. To date, a limited number of studies have investigated O-HBV mutations, in relatively few individuals [17–24]. Here, we describe a detailed evaluation of HBV genomic sequences from chronic (HBsAg+) and occult (HBsAg−) HBV-infected individuals in the same cohort and assess the presence of HBsAg mutations associated with O-HBV infection.

MATERIALS AND METHODS

Patient population

This prospective HIV-positive cohort was previously described in an analysis of O-HBV infection [4]. HBV DNA levels were determined by real-time PCR [lower limit of detection (LLD) = 67 copies/mL or 100 IU/mL] and HBV serologic markers, HBsAg, anti-HBc and anti-HBs, were evaluated by ELISA (LLD = 0.5 ng/mL, 2 NCU/mL and 10 mIU/mL, respectively) (Biochain, Hayward, CA, USA). HCV serostatus was determined by ELISA, measuring antibodies against HCV. As samples were collected between 1989 and 2004, HIV RNA levels were not available for all individuals. ALT levels above 92 U/L (two times the upper limit of local normal levels [25]) were considered elevated. Here, 23 C-HBV and six O-HBV serum samples were utilized, along with four HIV-positive, C-HBV samples from our serum repository.

PCR amplification

Hepatitis B virus DNA extracted from 200 to 400 μL of serum was used in separate nested PCRs for the PreS (549 bp) and S (339 bp) regions using primers [21,26] and amplification conditions listed in Supplemental Table S1. DNA amplification was performed using the GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Five microlitres of HBV DNA

initially served as template, while 1 μL of first round product served as template for the second round. A previously amplified HBV-positive patient serum sample served as a positive control, while HBV DNA-negative serum samples and a reaction without template served as negative controls.

Cloning and sequencing

PCR products were electrophoresed, purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). Ten clones per region per individual were sequenced bidirectionally. All sequences have been submitted to GenBank under accession numbers EU769235–EU769292.

Phylogenetic analysis

Sequence alignments were created for the PreS, S and their corresponding polymerase spacer [Pol(PS)] and RT [Pol(S)] regions, respectively, using the neighbour-joining method in CLUSTAL X [27]. Published references [28] and additional GenBank sequences were chosen at random to achieve five per genotype (Supplemental Table S2). For phylogenetic trees, the statistical robustness and reliability of the branching order were assessed using bootstrap analysis with 100 replicates. Calculations for genetic distance and non-synonymous–synonymous (dN–dS) mutation values [29] were performed using MEGA 3.1 [30]. Shannon entropy ($S_n = -\Sigma (p_i \ln p_i / \ln N)$) was calculated, where p_i was the frequency of each distinct nucleotide sequence and N was the total number of sequences analysed.

Mutational analysis

GenBank was searched for complete HBV genomes. One thousand nine hundred and ninety-three sequences were inspected, and all non-HBV, primate, recombinant and O-HBV genomes were excluded. The remaining full-length HBV sequences for genotypes A (n = 143), E (n = 58) and G (n = 23) were included in the mutational analysis.

GenBank references, as well as C-HBV sequences generated in this study, were compared to O-HBV sequences to identify distinct amino acid mutations in each genomic region that characterize O-HBV infections. Analyses were performed in a genotype-matched manner because of distinct biological and clinical differences among HBV genotypes [31]. The PreS and S regions analysed encode for all of PreS1 and PreS2, as well as amino acids 49–148 of S, and overlap with the entire polymerase spacer region, as well as amino acids 1–6 and 58–156 of the polymerase RT region (all numbered according to [32]). Mutations were considered to be associated with O-HBV infection if they were (i) identified in ≥1 O-HBV clonal sequence and (ii) not identified in any genotype-matched C-HBV sequences from this study or GenBank references analysed. Identified mutations were then compared to published mutations associated with O-HBV infection [17–24]. Mutation prevalence was also determined in all GenBank and C-HBV sequences after stratification by HBV genotype. To further identify signature amino acids, O-HBV sequences were compared to genotype-matched C-HBV sequences from this study using viral epidemiology signature pattern analysis [33] at a threshold of 0.5.

Statistical analysis

Wilcoxon rank-sum tests were performed on patient demographics, and P-values <0.05 were considered significant. For quasispecies parameters (genetic distance, entropy and dN–dS), values of each dependent variable were rank-ordered, and two-factor analysis of variance (ANOVA) tests were performed. *Infection status* was a between-subjects factor with two levels (occult or chronic), while *Region* was a within-subjects factor with four levels [PreS, S, Pol(S), Pol(PS)]. For each outcome, 12 planned pairwise comparisons of interest were

defined and utilized a t-type procedure where the mean difference in ranks for the conditions being compared was divided by the appropriate ANOVA error term (between-region comparisons using the within-subject error term and between-infection comparisons using the between-subject error term). A critical threshold for significance of $P = 0.0042$ ($P = 0.05/12$) was used to control for the overall Type I error rate at alpha = 0.05.

RESULTS

HBV region amplification

PreS was amplified and cloned from 19 of 27 (70.4%) *C*-HBV and 4 of 12 (33.3%) *O*-HBV infections. S was amplified and cloned from 25 of 27 (92.6%) *C*-HBV and 6 of 12 (50%) *O*-HBV infections. There were no statistically significant differences in patient demographics between *C*- and *O*-HBV-infected patients (Table 1), although median HBV DNA level was higher in *C*-HBV infections − 9.0×10^6 IU/mL – than in *O*-HBV infections − 1.4×10^4 IU/mL ($P < 0.05$), as reported previously [4].

Identification of HBV genotypes

Consensus nucleotide sequences were generated and aligned with reference sequences. In the PreS region, genotypes A ($n = 15$), D ($n = 1$) and G ($n = 2$) were identified in C-HBV subjects; genotypes A ($n = 1$) and G ($n = 2$) were identified in *O*-HBV subjects (Fig. 1a). Additionally, one mixed infection with genotypes A and G was identified (Occult 2). In S, genotypes A ($n = 17$), D ($n = 3$) and G ($n = 5$) were identified in *C*-HBV subjects, and genotypes A ($n = 2$), E ($n = 1$) and G ($n = 3$) were identified in *O*-HBV subjects, in agreement with PreS (Fig. 1b), although only genotype A was present for the mixed *O*-HBV infection.

Evaluation of quasispecies diversity

Clonal alignments were performed to assess quasispecies diversity. In PreS, all patients showed significant quasispecies diversity (≥2 distinct viral variants) (Supplemental Fig. S1). In S, while all patients with *O*-HBV showed significant quasispecies diversity, 5 of 27 *C*-HBV infections did not (Supplemental Fig. S2). No significant differences were found between *C*- and *O*-HBV infections with respect to genetic distance (Fig. 2a), entropy (Fig. 2b) or dN–dS (Fig. 2c), although dN–dS values were >0 – indicating positive selection – in three regions for *O*-HBV infections – PreS, S and Pol(PS) – compared to only Pol(PS) in *C*-HBV infections.

Among *C*-HBV patients, dN–dS values were significantly higher in Pol(PS) compared to PreS ($P = 0.001$). A signifi-cant increase in entropy was also noted in PreS and Pol(PS) compared to S and Pol(S), respectively (both, $P = 0.001$) (Fig. 2b).

Identification of occult hepatitis B virus sequence mutations

Genotype-matched sequence analysis was performed, identifying several novel mutations associated with *O*-HBV infection: five genotype A and 17 genotype G mutations in PreS, with three genotype A, one genotype E and six genotype G mutations, in addition to several previously published mutations in S (Table 2). Several S mutations identified reside within the antigenic determinant region of HBsAg (amino acids 100–165), suggesting that they may impact HBsAg detection.

Additional mutations associated with *O*-HBV were identified in the corresponding polymerase ORF (Table 3). In the spacer region, four genotype A and 13 genotype G mutations were identified, with three mutations for both genotypes A and G and two for genotype E in the RT region.

Prevalence of mutations associated with occult hepatitis B virus infection across genotypes

Occult hepatitis B virus mutation prevalence was also determined amongst distinct genotypes. Genotype A, E and G GenBank sequences and *C*-HBV sequences from this study were searched for all identified *O*-HBV mutations. Several OHBV mutations were identified at low frequencies in *C*-HBV sequences of different genotypes for the PreS1 (Fig. 3a), PreS2 (Fig. 3b), S (Fig. 3c) and polymerase spacer (Fig. 3d) regions. No RT mutations were identified in any other sequences.

Signature sequence analysis

Signature sequence analysis (SSA) was used to identify mutations more commonly found in *O*-HBV compared to *C*-HBV. SSA identified two PreS1 mutations in Occult 2 (genotype A), along with three corresponding polymerase spacer mutations, previously excluded because of low frequency in *C*-HBV sequences. In Occult 4 (genotype G), 50% of clones harboured a deletion of the first three N-terminal amino acids of PreS1. Another 40% of clones contained five mutations – M1I, G2R, L3I, S4P and W5R - below the 50% threshold for SSA. In the corresponding polymerase spacer, the first seven N-terminal amino acids were deleted in 50% of clones, and five mutations – G5Q, A6D, F7S, L8^{stop} and G10S - were present in another 40%.

In S, SSA identified three mutations in Occult 3 (genotype A) – M103I, K122R and G145A (bold, Table 2), present in all clones, although M103I and K122R were found at low levels in *C*-HBV sequences. In the corresponding polymerase RT region, two mutations – V112I and W153C – were identified in all clones, but only W153C was absent from chronic genotype A sequences (bold, Table 3). This mutation coincides with the G145A mutation in the S region; while other mutations have been reported at RT position 153 [22,34], none were a tryptophan-to-cysteine mutation.

DISCUSSION

Potential virological differences between *C*-HBV and *O*-HBV have not been well defined. It is clear that mutations in the S and Pol ORFs have the potential to (i) alter protein secretion from hepatocytes, (ii) alter protein structure, thereby inhibiting antibody binding in commercial HBsAg detection assays and/or (iii) decrease the overall replication efficacy of the virus. In this preliminary study, we examined genotype distribution and quasispecies diversity within HIV-positive patients previously identified with C-HBV or OHBV infection [4]. Similar to previous reports, genotype A was most common [35–37]; however, genotype G (24%) also exists within this cohort. While genotype G mono-infection has been reported previously [38], it is most commonly found in mixed infections with genotype A [37,39], of which we identified one in an *O*-HBV-infected individual. To our knowledge, only one mixed infection has been previously reported in the setting of *O*-HBV infection [20]. These data suggest that genotype G mono-infection may be more common in the United States than previously reported and further support the existence of mixed *O*-HBV infections.

Although HBV has a lower mutation rate than RNA viruses, it is higher than most DNA viruses. To date, few studies have examined intrapatient diversity of HBV [40,41], and none have included patients with *O*-HBV infection. Sequencing 10 clones has previously been demonstrated to effectively evaluate quasispecies diversity in HCV [42]; therefore, this number should also be sufficient for HBV given its lower mutation rate. Although our sample size is relatively small, dN-dS values were greater than zero for three of four genomic regions analysed. This indicates that positive immune selection pressures are acting against these regions in *O*-HBV infection, potentially resulting in mutations that may

adversely affect the production and/or detection of HBsAg. No significant differences in genetic distance, Shannon entropy or dN–dS were observed between *C*-HBV and *O*-HBV infections. When comparing regions within *C*-HBV infections, the significantly lower dN–dS for PreS vs Pol(PS) (P = 0.001) indicates increased synonymous mutation within PreS and highlights its intrinsic ability to tolerate mutations [43], although the spacer region is also highly tolerant of mutations/deletions. Significantly, higher entropies were also observed in PreS and Pol(PS) compared to S and Pol(S), respectively (*P* = 0.001), in *C*-HBV infections, again suggesting that functional constraints are less stringent for the PreS and spacer regions.

It is important to emphasize that mutations associated with *O*-HBV are frequently identified without a robust comparison with *C*-HBV infections in the same cohort, making it difficult to exclude natural polymorphisms and/or genotype-specific differences. To more effectively characterize virological differences between *C*- vs *O*-HBV, we performed genotype-matched sequence analysis of the PreS, S and polymerase spacer and RT regions to identify *O*-HBV mutations absent in *C*-HBV. Multiple mutations were identified, including several not previously described, although many were only found in one variant. New *O*-HBV mutations were particularly common for genotypes E and G, as previous investigations have focused primarily on the most common HBV genotypes (A–D). Because of the paucity of sequence data for these genotypes, a portion of these mutations could represent naturally polymorphic sites.

It is known that mutations within the S ORF affect the antigenicity [16,22] and detection of HBsAg [24,44,45]. Three *O*-HBV-infected individuals harboured mutations with direct importance to virus replication. Two sets of mutations in or near the antigenic determinant of HBsAg were of interest: Occult 3 (genotype A) – M103I, K122R and G145A – and Occult 6 (genotype E) – F85C, Y100S and G145R. While K122R is considered a polymorphism defining the d/y sub-serotypes, its effects in combination with the other two are unknown. The positions of these mutations – either alone or in combination – could alter the secondary structure of HBsAg and impair its detection. In particular, G145R resulted in undetectable HBsAg levels using three of four commercial assays [45]. RT mutations at position 153 – W153C and R153Q – result from the same nucleotide substitution as G145A and G145R in the S ORF. While G145A and G154R have been previously reported [18,21,22], these specific RT153 mutations have not been described. Although neither patient was receiving ART at the time, W153Q has been indicated in lamivudine resistance and was found to decrease replicative fitness in vitro along with additional mutations [34], although single RT mutations are also capable of decreasing HBV replication [46].

Most viral sequences for Occult 4 contained a defective or absent large HBsAg start codon. Although the M1I start codon mutation was also identified in 0.7% of genotype A GenBank reference sequences, these contain another potential start codon at position 12, where genotype G does not. Therefore, genotype G infections may not be able to overcome the consequences of this mutation. Defects in LHBsAg can lead to elimination of PreS1 synthesis or accumulation of large HBsAg (LHBsAg) within the endoplasmic reticulum of hepatocytes. Such mutations have been shown to arrest virion and subviral particle secretion [47,48], and could result in a lack of detectable HBsAg in the serum. Collectively, these data suggest that mutations at distinct amino acid positions in HBsAg or deletions at the N-terminus of the large HBsAg could play a role in persistence of *O*-HBV infection. Future studies are necessary to characterize additional genomic regions, and a functional analysis is ongoing to evaluate the effects of mutations identified here on HBsAg expression, retention and detection *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ART	antiretroviral therapy
C-HBV	chronic carriers of the hepatitis B virus
dN–dS	nonsynonymous–synonymous
HBc	HBV core protein
HBsAg	hepatitis B surface antigen
HCC	hepatocellular carcinoma
IDU	injection drug user
LLD	lower limit of detection
O-HBV	occult hepatitis B virus
ORF	open reading frame
PreS	PreSurface
RT	reverse transcriptase
SSA	signature sequence analysis
S	surface

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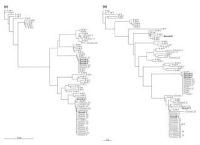

Fig. 1.
Phylogenetic trees for the (a) PreSurface (PreS) and (b) S regions of HBV. Consensus nucleotide sequences were generated from 10 clones per individual. One mixed infection was identified (Occult 2) in the PreS region containing both genotypes A and G (*).

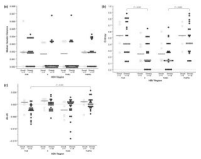

Fig. 2.

Dot plots for (a) median genetic distance, (b) Shannon entropy and (c) nonsynonymous–synonymous calculations. Although sequences were only available for four Occult hepatitis B virus (O-HBV)-infected patients for the PreSurface and Pol(PS) regions, $n = 5$ because of the mixed infection with genotypes A and G. (◯) represent values for O-HBV infections, while (•) represent values for chronic carriers of the hepatitis B virus (C-HBV) infections. Horizontal bars, black for O-HBV infections and grey for C-HBV infections, represent the median values for each group and dashed lines represent zero.

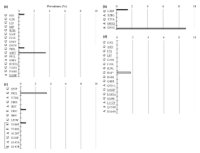

Fig. 3.

Prevalence of (a) PreS1, (b) PreS2, (c) S and (d) polymerase spacer [Pol(PS)] mutations identified in Occult hepatitis B virus infections in genotype A, E and G reference sequences. None of the identified polymerase reverse transcriptase mutations [Pol(S)] were found in any of the additional reference sequences. (*) Indicates a stop codon. Black bars indicate prevalence in genotype A sequences, white bars indicate prevalence in genotype E sequences, and grey bars indicate prevalence in genotype G sequences. The original genotype in which each mutation was identified is indicated on the left in bold. Underlined mutations could not be analysed in all genotypes because of genotype-specific sequence differences. Bracket in (c) indicates mutations present in the antigenic determinant of hepatitis B surface antigen (amino acids 100–165).

Table 1

Patient demographics. Patient demographic and clinical data were included for the 27 chronic carriers of the hepatitis B virus infections and six occult hepatitis B virus infections that could be amplified by nested PCR

HBV infection *n* = 33	**Chronic *n* = 27**	**Occult *n* = 6**
Age*	35.8 years (21.2–58)	35.4 years (26.7–42.2)
Race		
African-American	14 (51.9%)	3 (50.0%)
Caucasian	12 (44.4%)	2 (33.3%)
Other	1 (3.7%)	1 (16.7%)
Gender		
Male	27 (100%)	5 (83.3%)
Female	0	1 (16.7%)
ALT*,†	56.0 U/L	27.0 U/L
n = 31	(17–140)	(23–87)
HCV serostatus†	0	1 (25%)
n = 21		
HIV†		
Detectable	21/23 (91%)	5/5 (100%)
Viral load*	6.0×10^4 copies/mL (7.2×10^2–2.0×10^5)	1.7×10^5 copies/mL (1.0×10^4–3.7×10^5)
ART		
On therapy	6 (22.2%)	1 (16.7%)
With HBV activity	7‡ (100%)	0
$CD4^+$ count*,†	330.5 cells/mL (7–665)	141.0 cells/mL (6–559)
HBV DNA*	9.0×10^6 IU/mL (2.1×10^2–7.6×10^8)	1.4×10^4 IU/mL (1.1×10^3–7.6×10^5)

Local normal ALT levels were 5–46 U/L [25]; ALT levels are considered elevated when >92 U/L (two times the upper limit of normal). HCV serostatus was evaluated using anti-HCV ELISA. ART, antiretroviral therapy.

*Median values are given with the range in parentheses.

†Information was not available for all individuals (the number of patients with available information is listed under the heading); therefore, the median ALT and $CD4^+$ count and percent detectable HCV and HIV are given for the individuals tested.

‡One additional individual is on HBV mono-therapy but not ART.

Table 2

PreSurface (PreS) and surface (S) mutations. S open reading frame mutations identified in Occult hepatitis B virus-infected individuals

Surface region	HBV genotype	Newly identified		Previously identified	Reference
PreS1	A	N98Y			
		S109P			
	G	M1I	D41N		
		G2R	P88L		
		L3I	A89T		
		S4P	P93L		
		W5R	R102G		
		S16P	T105I		
		F23S	P106S		
		D26Y			
PreS2	A	S28G			
		H41Q			
		D51G			
	G	L20P			
		T37A			
Surface	A	Y72H			
		I82T		**G145A** †	[21]
		A128T†			
	E	Y100F†		F85C	[24]
				Y100S†	[24]
				G145R†	[18,22]
	G	S55P			
		P62L			
		F80S			
		I86V			
		L95W			
		S136P†			

Mutations also identified in the signature sequence analysis are indicated in bold.

† Mutations that reside within the antigenic determinant loops of hepatitis B surface antigen (AA 100–165).

Table 3

Polymerase mutations. P open reading frame mutations identified in occult hepatitis B virus-infected individuals

Polymerase region	HBV genotype	Newly identified	
Spacer	A	Q101L	Q150R
		L112P	H164N
	G	G5Q	R44*
		A6D	R44K
		F7S	Q48R
		L8*	S102P
		G10S	E105G
		F19S	S109F
		R29L	
RT	A	I103T	
		C136Y	
		W153C	
	E	L66F	
		R153Q	
	G	V63A	
		T70A	
		H94R	

Mutations also identified in the signature sequence analysis are indicated in bold. RT, reverse transcriptase.

* Indicates a stop codon.