Genetic Characterization of Hepatitis B Virus in Peripheral Blood Leukocytes: Evidence for Selection and Compartmentalization of Viral Variants with the Immune Escape G145R Mutation

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The compartmentalization of viral variants in distinct host tissues is a frequent event in many viral infections. Although hepatitis B virus (HBV) classically is considered hepatotropic, it has strong lymphotropic properties as well. However, unlike other viruses, molecular evolutionary studies to characterize HBV variants in compartments other than hepatocytes or sera have not been performed. The present work attempted to characterize HBV sequences from the peripheral blood leukocytes (PBL) of a large set of subjects, using advanced molecular biology and computational methods. The results of this study revealed the exclusive compartmentalization of HBV subgenotype Ae/A2-specific sequences with a potent immune escape G145R mutation in the PBL of the majority of the subjects. Interestingly, entirely different HBV genotypes/subgenotypes (C, D, or Aa/A1) were found to predominate in the sera of the same study populations. These results suggest that subgenotype Ae/A2 is selectively archived in the PBL, and the high prevalence of G145R indicates high immune pressure and high evolutionary rates of HBV DNA in the PBL. The results are analogous to available literature on the compartmentalization of other viruses. The present work thus provides evidence in favor of the compartment-specific abundance, evolution, and emergence of the potent immune escape mutant. These findings have important implications in the field of HBV molecular epidemiology, transmission, transfusion medicine, organ transplantation, and vaccination strategies.

Hepatitis B virus (HBV) is the prototype member of the Hepadnaviridae family and classically has been described to be hepatotropic, causing a wide range of clinical and subclinical manifestations of liver disease (57). Nevertheless, studies of HBV-infected human subjects and woodchucks infected with Woodchuck hepatitis virus (WHV; an animal model of hepadnaviral infection) have reported different molecular forms of replicative intermediates in the lymphatic cells and have established that hepadnaviruses are strongly lymphotropic in nature (29). Moreover, the results of studies of human subjects as well as with animal models have revealed that the life-long occult persistence of replication- and transmission-competent viruses in lymphatic cells is a strict consequence of hepadnaviral infections (29).

More interestingly, in animal models, lymphatic system-restricted occult hepadnaviral infection has been found to be transmissible vertically as an asymptomatic, serologically occult infection exclusively confined to the lymphatic system (29). Earlier we provided evidence that occult HBV persisting in the lymphatic cells are transmissible, specifically to the PBL through horizontal infrafamilial modes (9). These observations clearly indicate important immunological, pathogenic, and epidemiological implications of lymphatic system-restricted hepadnaviral infections. Although the involvement of specific viral variants has been suggested to explain this lymphatic system-restricted hepadnaviral infection and transmission (29), the classical belief that hepatocytes are the primary target and only reservoir of HBV has precluded the genetic characterization of hepadnaviruses from extrahepatic sites.

Fascinatingly, despite being classically considered a hepatotropic virus, hepatitis C virus (HCV), belonging to the family Flaviviridae, also shows occult persistence and lymphotropism very similar to that of hepadnaviruses (37). Similarly to WHV, HBV, and HCV, other viruses, including HIV (human immunodeficiency virus), small ruminant lentivirus, and Epstein-Barr virus, also have been shown to infect and persist in different anatomical compartments of the body in addition to their classical target cells (38, 40, 43, 45, 50). Furthermore, recent molecular evolutionary analyses based on envelope sequences of these viruses (e.g., HIV, HCV, small ruminant lentivirus, Epstein-Barr virus, etc.) have established clearly that these viruses undergo selection and independent evolution in diverse tissues, leading to the tissue-specific compart-
mentralization of viral populations (38, 40, 43, 45, 50). In contrast to other viruses, to the best of our knowledge, methodical molecular evolutionary studies to characterize HBV sequences isolated from extrahepatic sites of HBV-infected subjects have not been reported in the literature.

We hypothesized that similar to other viruses, HBV also undergo independent evolution in different compartments of the body under the influence of differential immune pressure. To examine our hypothesis, we used the most easily available lymphatic cells, the peripheral blood leukocytes (PBL), determined the HBV envelope sequences from HBV DNA isolated from these cells, and performed advanced genetic, phylogenetic, and mutational analysis. The results of this work demonstrate a highly compartment-specific preponderance of HBV genetic variants in serum and PBL of the same study population, providing evidence in favor of the compartmentalization of HBV genetic variants. The results and important implications of these findings are discussed in this work.

**MATERIALS AND METHODS**

**Study subjects.** The blood samples analyzed in this study were selected from a repository of blood samples (stored at −80°C) collected during our previous studies of an eastern Indian population. The samples were collected at different collection sites and at different times. Based on a detailed examination of the questionnaires, which were filled out during patient interviews, and on results of the serological assays, samples were carefully screened for inclusion in this study. Samples from subjects admitting activities related to multiple exposures to HBV (sexual interaction with multiple partners and/or commercial sex workers, intravenous drug abuse, etc.), alcoholism, or having markers for HCV and/or HIV coinfection were excluded. Only samples from incidentally detected, healthy, marker of past exposure to HBV) with normal levels of alanine aminotransferase were included in the study. These subjects also were naïve to antiviral therapy.

This work was a part of our study of the HBV genetic variability in eastern India and was approved by the institutional ethics committee. Informed consent was obtained from the donors before blood drawing.

**Seralogical tests.** Commercially available enzyme-linked immunosorbent assay-based kits were used for the detection of HBsAg (Biomerieux, Bontel, The Netherlands), anti-HBc (Biomerieux, Bontel, The Netherlands), anti-HCV (Ortho-HCV, Diagnostic, NJ), and anti-HIV (Biomerieux, Bontel, The Netherlands).

**DNA extraction and verification and HBV DNA detection.** Leukocytes were enriched and serum HBV DNA was depleted from the leukocytes by a selective erythrocyte lysis/leukocyte washing method. Briefly, 3 ml whole blood was incubated with erythrocyte lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM erythrocyte lysis/leukocyte washing method. To verify the efficiency of the erythrocyte lysis/leukocyte washing method was comparable to the enzymatic method in its efficiency to deplete serum HBV DNA from PBL DNA and also provides cells suitable for the sensitive detection of HBV genomes (data not shown). The concentration and purity of the extracted DNA was checked spec- torsometrically (Varian, Victoria, Australia), diluted appropriately, and stored at −20°C until further use. Simultaneously, DNA was extracted from 200 µl serum and from the supernatant of the last washing step of the PBL DNA extraction by the proteinase K, phenol-chloroform-isooamyl alcohol method and stored at −20°C until further use.

DNA extraction was verified by a PCR assay targeting the human interleukin-1β promoter region (primers pil-1H [5'-TGCCATTGATCGTGTCTCATC-3'] and pil-1bR [5'-TTTGGATGATCTCCCACCT-3']), producing an amplicon of 304 bp. For the detection of HBV DNA, nested PCR assays targeting the distalX/precore/core promoter region (3) and the surface gene region (described below) were used. HBV-specific cccDNA (covalently closed circular DNA) was detected by a previously described heminested PCR assay (7). Strict precautions were taken to avoid cross contamination (26), and appropriate no-template controls (water), negative controls (PBL DNA extracts from HBV/HBV-seronegative healthy subjects), and positive controls (PBL DNA/serum DNA extracts from HBsAg-positive subjects and/or cloned plasmid containing HBV genotype D genome) were included during each extraction, amplification, and cycle sequencing steps to rule out contamination.

**HBV genotyping, sequencing, and cloning.** First, HBV genotypes were determined by a rapid and highly sensitive nested PCR-fragment length polymorphism (PCR-RFLP) method, which we modified from a previously described single PCR-RFLP-based method for large-scale genotyping (27). Being a PCR-based amplification assay, our modified method was appropriate for the genotyping of HBV in HBsAg-negative samples with low viremia. Briefly, the surface gene region encoding the major hydrophilic loop was amplified by a nested PCR assay with primers 5'-ACCCCTGCTCGTGTTACAGGC-3' (sense; positions 184 to 204) and 5'-AAAGGCACAGACGTTGGGGGAAA-3' (antisense; positions 731 to 711) for the first round, as well as nested primers 5'-GACTCG GTGTTGACCTCCTC-3' (sense; positions 251 to 271) and 5'-TAAACTG AAAAGAGACAGATC-3' (antisense; positions 126 to 109) for the second round of PCR amplification, using a HotStart Taq polymerase (Applied Biosystems, Foster City, CA) and a thermal cycling profile, including incubation for 10 min at 95°C, 40 cycles (35 cycles for the second round) at 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s, followed by a final extension step at 72°C for 5 min. PCR products were stained by ethidium bromide and visualized under UV light (Fig. 1).

The restriction sites in the 429-bp partial surface gene amplicon were derived from the original report by Lindh et al. (27) and amplified by a modified method and a subsequent methodological analysis of 112 GenBank sequences of different HBV genotypes using BioEdit (19). It was observed that the banding pattern of 3Ps/091 was sufficient to distinguish between HBV genotypes A, C, and D, which are prevalent in our population. The specificity of the method was verified by using HBsAg-positive samples, which we genotyped previously by full-genome sequencing and phylogenetic analysis (1). After the digestion of the 429-bp amplicon with 3Ps/091 (at 65°C for 3 h), genotype A-specific amplicons yield three major bands (126, 109, and 90 bp), genotype C-specific amplicons yield two major bands (235 and 90 bp), and genotype D-specific amplicons also yield two major bands (173 and 109 bp). The RFLP genotyping patterns obtained in all of the samples were confirmed by direct sequencing and phylogenetic analysis.

**Surface gene PCR products were purified (Qiagen GmbH, Hilden, Germany) and sequenced (Big Dye 3.1; Applied Biosystems, Foster City, CA).** In this study, direct sequencing was carried out to identify sites under significant evolutionary pressures by virtue of analyzing multiple distinct peaks (sequencing mixtures) in a sequencing electropherogram (17, 42).

To verify the results of the direct sequencing and to scan the presence of a minor subpopulation of virus in different compartments, we randomly selected seven samples that had HBV DNA only in the PBL compartment for multiple clonal sequence analyses. Parallel PBL and serum DNA extracts from four subjects having HBV DNA in both compartments also were selected for multiple clonal sequence analyses. In addition, two sera DNA extracts having the subgenotype Aa/A1 (as determined in our previous studies) also were included as controls during the process of cloning, sequencing, and subsequent analysis to rule out cross contamination. The PCR products were cloned using a T/A cloning kit (Fermentas, Glen Burnie, MD), and 10 to 15 transformed colonies from each sample were randomly selected, amplified, sequenced, and analyzed phylogenetically.

**Molecular evolutionary analyses.** We aligned and edited the sequences using the BioEdit v7.0 program (19). We manually examined the direct sequence electropherograms and replaced the N residues in the alignment with appropriate ambiguous nucleotide codes by following International Union of Pure and Applied Chemistry conventions (2). HBV genotypes and subgenotypes were determined by phylogenetic analysis with HBV genotype- and subgenotype-specific reference sequences retrieved from GenBank according to a previous analysis (34). Pairwise evolutionary distances were calculated using the Kimura two-parameter model for the esti-
RESULTS

Prevalence of HBV DNA in serum and PBL. The results of the detection of HBV DNA in serum and PBL in subjects with HBsAg-negative and HBsAg-positive serology are presented in Table 1. Statistically there was no difference in the mean age of the HBsAg-positive and -negative subjects included in this study ($P = 0.135$). As expected, the detection rate of HBV DNA in serum was significantly higher in HBsAg-positive subjects than in HBsAg-negative subjects (23.3 and 5.5%, respectively; $P < 0.001$). The rate of HBV DNA detection in both serum and PBL together also was significantly higher in HBsAg-positive subjects than in HBsAg-negative subjects (30 and 14.8%, respectively; $P = 0.017$). In contrast, the rate of HBV DNA detection only in PBL was significantly higher in HBsAg-negative subjects than in HBsAg-positive subjects (61.1 and 40%, respectively; $P = 0.004$). HBV DNA was not detected in either serum or PBL in 6.6 and 18.5% of HBsAg-positive and HBsAg-negative subjects, respectively.

For studying the molecular characteristics of PBL-related HBV envelope sequences encoding the major hydrophilic loop region, 12 HBsAg-positive (designated group I) and 33 HBsAg-negative (designated group II) subjects having detectable HBV DNA exclusively in the PBL were selected and further studied in detail. This selection criterion was used to eliminate the possibility of the contamination of PBL DNA with HBV DNA circulating in the serum. In addition to comparing the HBV DNA sequences in the serum and PBL within the same individuals, eight HBsAg-negative subjects with HBV DNA detectable in both serum and PBL (designated group III) also were selected for detailed analysis. These samples had very low levels of HBV DNA in their sera ($10^2$ to $10^3$ copies ml$^{-1}$). In these cases, the stringent washing of the leukocyte pellets was performed to rule out the mixing of HBV DNA from serum and PBL, and the supernatant of the last wash step was used as a control. However, the HBsAg-positive subjects who had HBV DNA in both their serum and PBL were not included for detailed sequence analysis if they had very high levels of HBV DNA in their sera ($>10^6$ copies ml$^{-1}$); thus, the likelihood of the contamination of the PBL DNA with serum HBV DNA was much higher.

HBV cccDNA-specific amplicons were detectable in 25% (3/12), 18.2% (6/33), and 25% (2/8) of PBL DNA extracts from groups I, II, and III, respectively.

Genotypes and their distribution in serum and PBL. Results of the genotype-specific RFLP patterns were typical for geno-

FIG. 1. Results of representative nested PCR amplification. Shown is a gel of the 429-bp amplicon specific for HBV surface gene region in control and test samples. Lanes: 1, no-template control (NTC); 2 to 6, PBL extract from HBsAg-positive subjects (test samples); 7 and 9 to 12, PBL extracts from HBsAg-negative, anti-HBc positive subjects (test samples); 13, positive control (plasmid containing genotype D-specific HBV genome); 14, negative control (water); 15, PBL DNA extract from subject completely negative for HBV serological markers (mock sample); and 8 and 16, DNA molecular size markers (pUC19/MspI-digested DNA).

TABLE 1. Prevalence of HBV DNA in serum and PBL of HBsAg-positive and -negative subjects

<table>
<thead>
<tr>
<th>HBsAg Group</th>
<th>n</th>
<th>Age (yr)</th>
<th>No. (%) of samples positive for HBV DNA</th>
<th>No. (%) with no HBV DNA in serum and PBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>30</td>
<td>33.9 ± 13.4</td>
<td>7 (23.3)</td>
<td>9 (30.0)</td>
</tr>
<tr>
<td>Negative</td>
<td>54</td>
<td>29.1 ± 15.1</td>
<td>3 (5.5)</td>
<td>8 (14.8)</td>
</tr>
</tbody>
</table>

Nucleotide sequence accession numbers. The direct sequence data generated in this work have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under accession numbers EU275289 to EU275349.
type A, C, or D in all but five HBsAg-positive (group I) cases. In these five cases, the RFLP banding patterns were atypical, and the sequence electropherogram from these five subjects showed mixed peaks at a number of nucleotide positions (see Fig. S1 in the supplemental material). The comparison of the mixed peaks in these nucleotide sequences to consensus sequences of different HBV genotypes suggested the presence of two different genotypes of HBV in these extracts. Subsequently, the cloning of the amplicons from these five cases, followed by genotype analysis, revealed the presence of genotypes A and C in one sample, while genotypes A and D were detected in four isolates. Genotype A was detected in the majority of the clones from these subjects. Nucleotide sequences from the rest of the amplicons from group I, group II, and group III showed clearly distinct dye peaks (see Fig. S2 in the supplemental material), with mixed residues appearing at very few sites. The results of the phylogenetic analyses of these sequences were consistent with the results of the RFLP genotype results (data not shown).

Interestingly, the preponderance of genotype A was observed in PBL of groups I and II (Fig. 2a, b). This selective prevalence of HBV genotypes in the PBL was more evident in group III, where genotypes D and C were prevalent in the serum (Fig. 2c), significantly contrasting (P < 0.001) to the predominant of genotype A in the PBL (Fig. 2d). Upon comparing the paired samples (from serum and PBL) from eight subjects of group III, we found that in six of eight subjects the genotype circulating in the serum was either C or D, while genotype A was present invariably in the PBL of the same individuals, clearly indicating the specific preponderance of genotype A in the PBL over non-A genotypes in the serum.

Phylogenetic relatedness. In the phylogenetic tree, all of the sequences generated in the present study clustered with either genotype A, C, or D reference sequence clusters (Fig. 3). All of the genotype A sequences isolated from the PBL clustered with subgenotype Ae/A2 reference sequences and all of the genotype C sequences clustered with subgenotype Cs/C1 reference sequences, while genotype D was the most heterogeneous in subgenotypes, clustering with one of the subgenotype D1, D2, D3, and D5 reference sequences (Fig. 3).

As the occurrence of subgenotype Ae/A2 sequences was not in agreement with the results of our previous studies using serum HBV isolates, we aligned the PBL-specific surface gene sequences with serum-isolated HBV surface gene sequences from our previous studies and subjected them to phylogenetic analysis again. Genotype A sequences from PBL and serum formed clearly distinct clusters with subgenotype Ae/A2 and Aa/A1 reference sequences, respectively (Fig. 4).

The results of the analysis of multiple clones also confirmed the results of the direct sequencing. Among the three samples selected from group I, multiple clones from isolate PB58 showed a mix of subgenotype Ae/A2- and D1-specific sequences, and isolate PB74 showed only subgenotype D2-specific sequences (see Fig. S3 in the supplemental material). Of the four samples selected from group II, subgenotype Ae/A2 (isolate PB47)-, subgenotype D3 (isolate PB05)-, subgenotype Cs/C1 (isolate PB18)-, and subgenotype Ae/A2 (isolate PB24)-specific sequences were detected exclusively in the multiple clonal analysis (see Fig. S3 in the supplemental material). Of the four parallel serum and PBL isolates from group III samples selected for multiple clonal sequence analysis, isolate PB02 showed subgenotype Cs/C1 sequences in the serum and subgenotype Ae/A2 sequences in the PBL, isolate PB32 showed subgenotype D5 sequences in the serum and subgenotype Ae/A2 sequences in the PBL, isolate PB35 showed subgenotype D1 sequences in the serum and subgenotype Ae/A2 sequences in the PBL, and isolate PB36 showed subgenotype D5 sequences in the serum and subgenotype Ae/A2 sequences in the PBL (see Fig. S3 in the supplemental material). In the four isolates from group III described above, each of the subjects showed distinct HBV genotypes in the serum and PBL extracts. In these cases, the HBV genotype detected in the serum was not detected even as a minor subpopulation in the PBL, or vice versa, during the multiple clonal analyses. Moreover, all of the subgenotype Ae/A2-specific sequences detected in the PBL had an adenine at nucleotide position 587 (corresponding to G145R), confirming the results of the direct sequencing.

Genetic diversity and sequence characteristics of Ae/A2 sequences from PBL. On the calculation of the intergroup genetic distances, we found that the subgenotype Ae/A2 sequences found in group I were the most divergent, followed by sequences from groups III and II. Furthermore, the average of synonymous and nonsynonymous substitution distances and the ratio of synonymous and nonsynonymous substitution rates (dS/dN) also were found to correlate with the genetic distances (Table 2), signifying the higher rate of evolutionary pressure in the PBL of HBsAg-positive subjects compared to that of the HBsAg-negative subjects.

When the reference sequences from GenBank were analyzed based on the subgenotypes Aa/A1 and Ae/A2 for nucleotides 341 to 660 (the region analyzed in this study) of the surface gene, four positions were found to be phylogenetically informative in differentiating these two subgenotypes of A. Nucleotides 505T, 514C, 616A, and 619T represent subgenotype Aa/A1 strains from Asia, nucleotides 505T, 514C, 616A, and 619T represent subgenotype Ae/A2 strains from sub-Saharan Africa, and nucleotides 505T, 514A, 616T, and 619T were specific for subgenotype Ae/A2 sequences that are prevalent in northwestern Europe. In all of the present genotype A samples
amplified from PBL, these four informative sites had bases specific for subgenotype Ae/A2. In addition, all of the present genotype Ae/A2 sequences had two additional nucleotide substitutions, 358C and 587A, which were found to be coevolving sites.

The comparison of deduced amino acid sequence revealed that the four specific nucleotides that differed between subgenotype Aa/A1 and Ae/A2 sequences (nucleotides 505, 514, 616, and 619) were synonymous by nature. For the two other specific substitutions in the present Ae/A2 samples, the substitution at position 358 was found to be the wobble base of the codon encoding residue 68 of HBsAg. On the other hand, the nucleotide at position 587 is the first base of the codon specifying the amino acid at position 145 of HBsAg, and the mutation 587A results in a potent immune escape substitution from glycine to arginine (G145R).

Amino acid substitutions. The alignments of deduced amino acids and substitutions in the present samples are presented in Fig. 5. Analyses of the sequences showing mixed nucleotides revealed the changes to be mostly synonymous or genotype/serotype specific. Apart from the type-specific changes, in other sequences the substitution T125M was observed mostly in subgenotype D3 isolates and in one Cs isolate, and it was present with a P127T substitution. Interestingly, the G145R mutation was present invariably in all of the genotype A (subgenotype Ae/A2) isolates or the isolates showing mixed genotypes (group I isolates) and was found to be a PBL-specific HBsAg signature residue in VESPA. This mutation also was detected in one genotype C isolate from group II. Overall, the prevalence of determinant “a” mutations was low, except the specific association of G145R with genotype A isolates from the PBL. Remarkably, this mutation was not detected in any of the isolates from the serum in the present study.

DISCUSSION

The present study enlightens interesting and important facets of HBV lymphotropism, and it supports and extends earlier
observations that occult HBV DNA persistence in the lymphatic cells is an invariable outcome of HBV infection in the majority of subjects (7, 29, 53). Besides this persistence, cccDNA also was detected in the PBL, which suggests active HBV replication and transcription in these cells (60). However, due to the extremely small numbers of cccDNA molecules in the cells (60), the detection of this molecule has remained a challenge even in the era of sensitive PCR-based methods. Notably, in a previous study based on single-round PCR, Köck et al. (23) could not detect cccDNA in the PBL, and they concluded that the detection of viral nucleic acids in the PBL is attributable to adhered serum HBV particles (23). However, using a more sensitive PCR assay, we detected cccDNA in the PBL of subjects who lacked HBV DNA in their serum, in contrast to the conclusions of Köck et al. (23).

The frequent detection of HBV DNA in the PBL of HBsAg-negative anti-HBc-positive individuals is concordant with earlier studies of WHV (29). This phenomenon has been suggested to indicate low-rate viral replication and transcription in the PBL, which is necessary for the periodic stimulation of immune cells, creating a negative feedback loop for keeping the virus under strict control throughout life (29, 36). Thus, contrary to the classical belief of sterilizing immunity, the lifelong trace persistence of viruses in immunologically privileged sites seems to be common, at least in hepadnaviral infections.

In this study, we selected the HBV surface genetic region encoding the dominant B-cell epitope (the a determinant) for molecular evolutionary analysis. A strong anti-HBs humoral response is induced against this epitope; thus, this region has a high rate of evolution (49). Analogous genetic regions of the envelope gene frequently have been targeted for similar studies in other viruses (28, 38, 40, 43, 45, 50). Although the HBV x gene region shows considerable variability, the frequent loss and low genetic variability of this HBV region in our study population (3, 12) restricts its use for the robust molecular evolutionary analysis of HBV. We highlighted earlier that the HBV surface genetic region is an ideal candidate for molecular evolutionary analysis studies (11).

Interestingly, in analyzing different HBV genetic regions from serum isolates, we have documented the prevalence of HBV genotype D (subgenotypes D1, D2, D3, and D5), fol-

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**TABLE 2. Sd, Nd, and dS/dN calculated for HBV genotype A (subgenotype Ac/A2) sequences isolated from the PBL in this study**

<table>
<thead>
<tr>
<th>Group</th>
<th>Distance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sd</th>
<th>Nd</th>
<th>dS/dN</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (HBsAg positive, HBV DNA only in PBL)</td>
<td>4.58 ± 0.02</td>
<td>4.86</td>
<td>2.79</td>
<td>6.24</td>
</tr>
<tr>
<td>II (HBsAg negative, HBV DNA only in PBL)</td>
<td>1.30 ± 0.01</td>
<td>2.29</td>
<td>1.02</td>
<td>2.43</td>
</tr>
<tr>
<td>III (HBsAg negative, HBV DNA in PBL and serum)</td>
<td>1.39 ± 0.01</td>
<td>2.73</td>
<td>0.83</td>
<td>3.76</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of synonymous substitutions (Sd), nonsynonymous substitution (Nd) distances, and ratio of synonymous and nonsynonymous substitution rates (dS/dN) were calculated for the HBV genotype A (subgenotype Ac/A2) sequences isolated from the PBL in this study.

<sup>b</sup> Genetic distances are expressed as means ± standard deviations.
followed by genotypes C (subgenotype Cs/C1) and A (subgenotype Aa/A1) in our study population (1, 2, 3, 4, 12). Specifically, the incidence of HBV genotype Aa/A1 (the Afro-Asian subgenotype of genotype A) in the sera was found to be about 8 and 11% among the incidentally detected asymptomatic HBsAg-negative HBV carriers (occult HBV infection) and HBsAg-positive HBV carriers, respectively (2, 13). Surprisingly, PBL-associated HBV sequences analyzed in this study were found to be phylogenetically related to subgenotype Ae/A2 (the European subgenotype of genotype A). Moreover, in multiple clonal analyses of parallel serum and PBL isolates from the same individuals, subgenotype Ae/A2 sequences were detected exclusively in the PBL, but it was not detected in any of the serum samples analyzed. The presence of subgenotype Ae/A2 in the PBL and its absence from the sera thus underscores the confined presence of subgenotype Ae/A2 in the PBL. These observations also signify that different viral variants are present in the serum and PBL, and that viral variants

FIG. 5. Alignment of deduced amino acids showing substitutions detected in the present samples. A plus indicates sites where a nonsynonymous change was observed in the deduced amino acid sequence due to the presence of mixed nucleotides in the corresponding sequence. The genotype- and subgenotype-specific residues are evident in the alignment of the consensus sequences at the beginning of the alignment (indicated by the prefix con). The source of isolates (serum or PBL) and subgenotypes of the sequences, determined by phylogenetic analysis, is indicated after the sequence name. Paired sequences from the serum and PBL of individuals of group III have been kept adjacent to demonstrate the variation of viral genotypes in the two compartments of the same subject.
present in the PBL are not detectable in the sera. Earlier, two case studies of liver transplant patients also indicated that different HBV strains exist in PBL and in serum and that PBL acts as an important source of immune escape variants (5, 51). The results of the present study confirm and extend earlier studies.

The detection of subgenotype Ae/A2 sequences with specific immune escape variant G145R across individuals in this study was also surprising. However, the possible cross-contamination between samples or reagents was ruled out by including appropriate controls during all of the experimental steps and aptly following the PCR recommendations of Kwok and Higuchi (26). Moreover, subgenotype Ae/A2 has not been detected in any of the group III serum HBV isolates in this study, despite the fact that they were extracted and amplified simultaneously with the PBL samples. In addition, a number of our other studies focused on serum HBV isolates, and in parallel or after this study, we have detected diverse HBV subgenotypes, namely, Aa/A1, Cs/C1, D1, D2, D3, and D5 (1, 2, 4, 10, 12, 14), but subgenotype Ae/A2 with G145R was not detected, regardless of the fact that all of the studies were performed using the same experimental procedures, reagent sets, and laboratory facilities. These facts exclude the possibility that the detection of subgenotype Ae/A2, specifically in the PBL, across individuals in the present study was due to cross-contamination.

From the perspective of molecular epidemiology, the presence of European subgenotype Ae/A2 in the sera of the Indian population was quite anticipated due to India’s long history of European links (52). However, until now, studies of serum HBV isolates from different parts of India could not detect this subgenotype in the sera of the Indian population (1, 4, 16, 20, 34). Nevertheless, for the first time we reveal that subgenotype Ae/A2 sequences remain present in the leukocytes in our population. This observation is remarkably similar to the archiving of the fittest ancestral HIV sequences in different cellular or anatomical compartments independently of the viral genotypes dominant in the serum, a phenomenon defined as cellular or anatomical memory (6). In this study, we could not detect subgenotype Ae/A2 even after the clonal analysis of serum HBV isolates (the assay was sensitive enough to detect viral subpopulations as low as 8 to 10%), but it might be possible that subgenotype Ae/A2 circulates in a very small proportion of the sera that remains untraceable, either due to the sensitivity of the assay used or the predominating effect of other genotypes in the sera, similarly to the phenomenon previously explained in the case of HCV (32).

Recently, molecular evolutionary analyses of the hypervariable regions of the envelope sequences in different viruses clearly have demonstrated the independent evolution, selection, and emergence of phylogenetically related, yet distinct, viral variants in different anatomical compartments of the same individuals (33, 44, 45, 50, 58). Moreover, the compartment-specific predominance of particular HCV genotypes in the leukocytes while showing different genotype in the serum, even across individuals, also has been reported recently (15). Additionally, compartment-specific signature substitutions (such as a drug resistance substitution at position 308 of HIV gp160 in the cerebrospinal fluid-derived sequence) across individuals also have been well documented (39). Taking these results together, the detection of HBV subgenotype Ae/A2 with the immune escape substitution G145R in the PBL across individuals in this study supports earlier observations suggesting that similarities in the major histocompatibility complex-restricted host immune pressure across individuals of a specific population leads to the emergence of similar viral variants across individuals (39, 41).

It has been demonstrated that a strong antiviral humoral immune response against HBsAg expedites the emergence of G145R mutants (48). The high prevalence of G145R in the PBL in this study suggests that PBL-associated HBV DNA are under strong anti-HBs-specific humoral pressures. G145R is the most widely studied HBV immune escape mutation that emerges naturally or in response to vaccination or anti-HBs immunoglobulin G therapy (49) and can decrease the ability of neutralizing anti-HBs antibodies (8, 59). Interestingly, G145R does not cause any detrimental effect on virus morphogenesis and the stability of viral mRNAs, DNA, and antigens (21, 22). Moreover, HBV variants with G145R are infectious from cell to cell within a host and also are transmissible between hosts (9, 56). Thus, the selection of G145R seems to be an efficient strategy of the PBL-associated viruses to escape the anti-HBs humoral response without compromising its critical properties. The emergence, selection, persistence, and transmission of the acquired advantageous phenotype (G145R in this case) signifies the Lamarckian mechanism of the evolution of advantageous viral variants consistently following Darwinian principles and functioning at the molecular level, supporting the viral replicative homeostasis hypothesis of Sallie (46).

We have detected the compartmentalization of HBV subgenotype Ae/A2 in the PBL. This strain is well known to circulate in the sera/hepatocytes of European populations, but it has not been reported previously in Indian populations (34, 47). Thus, we could not identify a completely new genotype of HBV in the PBL, which is phylogenetically distinct from previously characterized HBV genotypes/subgenotypes (34, 47), supporting a recent observation that there exist no distinct lymphotropic variants of WHV that cannot infect the hepatocytes (31). This observation suggests that among the different HBV genotypes/subgenotypes, a particular one can persist in the sera/hepatocytes of one population, while the same may persist in the lymphatic cells in another population, indicating the significance of population-specific host immune factors in HBV compartmentalization. Recently, Mulrooney-Cousins and Michalak (31) repetitively passaged wild-type WHV in lymphocyte cultures 13 times but could not distinguish any specific lymphotropic variant, aside from a few random mutations. Interestingly, the random mutations accumulated during 13 repeated passages in lymphocyte cultures reverted to wild-type sequences in just a single passage of the virus in the animals. These observations clearly highlight the role of host immune pressure and signify that the selection and evolution of viral variants are more relevant in the context of immune pressure operating in vivo (28, 43) instead of in vitro cell cultures (18, 54).

HBV genotypes/subgenotypes are genetically related stable viral variants, having evolved under specific immune selection pressures in distinct geoethnic populations (47), and the genetic diversity of HBV in the sera of different geoethnic populations worldwide has been studied (34, 47). Recent studies
also have shown the differential clinical/virological significance of different HBV genotypes (13, 34, 47). The present study offers further evidence in favor of the differential clinical implications of HBV genetic variability in terms of the evolution of immune escape variants and compartmentalization. However, taking into account the key role of host-specific immune factors in evolution and the selection of HBV genotypes (47, 59), it is quite rational to anticipate different HBV genotypes with different immune escape variants in the PBL of different geoethnic populations. It is thus of great clinical and scientific substance to investigate the genetic diversity of HBV in the PBL and other extrahepatic sites, in different populations, to enlighten the more fascinating facets of HBV epidemiology, pathogenesis, and evolution.

Using the WHV model of hepadnaviral infection, Michalak and colleagues have established that the lymphatic system acts as an important reservoir of transmission-competent occult hepadnaviral infection that invariably infects the lymphatic system through parenteral or vertical routes but rarely infects liver (29, 30). In addition, we previously have shown that PBL-confined HBV infection also is capable of being transmitted exclusively to the PBL through nonparenteral, horizontal intrafamilial routes (9). Collectively, these observations provide a possible explanation for the compartment-specific dissemination and maintenance of PBL-specific HBV variants while it is absent from the sera of our study population. These observations signify the importance of leukocytes and encourage further investigation into other anatomic compartments to correlate the observed differences in the transmission modes among different populations where different HBV genotypes prevail (34, 47).

Recently, HBV infection has been found to be significantly associated with patients with non-Hodgkin’s lymphoma, and the oncogenic and lymphotropic properties of HBV have been suggested to contribute to the disease (35, 55). These observations indicate that the persistence of replicating virus has important implications in the induction of disorders that earlier were not considered to be related to hepadnaviral infection, and thus the further investigation of the pathogenic aspects of the extrahepatic persistence of HBV is extremely important.

In conclusion, the complete recognition of HBV strains that infect an individual is important to illuminate the essentials of HBV infection, including virus transmission, compartmentalization, persistence, and the development of extrahepatic manifestations, all of which still are poorly understood. To date, most of the data published on the genetic characterization of HBV were based on virus circulating in the sera or in the hepatocytes. Moreover, the studies of vaccine efficacy or antivirals have been performed mostly on isolates from sera and hepatocytes, but the present study clearly shows that the evolution of HBV in extrahepatic sites is important in the emergence of immune/vaccine escape variants and thus is clinically important. This is the first study of this kind to analyze the genetic diversity and evolution of HBV in the peripheral blood leukocytes, which opens an important field of investigation for extrahepatic HBV divergence. The findings of this study have extremely important implications in the field of transfusion medicine, transplantation, response to therapy, the emergence of vaccine escape mutants, and HBV transmission that are imperative for efficiently managing and designing strategies to reduce the burden of HBV infections.

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REFERENCES

synonymous mutation rates between sequences containing ambiguous nu-
cleotides (Syn-Scan). Bioinformatics 18:866–887.
turnover assay for human immunodeficiency virus type 1 replication selec-
tool and editor program for Windows 95/98/NT. Nucleic Acids Symp.
Meeting Ser. 41:95–98.
virus genotype A subtype assay that distinguishes subtype Aa from Ae and
immunodeficiency virus type 1 (HIV-1) env compartmentalization in the
culture of human peripheral blood mononuclear cells do not originate
from replicating virus. Hepatology 34:405–413.
assessing viral sequence relatedness. AIDS. Res. Hum. Retrovir. 8:1549–
1560.
molecular evolutionary genetics analysis and sequence alignment. Brief.
Bioinform. 5:150–163.
variants, and geographic origin of hepatitis B virus—large-scale analysis
hepadnavirus induce infection of the lymphatic system that does not engage
the liver. J. Virol. 78:1730–1738.
wild-type woodchuck hepatitis virus in lymphoid cells does not generate cell
type-specific variants or alter virus infectivity. J. Virol. 82:7540–7550.
terization of long-term cultures of hepatitis C virus. J. Virol. 70:3325–
3329.
Genetic diversity and tissue compartmentalization of the hepatitis C virus
infection in blood mononuclear cells, liver, and serum from chronic hepatitis
Mushahwar, B. H. Robertson, S. Locarnini, and L. G. Magnusi. 2004. Ge-
genetic diversity of hepatitis B virus strains derived worldwide: genotypes,
genotype subtypes, and HBsAg subtypes. Intervirology 47:289–309.
33. Park, S. C., S. H. Jeong, J. Kim, C. J. Han, Y. C. Kim, S. K. Choi, J. H. Cho,
Lee. 2008. High prevalence of hepatitis B virus infection in patients with
34. Penna, A., M. Artini, A. Cavalli, M. Levero, A. Bertoloetti, M. Pili, F. V.
Chisari, B. Rehermann, G. Del Prete, F. Fiaccadori, and C. Ferrari. 1996.
Long-lasting memory T cell responses following self-limited acute hepatitis
of hepatitis C virus infection. World J. Gastroenterol. 14:2789–2793.
Weiser. 2005. Human immunodeficiency virus type 1 genomic RNA se-
quenences in the female genital tract and blood: compartmentalization and
37. Pillai, K. S., S. L. K. Pond, Y. Liu, B. M. Good, M. C. Strain, R. J. Ellis, S.
McCutchan, D. D. Richman, and J. K. Wong. 2006. Genetic attributes of
of small ruminant lentinivirus between blood and colostrum in infected goats.
and A. J. Leigh Brown. 2006. Adaptation to different human populations by
and S. D. W. Frost. 2007. Adaptation to human populations is revealed by
Increased human immunodeficiency virus type 1 (HIV-1) env compartmental-
ization in the presence of HIV-1-associated dementia. J. Virol. 79:10830–
10834.
43. Roque-Alonso, A. M., D. Ducournouiller, G. Di Liberto, R. Kara, M. Gigou,
atitis C virus genotypes between serum and peripheral blood mononuclear
44. Sallie, R. 2005. Replicative homeostasis: a fundamental mechanism mediat-
45. Sallie, R. 2005. Replicative homeostasis: a fundamental mechanism mediat-
46. Sheldon, J., and Z. Guan. 2002. Compartmentalization of hep-
Profile, spectrum and significance of HBV genotypes in chronic liver disease
replication competence of hepatitis B virus DNA in peripheral blood mono-
nuclear cells from chronic hepatitis B patients and patients who have recov-
49. Voronin, Y., B. Chohan, M. Emerman, and J. Overbaugh. 2007. Primary
isolates of human immunodeficiency virus type 1 are usually dominated by
the major variants found in blood. J. Virol. 81:10232–10241.
50. Wang, F., R. Xu, B. Han, Y. Shi, H. Luo, W. Jiang, T. Lin, H. Huang, Z. Xia,
and Z. Guan. 2007. High incidence of hepatitis B virus infection in B-cell
subtypes non-Hodgkin lymphoma compared with other cancers. Cancer 109:
1360–1364.
51. Waters, J. A., M. Kennedy, P. Voelt, P. Hauser, J. Petre, W. Carman, and
52. Wieland, S. F., and F. V. Chisari. 2005. Stealth and cunning: hepatitis B and