

Comparison of hepatitis B virus core promoter sequences in peripheral blood mononuclear cells and serum from patients with hepatitis B

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It has been reported that hepatitis B virus (HBV) DNA is present in peripheral blood mononuclear cells (PBMCs), although it is unclear whether it actually replicates there or is adsorbed from serum. HBV DNA sequences from the core promoter and precore regions were amplified from PBMCs and serum taken from 13 patients with hepatitis B infection. Analysis by single strand conformation polymorphism, direct sequencing and cloning revealed identical HBV DNA sequences in both PBMCs and serum from five patients with acute hepatitis and in five out of eight patients with chronic hepatitis. However, in the remaining three chronic hepatitis cases, HBV DNA sequences in both PBMCs and serum were different: two patients harboured HBV DNA sequences from their PBMCs with deletions/insertions in the core promoter region and one patient harboured HBV DNA sequences from their PBMCs with two nucleotide substitutions. These findings point to a possible presence of independent HBV DNA replication in PBMCs.

It has been reported that hepatitis B virus (HBV) replicates at extrahepatic sites. Several groups have found viral DNA and RNA, as well as replicative intermediates, in peripheral blood mononuclear cells (PBMCs), kidney, pancreas, lymphatic nodes and skin (Pontisso *et al.*, 1984; Dejean *et al.*, 1984; Féray *et al.*, 1990). These extrahepatic sites were postulated to be the source of reinfection after liver transplantation or interferon treatment for chronic hepatitis B (Féray *et al.*, 1990). However, in contrast to avian hepadnaviruses, it is unclear whether

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complete HBV virions arise in tissues other than the liver as this infection does not seem to entail any injury to the tissues involved. In a recent study Köck *et al.* (1996) were unable to detect covalently closed circular (ccc) DNA, an early replicative form of HBV, in PBMCs and concluded that all previous observations could be explained by adsorption of the virus on these cells.

To help resolve these controversies, we compared HBV DNA sequences amplified from PBMCs with those amplified from the serum assuming that in the presence of passive adsorption they should be identical. For comparison we chose the core promoter region, which we found in our previous studies to be relatively variable (Laskus *et al.*, 1994).

Thirteen HBV infected patients who attended the Municipal Hospital for Infectious Diseases in Warsaw between June and August 1995 for their clinical care were studied: five had acute self-limited hepatitis and eight had biopsy-verified chronic hepatitis. None had received any antiviral therapy prior to the study. The likely source of infection was occupational exposure in two cases and hospitalization in five (two during surgery). In the remaining six patients the mode of infection was unclear. Informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 declaration of Helsinki. Subjects with acute hepatitis had a recent onset of clinical illness and were HBsAg and IgM anti-HBcAg positive. Chronic hepatitis patients had been HBsAg positive for at least 1 year and had liver biopsies compatible with chronic active hepatitis (with incipient cirrhosis occurring in three cases). None of the patients had serological evidence of hepatitis D virus, hepatitis C virus or human immunodeficiency type 1 virus (HIV) infection and none had a history of drug or alcohol abuse.

PBMCs were isolated by Ficoll-Hypaque (Pharmacia) density-gradient centrifugation, washed three times with PBS (pH 7.4), and stored frozen until use. DNA was extracted from 10^6 – 10^7 cells or 100 μ l serum with the Isoquick nucleic extraction kit (Microprobe) according to manufacturer's instructions and finally dissolved in 20 μ l of water. Ten μ l of



Fig. 1. Analysis by SSCP of HBV DNA sequences isolated and amplified from PBMCs and serum from ten patients with hepatitis B. Lanes A and B represent HBV DNA from PBMCs and serum, respectively. Patients 1–7 show identical band patterns in PBMCs and serum, patients 8–10 show different band patterns. The presence of identical and distinct HBV DNA sequences was verified by direct sequencing and/or sequencing after cloning.

this DNA solution was added to a 50 μ l PCR reaction and amplified with primers 5' GACCTTGAGGCATATTTCAAAGAC 3' (nt 1692–1715) and 5' GTAACCTCCACAGAA-GCTCCAAATTC 3' (nt 1946–1922) as described previously. Appropriate measures were used to prevent crossover contamination (Laskus *et al.*, 1994).

Amplified core promoter and precore regions (nt 1742–1900) were sequenced directly in both directions by the Sanger dideoxynucleotide chain termination method with a modified T7 DNA polymerase (Sequenase 2.0, USB). To reduce the probability of erroneous sequence determination due to Taq polymerase incorporation errors, whenever an HBV DNA sequence amplified from PBMCs differed from that amplified from serum, sequencing was repeated from a new PCR product. When deemed necessary the PCR products were cloned using a TA cloning kit (Invitrogen) and subsequently sequenced as described previously (Laskus *et al.*, 1994).

For single strand conformation polymorphism (SSCP) analysis 0.1 μ g of purified PCR product was subjected to non-denaturing PAGE in 1 \times Tris–borate–EDTA buffer and the bands were visualized with silver staining as described previously (Laskus *et al.*, 1996). By this technique we were routinely able to detect a minor variant admixture representing about 3% of the PCR product. Whenever the band pattern differed between PBMCs and serum derived HBV DNA sequences, the analysis was repeated with a new PCR product.

HBV DNA sequences were successfully amplified from all 13 serum–PBMC pairs. SSCP analysis of PCR products revealed the presence of indistinguishable band patterns from PBMCs and serum in ten patients, compatible with the presence of identical HBV DNA sequences, whereas in the remaining three patients (all of whom had chronic hepatitis) the band patterns from PBMCs and serum differed. These differences were similar in two independent experiments. Since spurious discrepancies between PBMCs and serum HBV DNA sequences might be expected if a low number of target copies were amplified, we quantified the amount of viral template in PBMCs and serum from these three patients. Tenfold serial dilutions of DNA extracted from these samples were amplified and compared with known amounts of HBV DNA viral target (plasmid with cloned full-length HBV DNA sequence). We found that the number of viral templates added to the PCR was 10^5 for PBMCs and 10^5 – 10^6 for serum.

By direct sequencing of PCR products we verified that the sequences in serum were indeed identical to sequences in PBMCs in all ten pairs with indistinguishable SSCP band patterns (not shown).

Of the three patients who had different SSCP band patterns one (patient number nine) had 2 nt substitutions (A \rightarrow T substitution at nt 1762 and G \rightarrow A transition at nt 1764) in HBV DNA sequence amplified from PBMCs when compared with sequence amplified from serum.

In patients number eight and ten, multiple bands on SSCP analysis suggested infection of PBMCs with a mixture of various HBV strains (Fig. 1); not unexpectedly, direct sequencing revealed unreadable, overlapping sequencing ladders. However, direct sequencing of serum HBV DNA was feasible in these patients. In both patients HBV DNA sequences amplified from PBMCs and serum were cloned and subsequently analysed by sequencing.

In patient number eight, 12 clones of HBV DNA from PBMCs and ten clones from serum HBV DNA were studied. All serum clones were identical to each other and to the 'Master sequence' determined by direct sequencing. However, all PBMCs clones were heterogeneous and different from serum sequence: seven were identical to wild-type strain sequence, two had an 8 nt deletion, another two had a 3 nt insertion (TTG) in the same region as the deletion and one had a 2 nt substitution (Fig. 2).

Analysis of serum HBV DNA sequence in patient number ten revealed that while 11 clones were identical or near identical to the wild-type sequence, two had an 18 nt deletion located just upstream of DR1 (Fig. 3). Of 12 clones from PBMCs eight were identical to the 'master' sequence from serum while the remaining four had an 8 nt deletion identical to that identified in patient number eight (Fig. 3).

A stop codon mutation at nt 1896 was not present in any of the HBV DNA sequences analysed, and all except three patients were infected with virus strains having a 'CCC' sequence at codon 15 in the precore ORF (typical for genotype A). Such strains are likely to be resistant to development of 'UAG' mutations at codon 28 due to disruption of G–C base-pairing in the stem–loop structure of the *cis*-encapsidation signal (Li *et al.*, 1993).

The likely mechanism behind the observed deletions/insertions is the action of DNA topoisomerase type I (topo I),

deletions/insertions further upstream suggesting that either the virus–virus religation is rare in this region or such variants are eliminated from replication. In our previous studies we identified ten patients harbouring HBV DNA sequences in their serum with deletions/insertions in the core promoter region but such changes were never located close to DR1 (Laskus *et al.*, 1994).

Other causes of the observed rearrangements in the core promoter region, such as the action of topo II (Han *et al.*, 1993), slipped mispairing between the template and progeny strand, or linear replication of HBV (Staprans *et al.*, 1991), are less likely, although they too would be more compatible with an active replication than with passive adsorption. Similarly, the presence in one patient of HBV strains with 2 nt substitutions in the PBMcs but not in the serum is compatible with independent virus replication at these extrahepatic sites. However, active DNA replication does not have to entail full virion production as this may require liver cell-specific factors (Ganem & Varmus, 1987). HBV DNA in PBMcs could provide a likely repository of the original infecting strain as illustrated in patient number eight in whom the 'wild-type' virus was present in the PBMcs but not in the serum.

Why topo I would affect HBV DNA sequences in PBMcs in the first place is unclear. The enzyme may reach high activities in human lymphocytes (Hwong *et al.*, 1993), and its action is commonly associated with DNA replication fork and active transcription (Wang, 1985; Liu & Wang, 1987). HBV replicates through cccDNA which is the template for pre-genomic transcript, the pool of which is constantly replenished through the action of reverse transcriptase (Tuttleman *et al.*, 1986). So far there is no evidence for semiconservative virus DNA replication; however, in nonpermissive cells virus replication could be different from that described for liver cells. An alternative explanation is that topo I might be recruited by reverse transcriptase complex as suggested for HIV and some other retroviruses (Takahashi *et al.*, 1995).

There remains a possibility that the amplified HBV DNA sequences with rearrangements in the core promoter region are derived from integrated viral sequences. However, there is no evidence, so far, that the virus actually integrates at this extrahepatic location and the integration event itself would be more likely in case of concomitant or antecedent active replication.

In summary, we identified differences between PBMcs and serum derived HBV sequences in some patients with chronic HBV infection. These discrepancies, most of which are likely due to the intracellular action of Topo I, are compatible with the presence of virus replication in PBMcs.

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