Clinical Studies

Virological profiles in hepatitis B virus inactive carriers: monthly evaluation in 1-year follow-up study


Abstract: Study subject: We longitudinally evaluated the virological behaviour and the hepatitis B virus (HBV) genomic variability in inactive HBV surface antigen (HBsAg) chronic carriers. Patients and methods: Fourteen HBsAg-positive healthy workers (13 inactive carriers and 1 with active HBV infection) were followed up for 12 months by monthly evaluation of aminotransferase, HBV DNA, and IgM anti HBV core antigen (IgM anti-HBc) values. Moreover, HBV serum isolates from each case were amplified, cloned and sequenced to evaluate the presence of the potentially clinical relevant core-promoter and precore mutations. The same technical procedures were used to examine the S gene of isolates from 3 randomly selected inactive carriers and the patients with active HBV infections. Results: Aminotransferase values were constantly normal in all cases. Viremia levels appear to fluctuate widely over time in each individual case, although the HBV DNA remained below 2 × 10^4 copies/ml in all samples. Only four serum samples from two inactive carriers had IgM anti-HBc values higher than the specific cut-off limit of the assay. Either wild type or core-promoter/precore HBV variants or a mixture of them were detected in the inactive carriers. S gene nucleotide homology among the clones from the three inactive carriers and the subject with active infection was 98.9%, 98.3%, 98.1% and 98.2%, respectively. Conclusions: The degree of suppression of HBV replication in inactive carriers is variable over time, and the entity and quality of HBV variability is comparable between active and inactive carriers.

Key words: core-promoter mutants – HBV replication – HBV variability – IgM anti-HBc – inactive HBsAg carriage – precore mutants

Hepatitis B virus (HBV) infection is a major health problem world wide, being one of the 10 leading causes of mortality and with an estimated 400 million people chronically infected at the turn of this Millennium (1, 2). Chronic HBV infection may be associated with a large spectrum of clinical forms, ranging from very mild and asymptomatic clinical pictures to the most severe liver diseases including cirrhosis and hepatocellular carcinoma (1, 3). The state of inactive HBV carrier is one of the most intriguing and common conditions that can be observed in chronic HBV surface antigen (HBsAg)-positive individuals. Inactive HBsAg carriers are invariably positive for antibody to HBV ‘e’ antigen (anti-HBe), and show the constant absence of clinical, histological, biochemical, and ultrasonographic signs of liver damage (reviewed in (4–6)). These individuals appear to have a favourable clinical outcome in the vast majority of the cases (7–10), and in fact were previously defined as ‘healthy’ HBsAg carriers (4, 5), so that performing a liver biopsy in such subjects is generally considered clinically useless and ethically debatable (7, 11). Nevertheless, several virological aspects of the inactive HBsAg carrier state are still unclear. First, we know that this status is always characterized by the suppression of the HBV replication and, consequently, by low levels of circulating viral genomes (4–6). However, there is no cut-off limit of serum HBV DNA level that

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makes it possible to surely distinguish between true inactive HBsAg/anti-HBe carriers and HBsAg/anti-HBe chronic hepatitis patients with only transient remission of the HBV replication (12, 13). In this context, both the two recent North-American and European consensus conferences on HBV infection have arbitrarily indicated a serum HBV DNA level of $10^5$ copies/ml to differentiate chronic hepatitis B patients from inactive carriers (5, 6). In contrast, a Greek team with extensive experience in the HBV field recently showed that the above reported cut-off value is too high and may lead to erroneously considering up to 13% of HBsAg/anti-HBe chronic hepatitis patients as inactive carriers. Consequently, they suggested classifying HBsAg positive individuals with serum HBV DNA levels below $3 \times 10^4$ copies/ml as inactive carriers (14).

A further aspect that remains undefined in the inactive HBsAg carrier status is whether the degree of suppression of the viral replication is constant or variable over time. In this regard, it appears relevant that reactivation of viral replication and liver disease may occur in individuals with inactive infection, particularly under immunosuppressive conditions (15–17), and this event is likely supported by the persistence of intrahepatic episomal viral DNA which in these subjects is quantitatively similar to that observed in patients with active infection, as we recently observed (18). Finally, another point insufficiently studied to date concerns the genomic heterogeneity of the viruses infecting inactive HBsAg carriers, and this aspect is worthy of elucidation considering the importance that the viral variability seems to have in the outcome of HBV-related liver diseases (reviewed in (19)).

We report here the results of a longitudinal study evaluating the serum HBV DNA levels and virus variability of isolates from inactive HBsAg health workers of our University Hospital at narrow time point controls.

At the time of the tests, 19 of the 27 HBsAg carriers had normal aminotransferase levels, while the remaining eight showed alanine aminotransferase (ALT) levels ranging from two to four times the normal values.

Thirteen of the 19 individuals showing normal ALT values (nine males and four females, mean age 53.6 years, range 40–62 years) had previously been diagnosed as chronic inactive HBV carriers. In fact, they were known to be HBsAg positive for at least 5 years (median 10 years, range 5–20 years), none had suffered from acute hepatitis, none had a history of any clinical, biochemical or ultrasonographic abnormalities that could imply liver dysfunction or serum HBV DNA higher than $2 \times 10^4$ copies/ml. Moreover, besides HBV infection, none had any viral, metabolic or toxic factor potentially able to induce liver damage. In particular, all of them were negative for markers of hepatitis C, hepatitis D, and immunodeficiency virus infections. All these subjects agreed to participate in this study. Moreover, one of the HBsAg-positive health workers (a 42-year-old woman) with altered liver biochemistry agreed to be included in the study as a control. For ethical reasons, we did not perform a liver biopsy in any of the inactive carriers, whereas the subject with features of liver disease refused to undergo the needle biopsy.

All participants were prospectively followed up on a monthly basis for 12 months (from April 2002 to March 2003) with determination of ALT values and collection of 2 ml of serum that was stored at $-80 \, ^{\circ}\text{C}$ for subsequent virological tests. Moreover, all these subjects underwent an ultrasound liver scan both at the time of the initial evaluation and at the end of the study.

The study was performed according to the principles of Declaration of Helsinki and written informed consent was obtained in every case.

Virological tests

At the end of the study, 12 frozen serum samples from each of the 14 cases were available. HBV DNA was quantified in all of them by the Amplicore HBV Monitor™ kit (Roche Diagnostics, Basel, Switzerland) that has a limit of detection of 1000 copies/ml. Moreover, each sample was tested by the IM × CORE-M kit (Abbott Laboratories, North Chicago, IL) that allows the semi-quantitative analysis of the IgM anti-HBV core antigen (IgM anti-HBc).

Real-time polymerase chain reaction (PCR) assay

To confirm the course of the viremia levels observed in the inactive carriers, real-time PCR

Patients and methods

Patients

From January 1999 to December 2000, 3880 professional health workers at the University Polyclinic of Messina were tested for HBV and hepatitis C virus (HCV) serum markers. Twenty-seven of these subjects (0.7%) were found to be positive for HBsAg, 36 (0.9%) were positive for the antibody to HCV (anti-HCV) and five (0.1%) were HBsAg/anti-HCV positive. Moreover, 275 (7.1%) individuals had serum markers of previous HBV infection (antibody to HBV core antigen ± antibody to HBsAg).
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Assay for quantification of serum HBV DNA was performed in two of them (cases 4 and 11) that appeared to have quite pronounced viremia fluctuations by the Amplicore kit. For this purpose, six serum samples from each case were tested by the use of the GeneAmp 5700 Sequence Detection System (Applera, Foster City, California). The 50 μl reaction mix contained 10 μl of DNA extracted from serum, 1× TaqMan Universal PCR Master Mix (Applera), 900 nM of forward primer HBSF1 and of reverse primer HBSR1 (Applera), 200 nM of TaqMan HBV probe (Applera). The PCR reaction was carried out as follows: 50 °C, 2 min, for one cycle; 95 °C, 10 min, for one cycle; 95 °C, 15 s, 60 °C, 1 min, for 45 cycles. Serial dilutions of plasmids containing the entire HBV genome of ayw subtype (Clonit, Milan, Italy) were used for validation of the real-time PCR assay and as external standards for quantitation. Each sample was tested in duplicate, and serial dilution of external standard DNA ranging from 10⁷ to 10⁰ copies were included in triplicate in each run. The HBV DNA content in each sample was derived by the interpolation of the mean C_t value with the external standard curve using the GeneAmp 5700 software, version 1.3.

Amplification, cloning, and sequencing of HBV genomic regions

To investigate the possible presence of variant strains commonly considered of potential clinical relevance, the core promoter and the precore genomic regions of the HBV isolates from serum samples collected at baseline from the 14 cases were amplified by nested PCR technique using the oligonucleotide primers HBV1–HBV2 (first PCR round) and HBV3–HBV4 (nested PCR). Analogously, to investigate the intra-individual HBV genomic variability, the entire pre-S–S gene of HBV isolates both from the first and the last serum samples of the series collected from three randomly selected inactive carriers (cases 1, 3, and 4) and from the patient with active HBV infection were amplified by nested PCR and the use of the primers HBV5–HBV6 (first PCR round) and HBV7–HBV8 (nested PCR). DNA extraction and amplification procedures were performed as previously described (20). These experiments allowed us to obtain amounts of PCR products adequate for the subsequent experiments in all the cases.

All the products of amplification were cloned using the TOPO TA cloning kit (Invitrogen, Paisley, UK) according to the manufacturer’s protocol. Seven different clones for each sample were sequenced using the M13 forward and reverse oligonucleotide primers (TOPO TA cloning kit) and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applera) according to the manufacturer’s instructions. In addition, the HBV-specific HBV9 and HBV10 primers were used for sequencing the more internal portion of the pre-S–S genes. The sequencing products were resolved in an automatic DNA sequencer (ABI PRISM 310 Genetic Analyser; Applera). The HBV sequences obtained were aligned and compared with those of the National Centre for Biotechnology Information data bank.

All the primers used were complementary to conserved regions of HBV genotype D at the following positions (from 5’ to 3’): HBV1: 1266–1286; HBV2: 2385–2366; HBV3: 1428–1477; HBV4: 2066–2048; HBV5: 2414–2431; HBV6: 1004–987; HBV7: 2815–2834; HBV8: 860–840; HBV9: 56–75; HBV10: 458–475.

Results

ALT, serum HBV DNA and IgM anti-HBc values detected at each time point for every individual included in the study are reported in Fig. 1. The abdominal ultrasonographic patterns – including liver, portal vein and spleen features – was normal before and at the end of the study both in all the inactive carriers and in the subject with active infection.

ALT values

ALT values were below the normal limit in all the samples of all the inactive HBsAg carriers. On the contrary, the patient with active infection had abnormal ALT values at nine time points, while those values were below the normal limit at four controls, independently of the corresponding levels of viremia and IgM anti-HBc.

HBV DNA quantification

Serum HBV DNA levels in inactive HBsAg carriers were below 2×10⁴ copies/ml in all tested samples. However, the mean amount of HBV DNA was different in each case compared with the others, and more interestingly quite varying values of virus DNA were detected in each individual case at the different time points of the study. In fact, comparing the highest and the lowest values of HBV DNA detected in each case during the 12 months of the study, and arbitrarily assigning a value of 100 copies/ml to samples with levels of HBV DNA below the detection limit of the kit (1000 copies/ml), we observed that in case 1 the highest value was eight times the lowest one (7920 vs 1020 copies/ml, median 2100 copies/ml); in case 2 that differ-
Fig. 1. Graphic representation of the ALT, IgM anti-HBc, and HBV DNA monthly levels detected in 13 inactive HBV carriers (cases 1–13) and one active HBV-infected patient (case 14). The dotted lines correspond – from the top to the bottom, respectively – to the limit of ALT normality (40 IU/l), the specificity cut off of the Abbott IgM anti-HBc assay used in the study (IMx index: 0.358), and the limit of detection of the Roche Amplicore HBV Monitor kit (1000 copies/ml). ALT, alanine aminotransferase; HBV, hepatitis B virus; IgM anti-HBc, IgM anti-HBV core antigen.
ence was five times (4320 vs 1040 copies/ml, median 2240 copies/ml); in case 3 it was 14 times (1440 vs 100 copies/ml, median 100 copies/ml); in case 4 it was 52 times (5280 vs 100 copies/ml, median 100 copies/ml); in case 5 it was 12 times (12,960 vs 1080 copies/ml, median 7920 copies/ml); in case 6 it was four times (4280 vs 1030 copies/ml, median 1380 copies/ml); in case 7 it was seven times (7240 vs 1060 copies/ml, median 3340 copies/ml); in case 8 it was 78 times (7800 vs 100 copies/ml, median 100 copies/ml); in case 9 it was 71 times (7120 vs 100 copies/ml, median 100 copies/ml); in case 10 it was four times (17,120 vs 4040 copies/ml, median 8060 copies/ml); in case 11 it was 72 times (7240 vs 100 copies/ml, median 3340 copies/ml); in case 12 it was 39 times (3920 vs 100 copies/ml, median 1325 copies/ml); in case 13 it was 19 times (5120 vs 100 copies/ml, median 1435 copies/ml). Thus, HBV viremia in inactive HBsAg carriers appears to fluctuate continuously over time, although it persistently remains within limits apparently not relevant in
terms of liver damage. To verify this fluctuation, we used the most sensitive real-time PCR technique to quantify the HBV DNA in six serially collected serum samples from two carriers (cases 4 and 11) (Fig. 2). The results obtained largely confirmed the fluctuation of HBV viremia over time.

Finally, the patient with active infection (case 14) had HBV DNA levels constantly and largely above \(10^5\) copies/ml, although she also showed a wide spectrum of viremia fluctuation at the various time points ranging from 532 200 to \(>40\) millions copies/ml.

IgM anti-HBc

A previous study using the same immunoassay that we utilized here for quantitative evaluation of IgM anti-HBc (IMx CORE-M) fixed the specificity cut off of the assay at 358 IMx index (21). All but four serum samples (three from case 8 and one from case 10) of our series of inactive HBV carriers had IgM anti-HBc values below that cut off. Curiously, case 8 was the one showing the widest viremia fluctuation profile with the most marked difference between the highest and the lowest HBV DNA level (78 times) during the 12 months of follow-up, whereas case 10 had the highest median of viremia (8060 copies/ml) and the highest value of HBV DNA that we detected in a single sample (17 120 copies/ml).

Case 14 showed IgM anti-HBc values above the specificity cut off in eight time points, and values slightly below that limit in four samples.

Patterns of HBV genomic heterogeneity

Firstly, we amplified, cloned and sequenced the core-promoter/precore genomic portion of HBV DNA in isolates from all the subjects included in the study with the clear aim of investigating the presence of HBV variants carrying the G1896A stop codon in the precore region (that enables the synthesis of the HBeAg), and/or the double A1762T-G1764A mutations in the core promoter region: both these variants have been presumed to be strictly associated with forms of progressive liver disease (19, 22–25). All the seven clones examined from cases 2, 6, and 7 had wild-type core-promoter/precore sequence, whereas all clones from cases 4, 5, 8–14 carried the three above-mentioned mutations. Moreover, a mixture of viral strains were detected in cases 1 and 3, each of whom showing five wild-type and two variant-type clones.

Subsequently, to investigate the intra-individual heterogeneity of HBV genomes, we amplified, cloned, and sequenced the pre-S/S gene of isolates obtained at both time points 1 and 12 from three randomly selected inactive carriers (cases 1, 3, and 4) and from case 14 that had active infection. In case 1, the mean homology among clones was 98.7% (range 97.8–99.6%) and 97.5% (range 96.8–99.4%) for isolates obtained at time points 1 and 12, respectively. When all the clones from both time points were compared with each other, a mean homology of 98.1% (range 96.5–99.6%) was observed. Case 3 showed a mean homology of 99.6% (range 99.4–99.9%) and 98.7% (range
97.9–100%) among clones from isolates of points 1 and 12, respectively, while the homology between all the clones from both time points was 99% (range 97.8–100%). In case 4, the mean homology among clones was 98.7% (range 98.1–99.7%) and 98.2% (range 97.2–99.7%) for isolates obtained at time points 1 and 12, respectively. When all the clones from both time points were compared with each other, a mean homology of 99.3% (range 97–99.7%) was found. Case 14 had a mean homology of 98.2% (range 97.1–98.5%) and 98.1% (range 97.5–98.8%) among clones from isolates of points 1 and 12, respectively, while the homology between all the clones from both time points was 98.2% (range 97.1–99.5%).

All the sequenced viral strains appeared to belong to the genotype D, as the vast majority of HBV isolates in Italy. Moreover, with the exception of the precore stop codon and core-promoter mutations, no genomic rearrangement apparently able to interfere with the virus activity was detected in any of the strains examined.

Discussion

Several prospective studies have been performed so far that evaluated the inactive HBV carrier state by means of long-term follow-up based on the examination of the patients at 6 or 12 months intervals (7–10). Here, we report the results of our prospective study designed to investigate the biochemical and virological behaviour of inactive HBsAg carriers by sequential and closed controls performed during a 1-year follow-up. Clear evidence emerged from this study that all our inactive carriers had serum HBV DNA levels invariably below 2 × 10⁴ copies/ml. This result seems to reinforce the hypothesis by Manesis et al. (14) who proposed 3 × 10⁴ instead of 10⁵ copies/ml as real cut off for distinguishing these carriers from chronic hepatitis patients. In our patients, the mean amount of HBV DNA was different in each case compared with the others, and more interestingly, quite varying values of viral DNA were detected in each individual case at the different time points of the study. Thus, HBV viremia in inactive HBsAg carriers appears to fluctuate continuously over time, although it persistently remains within limits apparently not relevant in terms of liver injury, as suggested by the ALT levels invariably within the normal values in all the serum samples examined. Considering that the serum HBV DNA level is commonly assumed to reflect the status of virus replication, these results indicate that HBV maintains its propensity to replicate also in inactive carriers. However, the host factors (immunologic?, epigenetic?, both?) responsible for its inhibition prevail, although their efficiency to block the viral activity appears to be variable over time. Consequently, the equilibrium between virus and host in inactive HBsAg carriers appears to be less stable than usually believed, and this might account for the prompt and efficient reactivation of the HBV replication – usually associated with a flare of hepatitis – that may occur either spontaneously or in the case of development of an immunosuppression condition. In this context, it appears of relevance that the patient with active infection included in the study showed differences in the HBV DNA levels at various time points even higher than 2 logs (an enormous difference in terms of number of virions!), confirming that all cases of chronic HBV infection may have patterns of HBV replication and/or secretion of viruses into the blood showing continuous variations over time.

We also tested all serum specimens for IgM anti-HBc which is considered an indirect marker of the active immune response against the HBV (26–28) and possibly correlates with the immunemediated liver injury produced by the virus (21, 29, 30). In our study only two cases tested occasionally positive for that antibody. Interestingly, these two subjects had shown the highest degree of viral activity in terms of serum HBV DNA values and fluctuation, confirming previous studies that proposed the IgM anti-HBc test as a surrogate marker of the HBV activity in chronic infection (21, 29, 31).

A further aspect that we analysed in the current study was the genomic heterogeneity of the viruses infecting the inactive HBsAg carriers. We found the precore and core-promoter mutations in the control-case with active infection but also in the majority (10/13) of the inactive carriers, while in the remaining three individuals we detected only wild-type strains. Since the core-mutant populations are usually absent in the first years of the chronic HBV infection (32), we might hypothesize that the host mechanisms responsible for the inhibition of the HBV activity had occurred during the early phases of the infection in the inactive HBsAg carriers with wild-type viruses, whereas in the other cases the HBV suppression likely occurred after a prolonged period of productive HBV replication, when the variants had already emerged and been selected. In any case, our results clearly confirm previous observations suggesting that infection by core-promoter and/or precore HBV variants is not invariably associated with the liver injury (33–35). Moreover, the analysis of the S-gene nucleotide sequences from HBV isolates demonstrated that...
there is a degree of 1–2% of intraindividual variability among viral strains infecting inactive carriers that is stable over time and quantitatively comparable with that observed in patients with active infection.

HBV is recognized as one of the most dangerous oncogenic agents (36, 37). What takes place in allowing the same virus to persist for decades in inactive carriers without producing any relevant liver damage is at present largely unknown. Our study has shown that, in this category of chronic HBsAg carriers, the HBV activity is inhibited but is not extinguished and that the virus is not genetically different from those detectable in cases with productive infection. The identification of the host mechanisms interplaying with the virus and maintaining its activity beyond the threshold where it becomes capable of inducing liver damage will be an important step for a better comprehension of the pathogenesis of the HBV-related liver disease, and hopefully to identify the best strategies to cure it.

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References


