RAPID COMMUNICATION

Hepatitis B Virus DNA in the Serum of Sardinian Blood Donors Negative for the Hepatitis B Surface Antigen

M.E. Lai, P. Farci, A. Figus, A. Balestrieri, M. Arnone, and G.N. Vyas

The high endemicity of hepatitis B virus (HBV) infection and liver disease in Sardinia led us to assess the occurrence of HBV DNA in 1,411 sera of two selected groups of hepatitis B surface antigen (HBsAg)-negative blood donors: 793 with abnormal serum alanine aminotransferase (ALT) and 618 with normal serum ALT values (determined during routine testing of their blood donation). HBV DNA sequences were detected by dot-blot hybridization in 68 of 793 subjects (9%) with abnormal ALT but only in three of 618 subjects (0.5%) with normal ALT. HBV-core antibody (anti-HBc) was detected in 338 of 793 subjects (43%) with normal ALT as well as in 125 of 618 subjects (20.2%).

NON-A, NON-B (NANB) HEPATITIS is a major clinical problem among patients who receive blood transfusions. A chronic asymptomatic carrier state exists in humans, especially in blood donors with abnormal serum alanine aminotransferase (ALT) levels, who are more likely to transmit NANB hepatitis to their recipients in comparison with the donors with normal serum ALT levels. More than one agent, including variants of hepatitis B virus (HBV), may cause this disease. Several investigators have also reported an association of posttransfusion hepatitis (PTH) with occurrence of HBV core antibodies (anti-HBc) in donors' blood. In the absence of serologic markers of NANB hepatitis, blood banks in the United States have introduced both ALT and anti-HBc as surrogate tests for screening blood before transfusion. However, it is not possible to use anti-HBc for screening donors' blood in areas where HBV infection is highly endemic, e.g., in Sardinia, only hepatitis B surface antigen (HBsAg) and ALT are used by blood banks for screening blood.

Several investigators have reported the presence of HBV DNA sequences in the liver and in the serum of HBsAg-negative patients by conventional radioimmunoassay (RIA), suggesting the possibility that some HBsAg-negative liver diseases may be related to an HBV infection. Most of such patients were originally reported from the Mediterranean basin, where HBV infection is endemic. Moreover, some of them lacked evidence of recent or past exposure to HBV, as shown by the absence of immunologic markers of HBV infection, suggesting that cryptic HBV carriers may exist. Although the prevalence of such carriers in different geographic areas is unknown due to a lack of molecular epidemiologic studies, it is likely to be high in those areas where HBV infection is endemic. To verify this concept, we surveyed Sardinian donor sera for the presence of HBV DNA sequences. A total of 1,411 specimens of serum from HBsAg-negative blood donors were selected on the basis of abnormal ALT levels among 793 and normal levels among 618 donations collected in the Blood Bank of Cagliari (South Sardinia).

MATERIALS AND METHODS

Human blood donors. Voluntary blood donors in Cagliari were enrolled in this study from March 1985 to November 1985. Blood donations were routinely screened for HBsAg by enzyme immunoassay (Abbott Laboratories, North Chicago) and for ALT by a kinetic method using the Astra analyzer (Beckman, Palo Alto, CA). Sera from 93 HBsAg-negative blood donors found to have elevated levels of serum ALT ranging from 48 to 346 IU/L and 618 HBsAg-negative donors found to have normal serum ALT were selected for investigation of molecular hybridization of HBV DNA; 918 donors were men and 493 (35%) were women; their mean age was 35.3 years, ranging from 18 to 63 years.

HBV DNA analysis. Serum HBV DNA was determined by the dot-blot hybridization technique using the following modification of the methods described by Berninger et al. Briefly, 50 μL of each serum was incubated for three hours at 37°C with 1 mg/mL Proteinase K (Boehringer Mannheim, Indianapolis) in 50 μL of lysis solution containing 0.2 mol/L NaCl, 4% sodium dodecyl sulfate, 0.1 mol/L Tris-HCl, 0.05 mol/L EDTA, pH 8.2. Nucleic acids were extracted with phenol/chloroform/isoamyl alcohol 25:24:1 (vol/vol) and denatured with 50 μL of 1 N NaOH for ten minutes at room temperature. Neutralization was performed using 50 μL of 3.0 mol/L NaCl and 0.1 mol/L HEPES buffer, pH 7. The total mixture was divided into two aliquots and filtered onto the nitrocellulose membrane under vacuum, using Schleicher & Schuell (Keene, NH) MiniFold apparatus.

To test the sensitivity of this method, graded dilutions of cloned and alkali-denatured HBV DNA, ranging from 50 pg to 0.1 pg, were filtered onto the nitrocellulose under the same conditions (Fig 1). The nitrocellulose filters were baked for two hours at 80°C under vacuum. The hybridization assay was carried out under conditions optimized for dot-blot hybridization.

From the University of Cagliari, Sardinia, Italy; the Blood Bank of Cagliari, Sardinia, Italy; and the Department of Laboratory Medicine, University of California, San Francisco.

Submitted June 14, 1988; accepted September 19, 1988.

This collaborative work was carried out principally in Cagliari and supported in part by Transfusion Medicine Program Project Grant No. PO1-HL 36589 from the National Heart, Lung, and Blood Institute.

Address reprint requests to G.N. Vyas, PhD, Transfusion Research Program, Department of Laboratory Medicine, University of California, San Francisco, CA 94143-0100.

The publication costs of this article were defrayed in part by charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc.

0006-4917/89/7301-0040$3.00/0

probe was labeled with alpha[32P] dCTP by the random primer previously described, using a probe of cloned HBV DNA purified from cloning vector pBR 322 by preparative gel electrophoresis. The probe was labeled with alpha[32P] dCTP by the random primer technique yielding a specific activity of 2 to 5 x 10^6 cpm/μg of HBV DNA. Each specimen was also hybridized in parallel with similarly labeled pBR 322 DNA containing no HBV DNA sequences. Subsequent to hybridization the nitrocellulose filters were washed, dried, and autoradiographed as previously described. A serum sample was considered HBV DNA positive when a strong or a weak but reproducible hybridization signal was detected (Fig 1).

All sera were tested for anti-HBc, and HBV DNA-containing sera were further analyzed for antibodies to HBsAg and the “e” antigen (anti-HBe) using the commercial radioimmunoassay kits (Abbott Laboratories, North Chicago).

RESULTS

The prevalence of HBsAg was 3% and abnormal ALT (>40 IU/L) was 4% among the 26,000 Sardinian voluntary blood donors fulfilling the clinical history and criteria for acceptance during 1985. Blood with either elevated ALT or reactive for HBsAg is not used for transfusion to patients. Among HBsAg-positive blood units, 10% have elevated ALT.

Detection of HBV DNA in dot-blot analysis, illustrated in Fig 1, was considered specific because none of the specimens showed hybridization with the pBR 322 DNA probe and the cloned HBV DNA probe showed a hybridization signal in a remarkably reproducible manner when retesting of each of the positive specimens was performed four to five times. Thus, the dot-blot assay is practical, specific, and reliable for HBV DNA hybridization analysis when a large number of serum specimens are assessed for molecular epidemiology of blood-borne viruses. As shown in Table 1, HBV DNA was detected in 68 of 793 donors (9%) with abnormal ALT compared with three of 618 (0.5%) with normal ALT levels (P < .01).

Among 1,411 HBsAg-negative donors overall, 71 specimens (5%) were HBV DNA positive. Since 49 of 71 specimens (69%) had no anti-HBc, anti-HBs, and anti-HBe, the presence of HBV DNA in a majority of sera was not associated with any immunologic marker of HBV infection.

The reported association of HBV DNA in 35% of the serum specimens reactive for HBsAg in a monoclonal antibody RIA warranted testing of both groups of HBV DNA-positive and -negative specimens under code. When the tests were performed in the laboratory of Dr Jack Wands, using monoclonal antibodies, HBsAg was detected in seven of 42 sera containing HBV DNA and also in five of 50 sera nonreactive for HBV DNA. Of the seven sera reactive for both monoclonal HBsAg and HBV DNA, five were anti-HBc positive, while anti-HBc was detected in four of five monoclonal HBsAg-positive but HBV DNA-negative sera.

Anti-HBc was detected in 463 of 1,411 HBsAg-negative donors (33%), which is consistent with high HBV exposure, since the prevalence of HBsAg reported in the general population of Sardinia is 6.5%. The prevalence of anti-HBc was 338 of 793 (43%) among the blood donors with abnormal ALT compared with 125 of 618 (20%) among the donors with normal ALT (P < .01) HBV DNA was detected in 21 of 338 (6%) sera with anti-HBc and abnormal ALT, in comparison with 47 of 455 (10%) anti-HBc–negative donors with abnormal ALT. Thus, the occurrence of HBV DNA is HBsAg-negative blood donors was simply correlated with elevated ALT, irrespective of their anti-HBc status.

DISCUSSION

Because HBV is highly endemic in Sardinia, this island may be an excellent location to search for HBV DNA in the serum of HBsAg-negative subjects. We have identified not only these viral sequences in 71 (5%) of the 1,411 HBsAg-negative blood donors, but also found that 68 (96%) of the 71 reactive specimens had elevated ALT. This remarkable correlation of HBV DNA and abnormal ALT is consistent with our hypothesis that a proportion of NANB PTH may be due to genetically similar but antigenically distinct HBV, termed “HBV-like.” The highest prevalence of HBV DNA was observed among the blood donors with elevated ALT levels. Since blood was elevated ALT is not transfused in Sardinia, we must have incidentally prevented transmission of HBV-like infection through transfusion. The presence of HBV DNA sequences in HBsAg-negative but anti-HBc positive subjects of Italian origin has been previously reported. In addition, HBV infectivity has been proven by

---

Table 1. Prevalence of HBV DNA and Anti-HBc Among HBsAg-Negative Donors With Normal vs Abnormal ALT Levels

<table>
<thead>
<tr>
<th></th>
<th>Normal ALT No. (%)</th>
<th>Elevated ALT No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sera tested</td>
<td>618</td>
<td>793</td>
</tr>
<tr>
<td>HBV DNA positive</td>
<td>3* (0.5)</td>
<td>68 (9)</td>
</tr>
<tr>
<td>HBV DNA negative</td>
<td>615 (99.5)</td>
<td>725 (91)</td>
</tr>
<tr>
<td>Anti-HBc</td>
<td>125 (20)</td>
<td>338 (43)</td>
</tr>
</tbody>
</table>

*While one had anti-HBc, two had no anti-HBc/anti-HBs/anti-HBe.
HBV DNA IN HBSAg-NEGATIVE BLOOD DONORS

inoculation of chimpanzees with serum containing anti-HBc and apparently lacking HBsAg.8

Replication of HBV-like agents without serologic expression of viral antigens and host immune response is suggested by the episomal HBV DNA found in the hepatocytes of patients with chronic liver disease.9 Because 96% of HBV DNA detection in this study was associated with ALT elevation and a majority of such sera had no immunologic markers of HBV infection, we suspect that a component of the NANB hepatitis reported before ALT screening may have been caused by HBV-like agents replicating in liver cells.5 We hypothesized that changes in the amino acids critically involved in expression of the viral antigens result from point mutations or nonlethal deletions in HBV genes.25,33,45 Such anomalous viral replication without expression of antigens may be revealed only by DNA hybridization conveniently performed with human serum. The possibility of HBV DNA amplification by polymerase chain reaction is currently under investigation by us to enhance our detection capability. It would be important to determine HBV DNA amplification in monoclonal HBSAg positive specimens nonreactive for HBV DNA. Analogous to the recent molecular characterization of a group of hepatitis viruses found in woodchucks,16 ground squirrels,7 and ducks,8 HBV-like agents from HBSAg-negative plasma must now be cloned molecularly. By sequencing the nucleotides of DNA cloned from such specimens, we can gain precise knowledge about the genetic basis of antigenic variations in HBV expression. Genetic variants of HBV must exist and be easily detectable in highly endemic areas, as exemplified by the recent report of “HBV2” from Dakar, Senegal.14

The presence of HBV DNA sequences in donors with normal ALT, although very uncommon, underscores the existence of a healthy HBV DNA carrier state that escapes current screening tests. We are planning to test chimpanzees for the infectivity of HBV DNA found in two donors with normal ALT and no serologic markers of HBV infection (see footnote to Table 1). The possibility that some posttransfusion hepatitis may be caused by HBV-like agents must be investigated systematically in Sardinia and other highly endemic areas of the world. Technologies for gene amplification and DNA hybridization lend molecular epidemiology a new set of tools for clinical investigation of PTH.

ACKNOWLEDGMENT

We thank Dr Jack Wands of Harvard Medical School for performing the unique monoclonal HBSAg RIA; Drs Philip Lane and George Garratty performed internal peer review of this manuscript before submission for publication.

REFERENCES

Hepatitis B virus DNA in the serum of Sardinian blood donors negative for the hepatitis B surface antigen

ME Lai, P Farci, A Figus, A Balestrieri, M Arnone and GN Vyas