Serum Hepatitis B Virus DNA Detects Cryptic Hepatitis B Virus Infections in Multitransfused Hemophilic Patients

By M.G. Rumi, M. Colombo, R. Romeo, G. Colucci, A. Gringeri, and P.M. Mannucci

The recognition of replicating hepatitis B virus (HBV) may be important to both define the cause of and know how to manage chronic liver disease in multitransfused hemophilic patients. Replicating HBV can be detected at the molecular level by methods for HBV-specific DNA (HBV-DNA), which are more sensitive than the immunologic methods for detecting hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg). Unselected hemophilic patients (260; 6% with HBsAg, 4% with isolated anti-hepatitis B core (anti-HBc), 52% with anti-HBs and anti-HBc, 26% with isolated anti-HBs, and 12% with no HBV marker) were investigated retrospectively with a dot spot hybridization technique that detects serum HBV-DNA down to 0.5 pg and by Southern blot analysis, which tests the specificity of the HBV-DNA reactions. Eighteen patients (7%; five with serum HBsAg and 13 HBsAg seronegative with antibodies to HBV) had serum HBV-DNA. Serum HBV-DNA was detected more frequently in HBsAg carriers than in seronegative patients (33% versus 6%, P < .01), and had no relationship to serum alanine aminotransferase. Serum HBV-DNA was more sensitive than the radioimmunoassay for HBeAg was for detecting replicating HBV (7% versus 1.1%, P < .01). These findings demonstrate that there is cryptic HBV infection in a number of hemophiliacs and that serum HBV-DNA may coexist with markers thought to reflect immunity against HBV.

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MATERIALS AND METHODS

Patients. We looked for HBV-DNA in serum samples from 260 unselected hemophilic patients attending the A. Bianchi Bonomi Hemophilia and Thrombosis Center of the University of Milan. They were all male, 2 to 71 years of age (mean, 28). Of the 260 patients, 209 had hemophilia A, 51 had hemophilia B. The clotting factor defect was severe (factor VIII or IX, less than 1%) in 176, moderate (between 1% and 5%) in 34, and mild (higher than 5%) in the remaining 50 patients. Table 1 shows other characteristics of these patients. Of 260 patients, 244 had been infused lifelong with clotting factor concentrates, as indicated by hepatitis B e antigen (HBeAg) in serum and hepatitis B core antigen (HBcAg) in the liver. This virologic profile, which, with few exceptions, is associated with HBV-dependent liver disease, may be time-limited by seroconversion to antibody to HBeAg (anti-HBe), which leads to suppression of HBV replication. For the other category, HBcAg carriers who have circulating anti-HBe, ie, a marker of suppressed HBV replication, the etiology of chronic liver disease is less certain. In these carriers, liver disease has been attributed to hepatotoxic factors other than HBV, such as the delta virus, the non-A, non-B viruses, drugs, autoimmunity, or alcohol. However, recent studies have clearly demonstrated that a subset of HBcAg carriers harbor HBV with circulating anti-HBe. In these, cryptic replication of HBV has been linked to preferential localization of HBeAg in the cytoplasm of infected hepatocytes and with the probability of progression of liver disease to cirrhosis. HBV-DNA sequences have also been found in the serum and liver of HBsAg-seronegative patients with chronic hepatitis by a molecular hybridization technique.

These and other findings suggest that the role of HBV in chronic hepatitis may be greater than previously thought. With the advent of antiviral treatment of chronic hepatitis B, detection of cryptic HBV infection may be important not only for definition of the etiology but also for the management of chronic liver disease in hemophiliacs. Therefore, we looked for HBV-DNA in the sera of 260 unselected hemophiliacs attending our clinic.
serum specimens were collected from patients during their regular annual clinical visits and kept frozen at -20°C until used. At the same time, liver function tests, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, alkaline phosphatase (AP), gamma-glutamyltransferase, and serum protein electrophoresis, were performed. HBV markers, including HBsAg, anti-HBs, and anti-HBc, were tested for by radioimmunoassay (RIA; Abbott Laboratories, North Chicago, IL). Anti-HDV was measured by enzyme-soaked in 20 x SSC, dried and baked in a vacuum oven at 80°C overnight. After staining with ethidium bromide, the gel was denaturated with 0.1% SDS, 0.1% dextran sulphate, and 10% formamide. Hybridization, carried out according to the instruction of the manufacturer. Electrophoresis (running buffer: 0.089 mol/L Tris, 0.089 mol/L boric acid, 0.002 mol/L EDTA) was carried out at 37°C overnight. After staining with ethidium bromide, the gel was exposed to Kodak X-Omat AR film for 12 to 20 hours at -80°C. The sensitivity of this technique was 0.5 pg, as assessed by the calibration curve that was constructed with known amounts of cloned HBV-DNA. Positive spots were graded from traces (+) to 4+. The samples graded as traces contained about 0.5 pg of HBV-DNA and were double retested. Grade 1+ corresponded to 1 pg; grade 2+, to 10 pg; grade 3+, to 50 pg; and grade 4+, to 100 pg of HBV-DNA (Fig 1a). Examples of HBV-DNA readings in patients' sera are shown in Fig 1b. The specificity of the HBV-DNA dot-blot was assessed by Southern blot hybridization. EcoRI-digested DNA samples (5 μg) were separated by electrophoresis on 0.8% and 1.5% agarose gel. Lambda HindIII DNA fragments were used as molecular weight markers. Digestion with restricted enzymes was carried out according to the instruction of the manufacturer. Electrophoresis (running buffer: 0.089 mol/L Tris, 0.089 mol/L boric acid, 0.002 mol/L EDTA) was carried out at 36 V, overnight. After staining with ethidium bromide, the gel was denaturated with 1 N NaOH for 40 minutes, neutralized with 1 mol/L Tris, pH 7.5, and processed for DNA transfer according to the EcoRI site in pBR325, was purified by preparative electrophoresis and radiolabeled by a random priming (Amersham Multiprime DNA labeling system, Little Chalfont, England) to yield a specific activity of 2 to 4 x 10^9 cpm/μg. After high stringency washes in 2 and 0.2 x SSC and 0.1% SDS at 68°C, nylon membranes were dried and exposed to Kodak X-Omat AR film for 12 to 20 hours at -80°C. The sensitivity of this technique was 0.5 pg, as assessed by the calibration curve that was constructed with known amounts of cloned HBV-DNA. Positive spots were graded from traces (+) to 4+. The samples graded as traces contained about 0.5 pg of HBV-DNA and were double retested. 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![Figure 1](http://www.bloodjournal.org)
Southern. The membrane was then dried, baked, hybridized, washed, and processed for autoradiography as previously described. To assess the molecular status of HBV-DNA in our samples, HBV-DNA isolated from circulating viral particles by phenol-chloroform extraction, and ethanol precipitation was used as control.

Statistical analysis. The prevalence of serum HBV-DNA in relation to other markers of HBV infection, severity of coagulation defect, intensity of previous treatments, ALT elevation, and presence of HIV infection were analyzed by Fisher's exact test. The prevalence of serum HBV-DNA in relation to patient age was analyzed by the Student's \( t \) test.

RESULTS

Eighteen patients (7%) tested repeatedly positive for serum HBV-DNA (Table 3). Five were HBsAg-positive (three with HBeAg, two with anti-HBe), six had both anti-HBs and anti-HBc, five had isolated anti-HBs (including two vaccinees), and two had isolated anti-HBc. HBV-DNA was detected in none of the 31 patients who did not have HBV markers. HBV-DNA was detected more frequently in HBsAg carriers than in HBsAg-negative patients with antibodies to HBV (5 of 15, 33%, versus 13 of 214, 6%, \( P < .01 \)). There was no relationship between serum HBV-DNA and ALT, with the prevalence of HBV-DNA in patients with elevated ALT similar to that of patients with normal ALT (15 of 170, 9%, versus 3 of 9, 33%, not statistically significant). HBV-DNA was more sensitive than the RIA for HBeAg for detecting patients with replicating HBV (18 of 260, 7%, versus 3 of 260, 1.1%, \( P < .01 \)). Serum levels of HBV-DNA were high (2+ to 4+) in three HBeAg-positive carriers, low (1+) in two anti-HBe-positive carriers, and traces (−) in 13 HBsAg seronegative patients with antibodies to HBV. The presence of free HBV genome was confirmed in all the HBV-DNA positive sera by Southern blot analysis (Fig 2). The prevalence of HBV-DNA was not influenced by patient age, severity of coagulation defect or intensity of previous treatments, nor by elevated levels of ALT or HIV infection (Table 3). Two patients with serum HBV-DNA as the only marker of HBV infection, who had been successfully vaccinated against hepatitis B when the results for HBV-DNA were not yet available, developed protective serum levels of anti-HBs 1 month after the fourth dose of the vaccine (greater than 120 mIU/mL). In one patient, HBV-DNA was detected during the course of vaccination. During 2 years of follow-up, this patient remained persistently negative for HBsAg and anti-HBc. The other patient was found to be HBV-DNA positive 2 years after the booster dose of vaccine, coincident with a period of heavy exposure to plasma concentrates.

DISCUSSION

Serum HBV-DNA is a sensitive and specific marker of HBV infection that is correlated quantitatively with the replicating activity of the hepatitis virus in the liver. Consistent with previous data for non-hemophilic patients, our study indicates that serum HBV-DNA is more sensitive than the RIA for HBeAg for detecting patients with replicating HBV (7% versus 1.1%, \( P < .01 \)). In fact, HBV-DNA was found not only in the three HBeAg-positive patients but also in two HBsAg-positive patients with serum anti-HBe, in two HBsAg-negative patients with anti-HBc, and in 11 with

<table>
<thead>
<tr>
<th>Serum Markers</th>
<th>No. Patients</th>
<th>Overall No. (%)</th>
<th>Normal ALT No. (%)</th>
<th>Elevated ALT No. (%)</th>
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<tr>
<td>HBsAg</td>
<td>15</td>
<td>5 (33)</td>
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<td>3/12 (25)</td>
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<td>Anti-HBs and anti-HBc</td>
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<td>6 (4)</td>
<td>1/4 (2)</td>
<td>5/94 (5)</td>
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<td>68</td>
<td>5 (7)</td>
<td>0/13 —</td>
<td>5/45 (11)</td>
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<tr>
<td>Anti-HBc</td>
<td>11</td>
<td>2 (18)</td>
<td>0/2 —</td>
<td>2/9 (22)</td>
</tr>
<tr>
<td>No HBV marker</td>
<td>31</td>
<td>0 —</td>
<td>0/21 —</td>
<td>0/10 —</td>
</tr>
<tr>
<td>Total</td>
<td>260</td>
<td>18 (7)</td>
<td>3/90 (3)</td>
<td>15/170 (9)</td>
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<td>85</td>
<td>9 (10)</td>
<td>4/19 (21)</td>
<td>5/66 (8)</td>
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<tr>
<td>Anti-HIV —</td>
<td>175</td>
<td>9 (6)</td>
<td>2/71 (3)</td>
<td>7/104 (7)</td>
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</table>
serum anti-HBs or anti-HBs and anti-HBc markers thought to indicate immunity against HBV.16

In this study, trace amounts of HBV-DNA were detected in 13 HBsAg-negative carriers. The interpretation of the results of the dot-blot assay graded as trace amounts of HBV-DNA was unequivocal, because the results were consistently reproducible, and dot-blot–positive serum samples were shown to contain HBV-DNA by Southern blot analysis. It was more difficult to interpret biologically these findings. Trace amounts of HBV-DNA were unlikely to be passively transferred from infusions with infected clotting factor concentrates, since the HBV-DNA assays were carried out in serum samples collected at least 2 weeks after the last infusion with concentrates. The serologic profile of HBV-DNA associated with anti-HBc only, present in two HBsAg-negative carriers, might signal either infection with HBV expressing subliminal serum levels of HBsAg or infection with an HBV strain defective for HBsAg synthesis. The existence of carriers with low serum levels of HBsAg is substantiated by the circumstantial evidence that a small proportion of blood donors with anti-HBc as the only marker of HBV infection transmit hepatitis B to the recipients of their blood donations.17 Recently, a DNA fragment that hybridized with HBV-DNA was extracted from the liver of an HBsAg-negative, anti-HBc-positive patient, demonstrating the existence of an HBV strain with a mutation within the sequence coding for HBsAg synthesis.18

The most intriguing finding of this study was the occurrence of serum HBV-DNA in six patients with anti-HBs and anti-HBc and in five patients isolated anti-HBs, including two vaccinees. Studies of immunoprophylaxis against HBV have indicated, with some exceptions, correlation of the presence of anti-HBs with immunity to subsequent hepatitis B infections.19 The first such exception was the finding in 1978 of Spero et al, who used an immunofluorescence technique for staining hepatic HBcAg in biopsy samples, that HBsAg-negative hemophiliacs with serum anti-HBs and anti-HBc can harbor replicating HBV.19 Another important exception was the report of well-documented cases of hepatitis B in non-hemophilic patients with pre-existing anti-HBs.20,21 These findings suggest that exposure to HBV may lead to convalescent anti-HBs with weak reactivity against subsequent HBV infection. The co-occurrence of HBV-DNA and isolated anti-HBs that we observed in five patients, including two vaccinees, might be explained by infection with HBV-related variants that are immunologically distinct from HBV and do not express core antigen.22,23 A similar explanation was offered for the occurrence of cryptic infection with HBV in successfully vaccinated newborns of chronically infected mothers and in adult family contacts.24 Taken together, these data confirm that in high risk patients vaccination does not give 100% protection against HBV. Whatever the significance of serum HBV-DNA in HBsAg-negative patients with antibodies to HBV, our data corroborate the evidence that HBV may have a greater role in chronic hepatitis of hemophiliacs than previously thought. This biologic profile is not unique to hemophiliacs, since it can also be found in other settings. Recently, HBV-DNA was detected in sera of 68 blood donors with elevated ALT who tested negative for HBsAg.25 These findings emphasize the importance of serum HBV-DNA as a test for detecting cryptic HBV infection. Assays for serum HBV-DNA should, therefore, be incorporated in the routine check-up of hemophilic patients, to better define the etiology of the underlying liver disease. These assays should also be employed as an adjunct to anti-HBc for detecting cryptic HBV infection in vaccinees.

REFERENCES


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