Occult hepatitis B virus infection as a cause of cirrhosis of liver in a region with intermediate endemicity

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**Background:** Serological tests may fail to identify hepatitis B virus (HBV) infection as a cause of liver cirrhosis in a proportion of patients. The frequency of such occult infection in regions with Intermediate HBV endemicity is not known. Such cases may be diagnosed by incremental testing for IgG anti-HBc, serum HBV DNA, and HBV DNA in liver tissue. **Methods:** We tested sera of 111 patients with cirrhosis, including 39 with history of significant alcohol ingestion, for HBsAg, anti-HBc and serum HBV DNA. In addition, in a subset of 14 patients, HBV DNA was looked for in liver tissue. **Results:** On HBsAg and anti-HBc testing, 66 patients had HBV infection. Serum HBV DNA testing identified HBV infection in 13 additional cases. Of 18 patients labeled as 'cryptogenic' on serological testing, HBV DNA was detected in the serum in 7 patients. Of 14 patients in whom paired liver tissue and serum specimens were tested, 4 additional patients with HBV infection were detected after liver biopsy analysis. **Conclusions:** Serological tests for HBsAg and anti-HBc antibody are insensitive in identifying HBV infection in patients with liver cirrhosis. HBV DNA testing in serum and liver can help in establishing HBV infection as etiology, either alone or in addition to another cause. [Indian J Gastroenterol 2003;22:127-131]

**Key words:** Cryptogenic cirrhosis, HBV DNA, polymerase chain reaction

In developed countries with a low prevalence of HBV infection in the general population, a significant proportion of patients with chronic liver disease who lack serological markers of this infection have been shown to have detectable viral HBV DNA in their serum or liver tissue. In regions with higher prevalence of HBV infection, such as the Far East and South Asia, the frequency of serology-negative, nucleic acid-positive patients may be expected to be higher; however, data from these regions are scanty. We therefore undertook a study of serological and molecular markers of HBV infection in patients with liver cirrhosis in India, which has HBsAg carrier rate in the intermediate endemicity range, viz., 2%-8%.9

**Methods**

Between December 1997 and March 1999, we studied 111 patients with cirrhosis of liver (aged 28-80 years, mean 47; 101 men) registered in the Department of Gastroenterology of our institute. A detailed clinical and laboratory evaluation was done and patients were classified as Child-Pugh class A, B or C.10 Cirrhosis was labeled as alcohol-related if alcohol intake exceeded 80 g per day for a period of over 5 years. Tests for diagnosing Wilson's disease and hemochromatosis were performed wherever there were clinical pointers to suspect these diseases.

Serum specimens were obtained from all patients and were stored at -20°C in multiple aliquots till further use. Post-mortem liver biopsy tissues were collected from 14 patients who died of end-stage liver disease. One piece of liver tissue was stored immediately in liquid nitrogen till further use. Another piece was fixed in formaldehyde (10%) and embedded in paraffin block; tissue sections were evaluated by a pathologist. The study was approved by our institution's Ethics Committee and informed consent was obtained from patients or their close family members, as appropriate.

**Enzyme immunoassay for HBV markers**

All sera were tested by EIA for HBsAg, antibodies to hepatitis B core antigen (anti-HBc) and antibodies to HBsAg (anti-HBs) (Organon Teknika, Boxtel, Netherlands). EIA were performed as per the manufacturers' instructions.
Immunofluorescence for antinuclear antibody

All sera were tested for antinuclear antibody using an indirect immunofluorescence assay. Briefly, cryostat sections of rat liver were incubated with test sera (diluted 1:10) at 37°C for 30 min. After washing thrice with phosphate buffered saline (PBS) for 15 min each, the sections were incubated with fluorescein isothiocyanate-conjugated rabbit anti-human polyclonal immunoglobulin (Dako, Copenhagen, Denmark) diluted 1:40 in PBS at 37°C for 30 min. The sections were then washed thrice with PBS, mounted and examined under a fluorescent microscope.

Detection of HBV DNA in serum

DNA was extracted from test sera using a standard protocol. In brief, 250 μL of serum was incubated overnight at 37°C with 250 mL of lysis buffer (250 μg/mL proteinase K [Sigma, St. Louis, MO, USA] in 150 mM NaCl, 10 mM EDTA, 10 mM Tris HCl [pH 7.5], 2% sodium dodecyl sulfate and 5 μg/mL salmon sperm DNA [Sigma, St. Louis, MO, USA]). This was followed by extraction with phenol and chloroform/isoamyl alcohol.

DNA was precipitated, washed once with cold 70% ethanol, and resuspended in 50 μL distilled water. PCR was done using 5 μL of DNA in a total reaction volume of 50 μL containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.25 mM deoxyribonucleotide triphosphates, 20 pmol each of forward (5’ CCT CGT TAT AAA GGG ACT CAC G 3’) and reverse (5’ CAG CGG TAT AAA GGG ACT CAC G 3’) primers corresponding to S (surface) gene of HBV12 and 0.6 unit of Taq polymerase. Reaction conditions were: 35 cycles of 94°C, 55°C and 72°C for 1 min each, followed by final extension at 72°C for 10 min. The expected 388-basepair PCR product was detected using 1.5% agarose gel electrophoresis and Southern hybridization with a digoxigenin-labeled full-length HBV DNA probe. The assay had a sensitivity of 20,000 HBV genome equivalents per milliliter of serum.

Detection of HBV DNA from liver biopsies

Liver biopsies (approximately 8 mg in weight) were homogenized in 750 μL of TRIZOL LS Reagent (GIBCO BRL) using a pestle (Tarsons, Kolkata) in 1.5 mL tubes. The homogenized samples were incubated at room temperature for 10 min to permit complete dissociation of nucleoprotein complex. After addition of 200 μL of chloroform, the tube was shaken vigorously and incubated at room temperature for a further 10 min. Samples were centrifuged at 12,000 g for 15 min at 4°C; the mixture separated into a lower red, phenol-chloroform phase, an interphase containing the DNA, and a colorless upper aqueous phase. The aqueous phase was discarded and DNA was extracted from the remaining mixture. DNA was precipitated with 300 μL of ethanol, followed by incubation at room temperature for 5 min and centrifugation at 2000 g at 4°C for 5 min. DNA pellet was washed thrice with 0.1 M sodium citrate in 10% ethanol for 30 min each, followed by a final wash with 70% ethanol. The DNA pellet was air-dried and dissolved in 20 μL of HPLC-grade water. The DNA was subjected to PCR for HBV DNA; the procedure followed was similar to that for serum.

Statistical analysis

Statistical analysis was done using chi-square test.

Results

Demographic details

Six patients were in Child-Pugh class A, five in class B and 100 in class C. Thirty-nine patients (35%) had history of alcohol intake adequate to cause chronic liver disease. Anti-nuclear antibodies were detected in one patient; however, the titer of these antibodies was low and this patient did not have any other feature of autoimmune liver disease. No patient had markers for Wilson's disease or hemochromatosis.

Serological tests

Of the 111 patients, 28 tested positive for HBSAg and an additional 38 patients were positive for anti-HBc. Thus, 66 (59%) patients had serological evidence of HBV infection. Anti-HCV antibodies were detected in 16 (14%) patients; of these, 8 had serological evidence of HBV infection (HBSAg alone 1, anti-HBc alone 6, both 1). Thus 74 patients had serological markers for infection with HBV, HCV or both.

Among patients with history of alcohol intake, frequency of serological markers of HBV infection was lower than that among those without such intake (18/39 [46%] vs. 48/72 [67%]; p<0.05); no such difference was observed for anti-HCV (3 [8%] vs. 13 [18%]). Of these, one alcoholic and 7 non-alcoholics had dual infection.

Eighteen patients lacked history of alcohol intake and serological evidence of HBV and HCV infection; these patients were thus classified as having cryptogenic cirrhosis, prior to testing for HBV DNA.

HBV DNA

Of 111 patients, 49 tested positive for HBV DNA (Table 1); these included 36 of 66 patients with detectable HBSAg, anti-HBc or both, and 13 of 45 patients who lacked such markers (Table 2); the HBV DNA positivity rate was higher among those with serological markers of

<table>
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<tr>
<th>Table 1: Distribution of serological markers of HBV among liver cirrhosis patients with (n=49) and without (n=62) HBV DNA</th>
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<tbody>
<tr>
<td>Markers of HBV infection</td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>n (56)</td>
</tr>
<tr>
<td>HBSAg + anti-HBc</td>
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<td>HBSAg + anti-HBc</td>
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<td>HBSAg + anti-HBc</td>
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<td>HBSAg + anti-HBc</td>
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Table 2: Change in etiological diagnosis on nucleic acid testing

<table>
<thead>
<tr>
<th>Etiology</th>
<th>Based on history and serology</th>
<th>After addition of molecular tests (*)</th>
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<tbody>
<tr>
<td>HBV</td>
<td>41</td>
<td>48</td>
</tr>
<tr>
<td>HCV</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>HBV + HCV</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Alcohol alone</td>
<td>19</td>
<td>13</td>
</tr>
<tr>
<td>Alcohol + HBV</td>
<td>17</td>
<td>22</td>
</tr>
<tr>
<td>Alcohol + HCV</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Alcohol + HBV + HCV</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>None (cryptogenic)</td>
<td>18</td>
<td>11</td>
</tr>
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*Total change in numbers owe to redistribution among different groups

HBV infection (Table 1, p<0.05).

Of 39 patients who were considered to be alcoholic based on history, 13 were positive for HBV DNA. Of these 13 patients, four had anti-HBc, one had HBsAg and two had both; thus in 6 patients, presence of HBV infection was detected only with HBV DNA. Of the 72 non-alcoholics, HBV DNA was detected in 36 patients. Of these, 13 tested positive for anti-HBc, 3 for HBsAg. 13 for both HBsAg and anti-HBc. In 7 patients, HBV DNA was the only marker of HBV infection. Frequency of HBV DNA positivity was similar among alcoholic and non-alcoholic patients (13/39 vs 36/72; p<0.05).

While only 28 (25%) patients were found to have HBV infection based on HBsAg alone, addition of anti-HBc led to detection of 38 additional cases with evidence of HBV infection, and molecular analysis added 13 more such cases (Fig 1). Thus, of 79 patients with HBV infection, 13 (16%) were picked up only on HBV DNA testing.

Relationship of serological and nucleic acid analysis

The frequency of etiological diagnoses for cirrhosis based on clinical history and serology but prior to HBV DNA testing, and also after addition of HBV DNA testing are shown in Table 2. Specific etiologic diagnoses could be reached in 100 patients after inclusion of nucleic acid detection tests as against in 93 patients based on histological and serological tests. Of the 18 patients diagnosed as having cryptogenic cirrhosis, seven (38%) were found to have a specific cause on HBV DNA testing.

Based on clinical and serological data, alcohol was the only identifiable cause of cirrhosis in 19 patients; of these, 6 (31%) were found to have HBV as an additional cause after HBV DNA detection.

Liver biopsy analysis

Of 14 patients in whom liver biopsies were available, 8 were classified as alcohol-related. In all these 8 patients, liver biopsy had histological features of alcoholic cirrhosis. Of these eight patients, one also tested positive for anti-HCV. Of six patients without evidence of alcoholic liver disease, one was positive for HBsAg and one for anti-HCV.

Sera from one of eight alcohol-related cases and one of six cases not related to alcohol tested positive for HBV DNA (Fig 2). Liver biopsy revealed HBV DNA in 4 additional patients (2 related to alcohol and 2 not related).

Discussion

In our study, a large proportion of patients with cirrhosis of liver were found to have HBV infection. In several patients, though there was no serological evidence of HBV infection, serum and liver biopsy specimens revealed presence of HBV DNA. Also, in many patients in whom alcohol or HCV was believed to be the only cause of cirrhosis, HBV DNA testing revealed HBV as an additional causative factor for liver disease.

Of the 79 patients with HBV-related cirrhosis in our study, HBsAg was positive in 28 (35%) patients and anti-HBc in an additional 38 patients; in the remaining 13 patients, HBV DNA was the only marker of HBV infection. Clinicians in the developing world frequently rely on HBsAg testing alone to diagnose or exclude
HBV infection as the cause of liver cirrhosis. Our data indicate that this test is too insensitive for this purpose and that anti-HBc and HBV DNA detection may be more appropriate. In recent years, HBV DNA has been detected in HBsAg-negative patients with chronic liver disease and HBsAg-negative blood donors. The term 'occult' HBV infection has been used to describe these patients who lack antigen and antibody markers for HBV but are HBV DNA positive. Most of such reports are from developed countries. Our data confirm that occult HBV infection is common even in regions with intermediate HBV endemicity.

In our study, HBV DNA was detected in the liver in 4 of 12 patients who lacked HBsAg, anti-HBc and HBV DNA in serum. It has previously been reported that HBV DNA may be present in liver biopsy and peripheral blood mononuclear cells of patients lacking serum markers of HBV infection. In a study from Israel, HBV DNA was detected in liver biopsies of 18 of 31 patients with liver cirrhosis whose sera tested negative for HBV DNA. A previous study from India however failed to detect HBV DNA in sera from patients with chronic liver disease lacking serological markers of HBV infection. The results of this study are at variance with those of the remaining published literature, and may have been related to a low sensitivity of HBV DNA assay used.

Our data suggest that additional tests, such as anti-HBc and/or HBV DNA in serum and liver tissue, may be necessary before HBV infection can be excluded as a cause of liver cirrhosis. Since HBV DNA testing is costly and not easily available in many centers, it may be acceptable to test only for anti-HBc, while bearing in mind that this approach will fail to detect HBV infection in some cases.

We found evidence of HBV infection in several patients who had another cause for liver cirrhosis like HCV infection or alcohol. In fact, HBV infection was as frequent in these patients as in those without alcoholism or HCV infection. Others have reported similar data. This suggests that even in patients with a discernible cause for liver cirrhosis it is important to perform these tests. This may be particularly relevant since HBV infection may potentiate the injurious effects of alcohol and HCV. Though the role of anti-viral treatment in patients with detectable HBV DNA but no HBsAg is currently unclear, specific treatment may be possible for such patients in future.

Cryptogenic cirrhosis is a diagnosis of exclusion and its frequency has varied from 5% to 30% in different studies, with higher rates being reported from developing countries than from developed countries. This variation may be related to differences in sensitivity of tests used for diagnosis of various etiologies.

Use of sensitive molecular techniques may significantly reduce the proportion of cryptogenic cases among patients with liver cirrhosis. In a recent European study of 1075 patients with chronic hepatitis with elevated transaminase levels, nucleic acid testing identified HBV or HCV as the cause in 28 of 101 patients with negative serological tests. In our study, using HBV DNA testing, etiological diagnosis could be achieved in most patients, and only 11 (10%) patients were labeled cryptogenic. Additional HCV RNA testing might have reduced this number even further.

To conclude, our data indicate that HBsAg testing alone may fail to identify HBV infection as a causative agent for cirrhosis. Inclusion of additional tests like anti-HBc and HBV DNA may help in establishment of HBV as the etiology of liver cirrhosis in a larger proportion of cases, and in detection of HBV infection as an additional factor in those with another known cause like HCV infection or alcohol. This may be particularly relevant in view of increasing therapeutic options for infection with hepatotropic viruses.

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News and Notices

Dr S R Naik Memorial Workshop on Scientific Communication will be held at Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, August 30-31, 2003

For details, contact: Dr Rakesh Aggarwal, Department of Gastroenterology, SGPGI, Lucknow 226 014.

Phone (522) 266 8800, Extn 2431/2400 (O), 2410 (R)

Fax: (522) 266 8078 or 266 8017. E-mail: rakesh@sgpgi.ac.in

The 18th Annual Conference of the Indian Association of Surgical Gastroenterology will be held in Bhopal September 18-21, 2003

For details, contact: Prof Subodh Varshney, Organizing Secretary, Department of Surgical Gastroenterology and Clinical Nutrition, Bhopal Memorial Hospital and Research Center, Bhopal (MP) 462 038.

Phone: (755) 274 2212. Fax: (755) 274 8309

E-mail: iasg2003@yahoo.com

The annual conference on Current Perspectives in Liver Diseases (theme: “Fatty Liver Disease”) will be held in Chandigarh October 18-19, 2003

For details, contact: Dr Yogesh Chawla, Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012.

Phone: (172) 74 7585-602, Ext 6334-5. Fax: (172) 74 4401

E-mail: pgihepat@glide.net.in, epld2k3@yahoo.co.in

The IXth annual conference of the Parenteral and Enteral Nutrition Society of Asia will be held in Goa, November 8-10, 2003

For details, contact: Dr Shivakumar Iyer, Organizing Secretary.

E-mail: s_iyer@vsnl.com, pens2003@indiatimes.com, ispenpune@vsnl.net

Website: www.ispen.org

The International Seminar and Workshop on Fermented Foods, Health Status and Social Well-being will be held in Anand, Gujarat, November 13-14, 2003

For details, contact: Prof J B Prajapati, Organizing Secretary, Department of Dairy Microbiology, SMC College of Dairy Science, Gujarat Agricultural University, Anand 388 110.

Phone: (2692) 26 1030, 26 1352. Fax: (2692) 26 1314

E-mail: jbprajapati@iagr.guj.nic.in, jbprajapati@lycos.com

The Asian Pacific Association for the Study of the Liver Biennial Conference will be held in New Delhi, December 11-15, 2004

For details, contact: Dr S K Sarin, President APASL, Room 201, Academic Block, Department of Gastroenterology, GB Pant Hospital, New Delhi 110 002.

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