Detection of hepatitis B virus in the liver by *in situ* hybridization (ISH) in HBsAg seropositive and seronegative patients

T. Caballero, M.A. Caballero, A. Ruiz-Extremera, Y. Barrios del Pino, F. O'Valle, F.J. Salmerón and G. Sánchez Salgado

Departments of Pathology and Gastroenterology, University Hospital, School of Medicine, Granada, Spain

**Summary.** The presence of hepatitis B virus (HBV) DNA in the liver of 119 patients was studied to assess the diagnostic value of in situ hybridization (ISH) and its relationship with viral replication and histological liver damage. Liver biopsies of 119 patients (55 hepatitis B surface antigen-HBsAg- seropositive and 64 HBsAg seronegative) were studied retrospectively. Among the HBsAg seropositive patients, the ISH was positive in 26 cases (47%) and negative in 29 (53%) and the former group had higher levels of serum transaminases. The hepatocyte number with positivity for HBsAg and hepatitis B core antigen (HBcAg) in the liver were similar in both ISH-positive and negative patients. The histological activity index (Knodell) was higher in ISH-positive patients (11 vs 7, p<0.001). Six patients out of 12 were positive by PCR. In the HBsAg seronegative patients, the ISH was negative in 75 cases and positive in 7. These 7 were positive for anti-HBs (5 cases) and/or anti-HBc (6 cases); 4 were confirmed by PCR. Thus, our data suggest that the ISH technique is useful for detecting viral nucleic acid in the liver, but that the HBV-DNA cannot always be considered as a replication marker, because we also show that some HBsAg seronegative patients with chronic liver disease do have HBV-DNA in their liver cells.

**Key words:** HBV, Immunohistochemistry, *in situ* hybridization, PCR, Viral replication

**Introduction**

The study of viral gene products of hepatitis B virus (HBV) in the liver is usually complementary to serological analysis; however, it is possible to find evidence of infection in the liver tissue of patients with negative serological markers, which confirms the importance of the analysis of virus markers in the liver: hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg) (Villa et al., 1980; Negro et al., 1984; Su et al., 1986; Cuccurullo et al., 1987; Naoumov et al., 1990; Raimondo et al., 1990) or HBV-DNA (Brechot et al., 1982, 1985, 1991; Rijnjtes et al., 1985; Lai et al., 1990; Lampertico et al., 1990; Diamantis et al., 1992; Loriot et al., 1992). HBV-DNA in hepatic cells, detected by *in situ* hybridization (ISH), is a useful complementary technique. ISH has some advantages in relation to other methods, such as preserving the cell detail and permitting the correlation of morphological characteristics with the presence of specific nucleic acids. This technology is very valuable in viral infections, as it detects both active and latent infections (Wolfe, 1988), and, finally, because retrospective material may be used (Grody et al., 1987; Höfler, 1987).

The aim of this study was to determine the relationship of ISH with viral replication and liver damage, and also to find out whether ISH is useful for detecting HBV nucleic acid in HBsAg negative patients.

**Materials and methods**

Liver biopsies from 119 patients were studied retrospectively: 55 HBsAg-positive and 64 HBsAg-negative. Conventional HBV markers were analyzed in serum by enzyme immunoassays. Serum HBV-DNA was analyzed by dot-blot technique in 35 HBsAg-positive patients. For each biopsy, besides conventional histology and histological activity index (HAI) (Knodell et al., 1981), we also carried out an immunohistochemical study to determine the presence of HBsAg and HBcAg using polyclonal antibodies (Dakopatts) (Dilutions: 1/16000 goat anti-HBs and 1/200 rabbit anti-HBc) and the peroxidase-antiperoxidase technique. The number of positive cells/mm² of tissue was quantified with the help of a grid placed in the eyepiece of an Olympus microscope.

ISH was done in formalin-fixed, paraffin-embedded...
ISH in HBsAg seropositive-negative patients

Liver tissue with a commercial biotinylated HBV-DNA probe (Pathogene kit; Enzo Biochemicals, NY). The sections (five microns thick) were placed on slides coated with APTS (3-aminopropyltriethoxyisilane; Sigma, St. Louis); paraffin-embedded tissue was deparaffinized and hydrated, and treated for 20 minutes with 0.1 mg/ml of proteinase K solution (Sigma) at 37 °C; endogenous peroxidase activity was blocked with 3% H₂O₂ for 30 minutes. The following procedures were carried out according to Naoumov et al. (1988) and the manufacturer’s instructions, with some modifications. Briefly, slides were dehydrated and air dried; a hybridization mixture was applied on slides placed onto the 96 °C heating block for 10 minutes and then incubated at 37 °C for 3 hours in a humidified box; the posthybridization reagent was applied for 20 minutes at 37 °C. The detection complex was avidin-peroxidase biotinylated and the chromogen reagent was aminoethylcarbazole; with this procedure we only detected HBV-DNA in the cytoplasm of hepatocytes (red deposits). A positive and negative control (a case previously checked, with and without DNA probe, respectively) were analyzed simultaneously.

In order to check the specificity of the ISH technique, polymerase chain reaction (PCR) was applied to 37 cases in paraffin-embedded tissue sections. Four-to-five micron sections were cut from the paraffin blocks and placed in a 1.5 ml Eppendorf tube, deparaffinized with xylene and hydrated with ethanol; the tissue was digested with 200 microliters of proteinase K solution overnight. After phenol-chloroform extraction and ethanol precipitation, the DNA pellet was resuspended in 50 microliters of TE buffer. Five microliters were amplified with primers 1763 and 2032R first, and reamplified with primers 1778E and 2017R-B following previously reported nested-PCR protocols (Fugiyama et al., 1983; Kaneko et al., 1990). Samples were also amplified with primers for the cystic fibrosis gene as a control for the presence of human DNA in all the samples.

Statistical methods were determined by the Student’s t-test, Chi square-test, percentage comparisons, analysis of variance (ANOVA), and Pearson’s correlation test.

Results

HBsAg-seropositive patients

Twenty-eight out of 55 HBsAg-positive patients were hepatitis B e antigen (HBeAg) seropositive, and HBV-DNA was positive in 17 out of the 35 patients in which dot-blot was performed. The biopsy study showed 15 non-specific reactive hepatitis (NSRH), 2 chronic persistent hepatitis (CPH), 27 chronic active hepatitis (CAH) and 11 cirrhosis.

1) HBsAg and HBcAg in the liver:

HBeAg was positive in 21 cases (38%), all of them HBcAg-positive and none negative. Sixteen out of 17 seropositive HBV-DNA patients had HBcAg in the liver. In 15 (71%) the location was nuclear (N) and in 6 (29%) nuclear plus cytoplasmic (N-C) (Fig. 1); the HAI was higher in the N-C cases than in the N ones (12 vs 8.2). The number of positive cells/mm² was higher when HBcAg was N-C than when it was N (194 vs 168; not significant -NS-), and was correlated with HBeAg and HBV-DNA (p<0.01).

Fig. 1. Liver stained with polyclonal antiHBc antibody: a nuclear and/or cytoplasmic brown stain is shown in some hepatocytes. PAP. x 100
HBsAg was detected in 46 biopsies (83%), with most of them showing cytoplasmic distribution (C) (66%) and the rest being membranous (M), independently of whether they were HBeAg- or HBV-DNA-positive or negative. The percentage of cases with HBsAg M was higher when HBeAg was negative (53% vs 25%). The number of HBsAg-positive hepatocytes/mm² was higher when HBeAg (p<0.05) and HBV-DNA were negative; an inverse relationship was noted between the number of HBsAg + hepatocytes/mm² and the HAI and aspartate

Fig. 2. In situ hybridization technique using a biotinylated HBV-DNA probe: red granular deposits are seen in the cytoplasm of the hepatocytes. ISH x 100

Fig. 3. A representative ethidium-bromide-stained agarose gel of PCR re-amplification products. A 239 bp band, between 270 and 234 bp markers, on lanes 2 (weakly positive), 3, 4, 7, 9, 14, 16 and 17 is present. M = standard molecular weights.
aaminotransferase (AST) (r=0.32 and -0.30 respectively, p<0.05). The number of positive cells was higher in those with M distribution of HBsAg (350 vs 168, p<0.05) and mild histological injury.

2) ISH:

ISH was positive in 26 (47%) biopsies (3 NSRH, 2 CPH, 15 CAH, 6 cirrhosis) and negative in 29 (53%) (12 NSRH, 12 CAH, 5 cirrhosis). In positive cases, red granular deposits were generally seen in the cytoplasm of small groups of hepatocytes (Fig. 2) except in 4 patients, who had granular deposits in many of them.

Transaminases were high in the ISH-positive group (alanine aminotransferase -ALT- 217 international units per liter -IU- vs 139 IU; AST 142 IU vs 81 IU) and a high percentage of ISH-positive cases was observed in HBeAg and HBV-DNA seropositive patients (54% vs 42% and 47% vs 41%, respectively).

HBsAg distribution in the liver was similar in both groups. Nine out of 21 cases with HBcAg in the liver were ISH positive. In 67% of the cases with positive ISH, the HBcAg distribution was C-N, while in the ISH-negative group the expression was only N type (p<0.001). The number of positive cells/mm² was higher in the ISH-negative group but not significant (HBsAg: 211 vs 181; HBcAg: 80 vs 52). HAI was higher in ISH-positive patients (11 vs 7, p<0.001).

**HBsAg-seronegative patients**

ISH was negative in 57 cases (1 NSRH, 3 CPH, 36 CAH, 17 cirrhosis) and positive in 7 (1 CPH, 4 CAH, 2 cirrhosis). HBsAg and HBcAg in the liver were negative in all cases. In both groups there was evidence of contact with HBV (anti-HBs and/or anti-HBc); however, the rate was higher when ISH was positive (anti-HBs: 71% vs 36%; anti-HBc: 86% vs 42%; p<0.01), so that all of the ISH-positive had anti-HBs (5 cases) and/or anti-HBc (6 cases). Transaminases were higher in the ISH-positive patients (ALT: 269 vs 121; AST: 242 vs 83, p<0.05). Finally, HAI was higher in the positive group (13 vs 10).

**PCR**

PCR was studied in 37 biopsies: 12 HBsAg-seropositive and 25 HBsAg-seronegative patients; the last group included the 7 cases of HBsAg-negative patients with ISH positive in the biopsy. A visible DNA amplification band of the expected molecular size was observed (Fig. 3) in 21 patients.

In seven biopsies (58%) of 12 HBsAg-positive patients the PCR was positive and ISH was positive in 6 (50%) (Table 1). However, in seronegative patients the PCR showed a higher rate of positive cases (56%) than ISH (28%). PCR was also positive in 4 out of 7 patients with ISH positive and HBsAg negative (Table 1).

**Discussion**

The study of viral gene products of HBV in the liver and its relationship with serum markers is an interesting subject, and has given rise to many publications which have tried to explain contradictory situations. Among the histology-based techniques, it is currently possible to determine HBsAg and HBcAg by immunoperoxidase and HBV-DNA by ISH.

Hepatic HBcAg is a good marker of viral replication (Chu and Liaw, 1987; Hsu et al., 1987; Naoumov et al., 1990, 1993), and when it is associated with HBeAg it can be safely asserted that the patient is also HBV-DNA seropositive. We found that the number of cells/mm² and the HAI were higher in cases with N-C location than in cases with only N location, although the difference was not significant. This type of location may be associated with more severe hepatic illness (Chu and Liaw, 1987; Hsu et al., 1987; Ramalho et al., 1988; Kakumu et al., 1989; Naoumov et al., 1990, 1993). Some authors consider that the membranous HBsAg may also be related with replication (Ramalho et al., 1988), but this correlation is less evident, as may be seen from this and other studies (Dienes et al., 1990).

An interesting point is that some patients without circulating HBsAg have been reported to have positive viral markers in liver tissue (Villa et al., 1980; Negro et al., 1984; Su et al., 1986; Cuccurullo et al., 1987; Naoumov et al., 1990; Raimondo et al., 1990). Such patients usually have high serum concentrations of anti-HBc. Several explanations for this phenomenon have been suggested, including low HBsAg concentration undetectable by traditional diagnostic methods and immunocomplex formation with circulating antibodies. These studies and others suggest that it is highly unlikely that a seronegative patient will display HBsAg or HBcAg in the liver (Escobar et al., 1989; Naoumov et al., 1993).

To detect HBV-DNA in hepatic tissue, several hybridization techniques have commonly been used, such as Southern blot, which has a high sensitivity for analyzing small amounts of sequences, differentiating between free and integrated forms (Brenchot et al., 1981, 1982, 1985; Burrell et al., 1982; Yokosuka et al., 1985). On the other hand, with ISH technique, such as it was used in this study, viral DNA was found to be present only in the cytoplasm (Rijntjes et al., 1985; Herrmann and Hübner, 1987; Naoumov et al.,

---

**Table 1. Comparison between ISH and PCR paraffin-embedded liver tissue in 37 HBsAg seropositive and seronegative patients.**

<table>
<thead>
<tr>
<th>HBsAg SERUM</th>
<th>No. CASES</th>
<th>ISH positive/negative</th>
<th>PCR positive/negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>12</td>
<td>6/6</td>
<td>7/5</td>
</tr>
<tr>
<td>Negative</td>
<td>25</td>
<td>7/18</td>
<td>14/11</td>
</tr>
<tr>
<td>TOTAL</td>
<td>37</td>
<td>13/24</td>
<td>21/16</td>
</tr>
</tbody>
</table>

*: in four cases the ISH was positive; **: in four cases the PCR was positive.
ISH in HBsAg seropositive-negative patients

1988, 1993); the distribution pattern is in general focal (Rijnjtes et al., 1985; Herrmann and Hübner, 1987; Naoumov et al., 1993), as in our cases, where only 4 had a high positivity with a wide-spread pattern.

In order to avoid the non-specific staining caused by endogenous-biotin, proteolytic enzymes (proteinase k, pronase) under different concentrations were used (Naoumov et al., 1988).

With the commercially available probe used we obtained 47% HBV-DNA positive in HBsAg-positive patients and 11% in negative ones. According to Rijnjtes et al. (1985), the rate is much higher: 100% in HBsAg-positive and 24% in negative; however, he does not specify the HBeAg nor does he correlate it to other hepat markers. In the study of Herrmann and Hübner (1987), the HBV-DNA by ISH was positive in all patients with HBsAg and in 78% of the negatives. This data shows a higher rate of positives than would be expected by immunohistochemistry. Although our results show a smaller proportion of positive cases, we found a high correlation to PCR; in the above-mentioned studies a confirmation test was not done, and it is possible that some cases were false positives.

It is not easy to explain the HBV-DNA presence in HBsAg-negative patients; the appearance of viral mutants or genome deletion could account for this situation (Shafritz, 1985; Brunetto et al., 1990; Lai et al., 1990; Liang et al., 1990; Brown et al., 1992; Carman and Thomas, 1992). Alternatively, a small or intermittent production of HBsAg that could avoid detection (Shafritz, 1985; Brown et al., 1992) has been proposed. However, with more sensitive techniques such as hybridization, it should be possible to determine the HBV-DNA. Another possibility is that they are false positives (Shafritz, 1985); consequently, it would be necessary to do specificity controls (Grody et al., 1987) such us the PCR technique applied to paraffin sections, which has a high sensitivity (Lo et al., 1989; Wright and Wynford-Thomas, 1990; Chen et al., 1991; Brown et al., 1992). This procedure has shown HBV-DNA in patients without any serum or tissue markers of HBV infection for or with anti-HBc as the only marker (Brechot et al., 1991; Diamantis et al., 1992). As hepatic illness may be produced by an HBV variant with genomic modification, it is necessary to use several primers in the PCR (Brechot et al., 1991), as in this study.

Our results of PCR-positive rate were lower than in other studies (Diamantis et al., 1992; Kuhns et al., 1992) in HBsAg sero-positive and -negative patients. This could be attributed to the quality of material used. When PCR assays were used for frozen liver tissue, the percentage of cases with viral DNA-HBV among surface antigen-negative patients was higher (Kuhns et al., 1992; Fong et al., 1993).

The fact that 4 HBsAg-negative patients had HBV-DNA in the liver, confirmed by ISH and PCR, shows that it is possible to detect viral DNA in patients without serological signs of infection. DNA-HBV in HBsAg-negative patients could represent non-replicative or inactive forms and it should not be considered as a replication marker.

Acknowledgements. This research was supported in part by Shering Plough and Roche Laboratories.

References


