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Membrane staining for hepatitis B surface antigen on hepatocytes: a sensitive and specific marker of active viral replication in hepatitis B

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Abstract

***Aims*—To test the hypothesis that membranous staining of hepatitis B surface antigen (HBsAg) on the hepatocyte is a marker of active viral replication in chronic hepatitis B virus (HBV) infection.**

***Methods*—Intrahepatic expression of HBsAg and hepatitis B core antigen (HBcAg) was studied by indirect immunofluorescence on frozen sections of liver specimens from 75 patients with chronic hepatitis B, and the results were correlated with serum levels of HBV-DNA assayed by spot hybridisation.**

***Results*—Hepatocyte HBcAg was detected in all of 20 patients with serum levels of HBV-DNA >1000 pg/ml, 18 (75%) of 24 patients with levels of HBV-DNA ≤1000 pg/ml, and two (6·5%) of 31 patients without detectable serum HBV-DNA. The concordance between hepatocyte HBcAg and serum HBV-DNA was 89·3% (67/75). There were six patients (8%) who had detectable serum HBV-DNA but without hepatocyte HBcAg, and two patients (2·7%) who had detectable hepatocyte HBcAg but without serum HBV-DNA. Membranous staining of HBsAg associated with variable degrees of cytoplasmic HBsAg was found in all but one of 44 patients with serum HBV-DNA, irrespective of the levels, but in none of the 31 patients without serum HBV-DNA. Of the latter, HBsAg was distributed solely in the cytoplasm. In addition, there is an inverse correlation between serum levels of HBV-DNA and the degrees of cytoplasmic staining of HBsAg. The concordance between membranous staining of HBsAg and serum HBV-DNA was 98·7% (74/75), significantly higher than that between hepatocyte HBcAg and serum HBV-DNA.**

***Conclusions*—Membranous staining of HBsAg on the hepatocyte correlated excellently with serum HBV-DNA and thus can be recognised as a sensitive and specific marker of active hepatitis B virus replication.**

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Keywords: Hepatitis B, HBV-DNA, HBsAg, HBcAg, immunostaining.

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Previous immunohistochemical studies have suggested that hepatocyte expression of hepatitis B core antigen (HBcAg) had a significant diagnostic and prognostic implication in patients with chronic hepatitis B virus (HBV) infection.[1–8] The demonstration of HBcAg in liver usually correlated with active viral replication and was associated with variable degrees of inflammatory activity in the liver, while failure to detect HBcAg in liver usually indicated low levels of viral replication and was associated with little or no inflammatory activity.[1–8] Assays of HBV-DNA in serum by molecular hybridisation techniques have long been recognised as a sensitive and specific method of evaluating viral replication in chronic hepatitis B virus infection.[9–11] However, these earlier data have shown that the discordance between serum HBV-DNA and hepatocyte expression of HBcAg ranged from 12% to 23%, including (1) 9–23% of patients who were positive for serum HBV-DNA without hepatocyte HBcAg and (2) 0–3% of patients who had hepatocyte expression of HBcAg without serum HBV-DNA.[12–15] One possible reason for the former might be sampling error in the biopsy specimens because hepatocyte expression of HBcAg could be only focal in certain patients with chronic hepatitis B[16,17]; in the case of the latter, false positive immunostaining of HBcAg cannot be excluded.[18]

It has been shown that there is membranous staining of HBsAg associated with variable degrees of cytoplasmic staining of HBsAg during the phase of active hepatitis B virus replication and, by the contrast, solely cytoplasmic staining of HBsAg during the non-replicative phase.[1,2,5,19] Of note is that the distribution of membranous staining of HBsAg is usually diffuse and the staining intensity is rather strong during active viral replication.[1,2,5,19] It is therefore postulated that immunostaining of HBsAg on the hepatocyte membrane might be more sensitive and specific than immunostaining of HBcAg as a marker of active hepatitis B virus replication. To test this hypothesis, serum levels of HBV-DNA were correlated with membranous staining of HBsAg on the hepatocyte versus hepatocyte staining of HBcAg in 75 patients with chronic hepatitis B.

Methods

Seventy five patients with chronic HBV infection were studied. All had been HBsAg positive for more than six months before a histological diagnosis of chronic hepatitis was made. Forty four patients were seropositive for HBeAg and the other 31 were seropositive for antibody against HBeAg (anti-HBe). All were

Table 1 Mean (SEM) clinical and laboratory data of patients studied

Category	No of cases	Age (years)	Sex (M:F)	AST (IU/l) (n <40)	ALT (IU/l) (N <40)
HBeAg positive CPH	22	24 (3)	18:4	48 (2)	60 (3)
HBeAg positive CAH	22	29 (2)	20:2	84 (8)	202 (18)
Anti-HBe positive CAH	5	33 (2)	5:0	80 (30)	180 (40)
Anti-HBe positive MHC	26	35 (1)	24:2	29 (3)	34 (3)

HBeAg = hepatitis B e antigen; anti-HBe = antibody against HBeAg; CPH = chronic persistent hepatitis; CAH = chronic active hepatitis; MHC = minimal histological changes.

negative for antibody against hepatitis δ virus (anti-HDV). None admitted intravenous drug abuse, nor had they ever received antiviral or immunosuppressive treatment. The histological diagnosis of chronic hepatitis was made according to standard criteria.[20] The clinical and laboratory data of the patients studied are listed in table 1.

Serum aspartate transferase (AST) and alanine transaminase (ALT) were measured by sequential multiple autoanalysers. Serum HBsAg, HBeAg, anti-HBe, and anti-HDV were assayed using commercially available radioimmunoassay kits (Ausria-II, HBeAg-RIA, and anti-delta, Abbot Laboratories).

Serum specimens for test of HBV-DNA were collected on the same day of liver biopsy and stored at −70°C until use. Serum HBV-DNA was assayed by spot hybridisation techniques using ^{32}P labelled cloned HBV-DNA, as reported previously.[21]

Liver specimens were obtained by percutaneous needle biopsy with a Menghini needle. Fragments of specimens were snap frozen in isopentane cooled with liquid nitrogen and stored at −70°C until use. Samples of the same biopsy specimens were also fixed in 10% formaldehyde and embedded in paraffin wax for routine histological diagnosis. Cryostat sections (5 μm) were dried overnight at room temperature and fixed in carbon tetrachloride at 4°C for 10 minutes, followed by an extensive wash with phosphate buffered saline (pH 7·2) before staining. Intrahepatic expression of HBcAg was studied by indirect immunofluorescence using rabbit anti-HBc (Dako), followed by fluorescein isothiocyanate (FITC) labelled swine anti-rabbit immunoglobulin G. Intrahepatic expression of HBsAg was studied by indirect immunofluorescence using murine monoclonal anti-HBs (Chemicon), followed by FITC conjugated rabbit anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories).

Statistical analyses were performed using χ^2 test with Yate's correction, Wilcoxon rank sum test, or Spearman's rank correlation where appropriate.

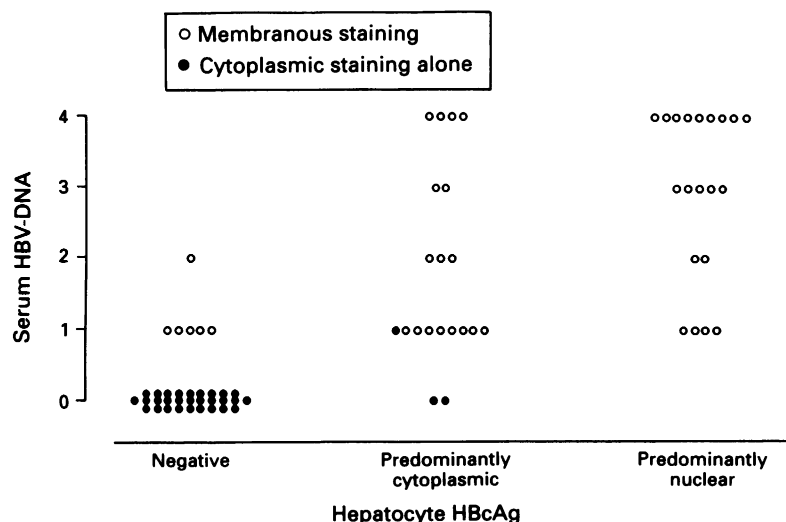

Figure 1 Correlation between serum levels of HBV-DNA and hepatocyte staining of HBcAg in 75 patients with chronic hepatitis B. Serum levels of HBV-DNA were semiquantitatively scored on a 0 to 4+ scale, corresponding to undetectable, ≤500 pg/ml, 501–1000 pg/ml, 1001–2000 pg/ml, and >2000 pg/ml. The empty circles indicate patients with membranous staining of HBsAg and the filled circles indicate patients with solely cytoplasmic staining of HBsAg. Spearman's rank correlation coefficient = −0·64, p<0·001.

Results

Hepatocyte staining of HBcAg was detected in 40 patients, including 20 with predominantly nuclear staining and 20 with predominantly cytoplasmic staining. Serum HBV-DNA was detected in 44 patients, including 20 with levels >1000 pg/ml and 24 with levels ≤1000 pg/ml. As shown in table 2, the concordance between serum HBV-DNA and hepatocyte staining of HBcAg was 89·3% (67/75).

Figure 1 summarises the correlation between serum levels of HBV-DNA and hepatocyte staining of HBcAg. Of the 44 patients positive for serum HBV-DNA, 38 (86·4%) had HBcAg detectable in liver. All of the 20 patients with serum levels of HBV-DNA >1000 pg/ml had detectable HBcAg in liver, with 70% (14/20) predominantly in the nucleus and 30% (6/20) predominantly in the cytoplasm, whereas only 75% (18/24) of those with serum levels of HBV-DNA ≤1000 pg/ml had detectable HBcAg in liver (p<0·05), with 67% (12/18) predominantly in the cytoplasm and 33% (6/18) predominantly in the nucleus (p<0·05). Serum levels of HBV-DNA were significantly higher in patients with predominantly nuclear staining of HBcAg than in those with predominantly cytoplasmic staining of HBcAg (p<0·01). Five of six patients who had HBV-DNA detectable in serum without hepatocyte staining of HBcAg had levels of HBV-DNA ≤500 pg/ml.

Hepatocyte staining of HBsAg was detected in all of the 75 study patients, including six with purely membranous staining, 32 with purely cytoplasmic staining, and 37 with mixed membranous and cytoplasmic staining. Figure 2 summarises the correlation between serum levels of HBV-DNA and hepatocyte staining of HBsAg. All but one of 44 patients with serum HBV-DNA, including the six patients who had

Table 2 Results of immunostaining of HBcAg on hepatocyte and serum HBV-DNA in 75 patients with chronic type B hepatitis

Category	Hepatocyte HBcAg/serum HBV-DNA			
	+/−	−/−	−/+	+/−
HBeAg positive CPH	20	1	1	0
HBeAg positive CAH	15	3	4	0
Anti-HBe positive CAH	3	1	1	0
Anti-HBe positive MHC	0	24	0	2
Total	38	29	6	2

HBcAg = hepatitis B core antigen; HBV = hepatitis B virus. For other abbreviations see table 1.

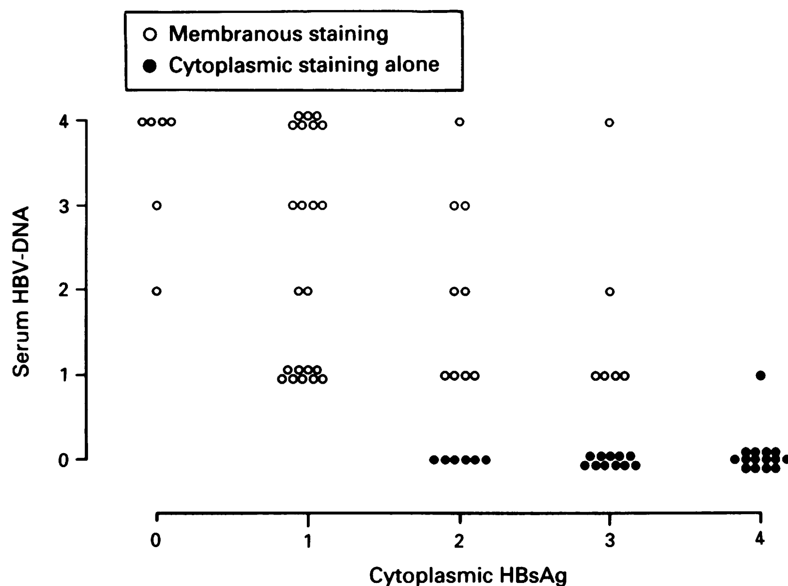

Figure 2 Correlation between serum levels of HBV-DNA and hepatocyte staining of HBcAg in 75 patients with chronic hepatitis B. Serum levels of HBV-DNA were semiquantitatively scored on a 0 to 4+ scale, corresponding to undetectable, ≤500 pg/ml, 501–1000 pg/ml, 1001–2000 pg/ml, and >2000 pg/ml. The degree of cytoplasmic staining of HBsAg was semiquantitatively scored on a 0 to 4+ scale, corresponding to positivity in 0%, 1–10%, 11–25%, 26–50%, and >50% of hepatocytes examined. The empty circles indicate patients with membranous staining of HBsAg and the filled circles indicate patients with solely cytoplasmic staining of HBsAg. Spearman's rank correlation coefficient = −0·64, p<0·001.

no detectable HBcAg in liver, had membranous staining of HBsAg associated with variable degrees of cytoplasmic staining of HBsAg, irrespective of the levels of HBV-DNA. In contrast, all of the 31 patients without serum HBV-DNA, including the two patients with hepatocyte staining of HBcAg, had solely cytoplasmic staining of HBsAg. The concordance between membranous staining of HBsAg and serum HBV-DNA was 98·7% (74/75), significantly higher than that between hepatocyte staining of HBcAg and serum HBV-DNA (p<0·05). In addition, there was an inverse correlation between serum levels of HBV-DNA and the degrees of cytoplasmic staining of HBsAg.

Discussion

The data reported previously have revealed that the discordance between hepatocyte staining of HBcAg and serum HBV-DNA in patients with chronic hepatitis B was not infrequent, ranging from 12 to 23%.[12–15] The present results indicated that intrahepatic expression and topographical distribution of HBcAg were closely related to serum levels of HBV-DNA. All of the patients with high levels of HBV-DNA in serum had detectable HBcAg, which was predominantly distributed in the nucleus, whereas only 75% of patients with low levels of HBV-DNA in serum had detectable HBcAg, which was predominantly distributed in the cytoplasm. Moreover, serum levels of HBV-DNA was significantly higher in patients with predominantly nuclear staining of HBcAg than in those with predominantly cytoplasmic staining of HBcAg. These findings are in keeping with the suggestion by Ou *et al*[22] that one possible function of the nuclear localisation of HBcAg might be to transport daughter viral genomes from the cytosol to the nucleus and then direct the synthesis of additional viral RNA and eventually increase the number of progeny particles produced by the infected cells.

About 8% of patients in the present series had HBV-DNA detectable in serum without hepatocyte staining of HBcAg. This figure is relatively lower compared with the 9–23% reported previously by others.[12–15] As shown in fig 1, serum levels of HBV-DNA in these patients were usually relatively low compared with those with detectable hepatocyte HBcAg. The possibility of focal predominantly cytoplasmic staining of HBcAg cannot be excluded. In addition, the indirect immunofluorescence techniques on frozen sections of liver specimens used in the present study have been shown to have improved sensitivity to detect HBcAg in the cytoplasm[23] and thus might have less sampling error compared with other studies.[12–15] Another 3% of patients in the present series had HBcAg staining in the liver without detectable HBV-DNA in serum using spot hybridisation techniques. This figure is similar to the 0–3% reported previously by others.[12–15] Although the possibility of false positive immunostaining of HBcAg cannot be excluded,[18] the presence of low levels of viral replication in these patients should be further investigated by serum HBV-DNA assay using polymerase chain reaction.

In keeping with the previous observations,[1,2,5,19] the present data showed that HBsAg was stained diffusely on the hepatocyte membrane, in association with variable degrees of cytoplasmic staining during the phase of active viral replication, while HBsAg was distributed solely in the cytoplasm during the non-replicative phase. Perhaps the more important finding of the present study is that serum levels of HBV-DNA correlated inversely with the degrees of cytoplasmic staining of HBsAg (fig 2). These findings suggested that there is efficient export of intracellular HBsAg packed with HBV-DNA and nucleocapsid protein as a complete viron during active viral replication, and reduced export of HBsAg resulting in accumulation of intracellular HBsAg when viral replication decreases. Another important finding of the present study is that the concordance between membranous staining of HBsAg on the hepatocyte and serum HBV-DNA was significantly higher than that between hepatocyte expression of HBcAg and serum HBV-DNA, suggesting that hepatocyte membranous staining of HBsAg is more sensitive and specific than hepatocyte staining of HBcAg as a marker of active hepatitis B virus replication. The mechanism of membranous staining of HBsAg during active viral replication is uncertain. Of note is that the distribution of membranous staining of HBsAg is usually very diffuse. The population of cells with membranous staining of HBsAg should thus be larger than the population of cells in which the virus replicates. It seems less likely that membranous staining of HBsAg indicates expression of viral antigen on the cell membrane of infected hepatocytes.

Instead it is suggested that the diffuse membranous staining of HBsAg during active HBV replication indicates absorption of circulating hepatitis B virus virons on the liver cell surface; the virons are surrounded by HBsAg which contains polymerised human albumin receptor and could thus be absorbed on the liver cell surface.[24 25]

In conclusion, membranous staining of HBsAg on the hepatocyte correlated excellently with serum HBV-DNA and could be recognised as a marker of active hepatitis B virus replication. It was suggested that membranous staining of HBsAg possibly indicates absorption of circulating hepatitis B virus virons on the cell surface rather than expression of viral proteins on the cell membrane of infected cells.

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