Hepatitis B-DNA replication and histological patterns in liver biopsy specimens of chronic HBsAg positive patients with and without hepatitis delta virus superinfection

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SUMMARY The role of active hepatitis B virus (HBV) infection in chronic HBsAg positive hepatitis with and without hepatitis delta virus (HDV) superinfection was analysed in percutaneous liver biopsy specimens from 50 patients. Each specimen was divided into two—one part for histological evaluation and for the detection of HBcAg and delta antigen; the other part was tested for HBV-DNA using Southern blotting. Ten cases were of chronic lobular hepatitis, 10 of chronic persistent hepatitis, and 30 of chronic active hepatitis. Ten cases were delta antigen positive and showed high grade lobular activity but no evidence of HBV-DNA episomal forms or HBcAg reactivity. Twenty one cases showed HBV-DNA replicative intermediate forms; 19 had high grade lobular activity, which occurred in five cases without evidence of free viral DNA. Of the 21 biopsy specimens with HBV-DNA episomal forms, 14 were positive for HBcAg; only one of the 19 cases without detectable viral DNA was positive for such antigen.

These data indicate that the presence of HBV or HDV active infection correlates with the histological finding of prominent lobular necrosis. Moreover, intrahepatic HBV-DNA seems to be a more sensitive marker than the presence of viral antigens for indicating HBV replication.

Hepatic damage in chronic carriers of hepatitis B surface antigen (HBsAg) seems to be strictly related to the presence of hepatitis B virus (HBV) active infection or delta virus (HDV) superinfection.¹⁻⁶

In most of the studies performed so far active replication of HBV has been evaluated by immunohistochemical detection of hepatitis B core antigen (HBcAg) in liver tissue and using serological markers.^{3,7,8} The development of molecular biological techniques has allowed specific HBV-DNA sequences in liver biopsy specimens to be detected, thus providing direct evidence of HBV replication.^{1,9}

This study aimed to investigate the association between the presence of HBV-DNA episomal forms and the different patterns of histological activity within the liver tissue of chronic HBsAg positive patients with and without HDV superinfection. We also compared the results of the molecular analysis

with the immunohistochemical markers of viral activity.

Material and methods

Percutaneous liver biopsy specimens from 50 chronic HBsAg carriers consecutively admitted to hospital between 1983 and 1987 were studied. Thirteen of them were positive for hepatitis B e antigen (HBeAg) and 37 for the corresponding antibodies (anti-HBe); 11 HBeAg and five anti-HBe patients had HBV-DNA in their serum. None was found to be positive for IgM antibodies to HBcAg (IgM anti-HBc), indicating chronic HBV infection. Ten patients were positive for antibodies to delta antigen.

Serological markers of HBV and HDV infection were measured by commercial radioimmunoassays (AUK 3, AB AUK 3, CORE IGMK, AB DELTA, Sorin Biomedica, Saluggia, Italy). Serum HBV-DNA was detected by a molecular hybridisation technique, as previously described.¹⁰

Fig 1 *An example of low lobular activity. Few inflammatory infiltrates are present in this lobule. (Haematoxylin and eosin.)*

Fig 2 *An example of high lobular activity. Inflammatory infiltrates and degenerative changes are visible in the lobule. (Haematoxylin and eosin.)*

None of the patients had ever been treated with antiviral or immunosuppressive drugs.

Each liver biopsy sample was divided into two parts: one was fixed in formalin and embedded in paraffin wax for histological and immunohistochemical evaluation; the other was immediately frozen and stored at -70°C until further molecular analysis. The specimens were stained with haematoxylin and eosin and the diagnosis was made according to generally accepted criteria.¹¹ The degree of lobular activity was evaluated by analysing mesenchymal cell reaction, foci of necrosis, inflammatory infiltrates and degenerative and regenerative liver cell changes. The presence of only a few foci of necrosis and the absence of cellular changes were regarded as indicative of a low degree of lobular activity (fig 1). Foci of inflammatory necrosis were indicative of a high degree of lobular activity and cellular modifications observed in each lobule (fig 2).

Immunoperoxidase staining for HBcAg and delta antigen was done on paraffin wax sections of each liver biopsy specimen; for HBcAg a peroxidase-anti-peroxidase kit was used (HBcAg PAP kit system, Dako Corporation, Santa Barbara, California, USA); delta antigen was detected by a peroxidase labelled specific antiserum, kindly donated by Drs M Rizzetto and C Lavarini.

For hybridisation, DNA was extracted from tissue and analysed by the Southern blot technique, as previously described¹²; in brief, liver biopsy specimens were homogenised in 150 mM NaCl and 50 mM Tris-HCl (pH 7.4) and 10 mM EDTA 0.1% sodium dodecyl sulphate and incubated overnight with proteinase K (800 $\mu\text{g}/\text{ml}$) at 37°C . After extraction with phenol: chloroform (1:1 v/v) the nucleic acid was precipitated

in three volumes of pure cold ethanol. The nucleic acid was resuspended and digested with pancreatic ribonuclease (100 $\mu\text{g}/\text{ml}$), followed by digestion with proteinase K (100 $\mu\text{g}/\text{ml}$), extraction with phenol: chloroform and reprecipitation in pure cold ethanol. The DNA was resuspended in 10 mM Tris-HCl (pH 7.4) – 1 mM EDTA, and the concentration of nucleic acid in solution was determined by spectrophotometry at 260 nm; after digestion with restriction endonuclease *Hind III* 10–15 μg of DNA were electrophoresed through 0.8% agarose gel. The separated DNA was transferred to microcellulose filter sheets and the filters baked for two hours at 80°C . The filters were prehybridised with 5x SSC-0.1% sodium dodecyl sulphate-50% formamide-0.1% Denhardt's solution and denatured salmon sperm DNA (200 $\mu\text{g}/\text{ml}$) at 42°C overnight, and then hybridised under similar conditions for 16 hours using double stranded, cloned repurified HBV-DNA (3200 base pairs). Purified HBV-DNA was labelled with ^{32}P to high specificity ($2-4 \times 10^8$ cpm/ μg DNA) by using primer extension, as described by Summers.¹³ After hybridisation the nitrocellulose filter was washed, dried, and autoradiographed.

Results

Of the 50 cases examined, 10 (20%) had chronic lobular hepatitis (group 1), 10 (20%) had chronic persistent hepatitis (group 2), and 30 (60%) had chronic active hepatitis (group 3) (table 1).

The evaluation of the lobular activity showed high grade necrosis and inflammation in all group 1 cases and in 23 group 3 cases (table 1). Ten cases, three group 1 and seven group 3, were positive for delta antigen, and all showed high grade lobular activity; none of them showed HBV-DNA or HBcAg reactivity (tables 1–3). HBV-DNA replicative forms were found in 21 cases (five group 1, 16 group 3) (fig 3), and 19 showed high grade lobular activity; HBcAg was detected in 14 of these cases (tables 1–3) with an exclusively nuclear reactivity in 10 and nuclear and cytoplasmic staining in four.

Nineteen liver biopsy specimens were negative for

Table 1 Histological patterns in liver biopsy specimens from 50 chronic HBsAg positive patients

	Total No of cases	High grade lobular necrosis	Low grade lobular necrosis
Group 1	10	10	0
Group 2	10	0	10
Group 3	30	23	7

Table 2 Markers of active HBV and HDV replication in liver tissue of 33 chronic HBsAg positive cases with high grade lobular activity

	Delta antigen	†HBV-DNA* HBcAg*	†HBV-DNA* HBcAg ⁻	†HBV-DNA ⁻ HBcAg*	†HBV-DNA ⁻ HBcAg ⁻
Group 1	3	4	1	0	2
Group 2	0	0	0	0	0
Group 3	7	10	4	0	2

*HBcAg and HBV-DNA negative.

†Replicative intermediate forms.

⁻ negative, * positive.

Table 3 Markers of active HBV and HDV replication in liver tissue of 17 chronic HBsAg positive cases with low grade lobular activity

	*Delta antigen ⁺	†HBV-DNA ⁺ HBcAg ⁺	†HBV-DNA ⁺ HBcAg ⁻	†HBV-DNA ⁻ HBcAg ⁺	†HBV-DNA ⁻ HBcAg ⁻
Group 1	0	0	0	0	0
Group 2	0	0	0	0	10
Group 3	0	0	2	1	4

delta antigen and HBV-DNA (two group 1, 10 group 2, seven group 3) and only one of them was positive for HBcAg with a pattern of nuclear and focal reactivity; 15 of these 19 cases showed low grade of lobular activity (table 3). All 16 patients seropositive for HBV-DNA showed free viral forms in the liver; 13 of these cases were positive and three negative for HBcAg.

Discussion

The detection of markers for HBV and HDV infection in liver tissue seems to be the best way of identifying active viral replication. The determination of conventional serological markers gives only indirect information about the state of infection, and the detection of specific nucleic acid sequences in the serum can be misleading because viraemia often fluctuates.

Fig 3 Southern blot hybridisation analysis for HBV-DNA sequences in total DNA extracts from three percutaneous liver biopsy specimens (lanes B–D) showing typical pattern of HBV-DNA replication. The diagraphic band in lane A marks the position of 3.2 kilobase linear HBV-DNA.

HDV is a defective virus with a putative RNA genome whose replication requires the helper function of HBV.⁶ The examination of liver biopsy specimens for HDV-DNA still presents some problems, because of the difficulty of preserving the RNA properly and also because the amount of the RNA obtained from small fractions of tissue is often not sufficient for the test. The best method, therefore, for the diagnosis of active HDV infection remains finding delta antigen in the liver tissue. On the other hand, molecular hybridisation techniques have been successfully used to detect HBV-DNA sequences in liver specimens^{9 12 14} and they give clinically important information about the HBV infection.

Ten of the cases studied were positive for delta antigen in the liver. Because the pathogenicity of delta virus probably results from direct cell damage, it is not surprising that in all the delta positive biopsy specimens that we studied pronounced lobular necrosis was observed. Moreover, there is strong evidence to suggest that delta virus infection plays an important part in aggravating liver disease in chronic HBsAg carriers; seven of our HDV positive patients had severe chronic hepatitis.^{4 6} The other three patients with delta antigen in their livers had chronic lobular hepatitis: in these subjects delta virus superinfection may be a recent event and the disease might progress to a more severe form of chronic hepatitis.⁴ The absence of HBcAg and HBV-DNA episomal forms in the liver tissue of such patients confirms that HDV inhibits HBV replication.^{15 16}

Of the 40 biopsy specimens negative for delta antigen, 21 showed replication of intermediate forms of HBV-DNA; only 14 of them were also positive for HBcAg. These data seem to show that the analysis of hepatic DNA is more sensitive than the study of viral antigens for showing active HBV replication. The inhibition of production of core proteins may be the first step in a process which eventually halts active HBV-DNA replication.¹⁷

As we observed in the liver biopsy specimens positive for delta antigen, the presence of intrahepatic HBV-DNA replicative forms seems to be associated only with the histological finding of high grade lobular activity. In fact, 19 of 21 cases with HBV-DNA free forms showed prominent necrosis, while in 15 of the 19

cases in which the HBV-DNA was absent, none or very few necrotic foci were observed. These data indicate that necrosis in chronic HBV hepatitis is related to the formation of complete virion particles, even if hepatocyte injury is probably mediated by host immune response.

Recent observations suggest that mild forms of chronic hepatitis may progress to more severe chronic disease if active viral replication persists.^{4,18} In chronic HBsAg positive patients, therefore, the analysis of liver biopsy specimens for HBV and HDV markers of active infection seems to be helpful for correct diagnosis, prognosis, and appropriate management.

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References

- Hadziyannis SJ, Lieberman HM, Karvountzis GG, Shafritz DA. Analysis of liver disease, nuclear HBcAg, viral replication and hepatitis B virus DNA in liver and serum of HBeAg Vs. anti-HBe positive carriers of hepatitis B virus. *Hepatology* 1983;3:656-62.
- Bonino F, Rosina F, Rizzetto M, et al. Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. *Gastroenterology* 1986;90:1268-73.
- Ramalbo F, Brunetto MR, Rocca FG, et al. Serum markers of hepatitis B virus replication, liver histology and intrahepatic expression of hepatitis B core antigen. *J Hepatol* 1988;7:14-20.
- Rizzetto M, Verme G, Recchia S, et al. Chronic hepatitis in carriers of hepatitis B surface antigen with intrahepatic expression of the delta agent. *Ann Int Med* 1983;98:437-41.
- Craxi A, Raimondo G, Longo G, et al. Delta agent infection in acute hepatitis and chronic HBsAg carriers with and without liver disease. *Gut* 1984;25:1288-90.
- Rizzetto M, Verme G. Delta hepatitis: present status. *J Hepatol* 1985;1:187-93.
- Chu CM, Liaw YF. Intrahepatic distribution of hepatitis B surface and core antigens in chronic hepatitis B virus infection. *Gastroenterology* 1987;92:220-5.
- Yoo JY, Howard R, Waggoner JG, Hoffnagle JH. Peroxidase-anti-peroxidase detection of hepatitis B surface and core antigens in liver biopsy specimens from patients with chronic type B hepatitis. *J Med Virol* 1987;23:273-81.
- Bonino F. The importance of hepatitis B viral DNA in serum and liver. *J Hepatol* 1986;3:136-41.
- Lieberman HM, LaBrecque DR, Kew MC, Hadziyannis SJ, Shafritz DA. Detection of hepatitis B virus DNA in human serum by a simplified molecular hybridisation test: comparison to HBeAg/anti-HBe status in HBsAg carriers. *Hepatology* 1983;3:285-91.
- Phillips MJ, Poucell S. Modern aspects of the morphology of viral hepatitis. *Hum Pathol* 1981;12:1060-84.
- Shafritz DA, Shouval D, Sherman HI, Hadziyannis SJ, Kew MC. Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. *N Engl J Med* 1981;305:1067-73.
- Summers J. Physical map of polyoma viral DNA fragments produced by cleavage with a restriction enzyme from Haemophilus aegyptius endonuclease R Hae III. *J Virol* 1975;15:946-53.
- Brechot C, Hadchouel M, Scott J, et al. Detection of hepatitis B virus DNA in liver and serum: a direct appraisal of the chronic carrier state. *Lancet* 1981;ii:765-8.
- Negro F, Chaberge E, Olivero S, et al. Hepatitis B virus DNA (HBV-DNA) in anti-HBe positive sera. *Liver* 1984;4:177-83.
- Hadziyannis SJ, Sherman M, Lieberman HM, Shafritz DA. Liver disease activity and hepatitis B virus replication in chronic delta antigen positive B virus carriers. *Hepatology* 1985;5:544-8.
- Raimondo G, Burk RD, Lieberman HM, et al. Interrupted replication of hepatitis B virus in liver tissue of HBsAg carriers with hepatocellular carcinoma. *Virology* 1988;166:103-12.
- Paz MOA, Brenes F, Karayiannis P, et al. Chronic hepatitis B virus infection. *J Hepatol* 1986;3:371-7.

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