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Detection of HBV-DNA in liver biopsy and serum: its significance in the selection of hepatitis B patients for antiviral therapy

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Summary

We have characterized the hepatitis B virus state in liver and serum of 38 HBsAg-positive chronic hepatitis patients (chronic active hepatitis (CAH), 19; chronic persistent hepatitis (CPH), 7; cirrhosis, 11; 'normal' carrier, 1) and 21 HBsAg-negative patients. Episomal HBV-DNA in liver, without detectable integrated HBV-DNA sequences, concomitant with HBV-DNA in serum was found in 19 HBeAg-positive patients (CAH, 16; CPH, 1; cirrhosis, 2). Integrated sequences were detected in 13 HBsAg-positive HBeAg-negative patients (CAH, 1; CPH, 5; cirrhosis, 7) and in 1 HBsAg-negative patient. Episomal HBV-DNA and integrated HBV-DNA sequences were observed simultaneously in 1 HBsAg-positive HBeAg-negative CPH patient and in 4 HBsAg-positive cirrhosis patients (2 HBeAg-positive, 2 HBeAg-negative). The presence of HBcAg immunofluorescence corresponded well with that of episomal HBV-DNA. Antiviral therapy is advised for HBsAg-positive chronic hepatitis patients with episomal HBV-DNA, irrespective of the presence of integrated sequences. Since the presence of episomal HBV-DNA in liver is not always accompanied by the presence of serum HBV-DNA, procedures for the selection and evaluation of patients for antiviral therapy should be extended by characterization of the HBV-DNA state in liver biopsies.

Hepatitis B virus; Chronic hepatitis; HBV-DNA; Integration; Antiviral therapy

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Introduction

Acute hepatitis B virus (HBV) infection may progress to chronic liver disease, which in turn may lead to liver failure or hepatocellular carcinoma (HCC). Preventive measures, such as passive and active immunization reduces the incidence of HBV infection and the subsequent development of chronic liver disease. However, at this time about 200 million carriers remain at risk for the said complications of chronic hepatitis B [38].

Therapeutic trials with antiviral agents like interferon [10,25,29,36,39,41], ara-A [1,17,22,26,43], and acyclovir [42], given as a single agent or in combination [30,31] are in progress. Patients selected for these trials have generally been chronic hepatitis patients with active viral replication (HBeAg-positivity and demonstrable DNA polymerase activity in serum). Since disappearance of HBeAg and DNA polymerase activity from the serum is usually followed by reduction of inflammatory activity in the liver, effective antiviral therapy may inhibit the progression to cirrhosis and HCC.

Independent of antiviral therapy trials, the HBV-DNA state in liver and serum has been a subject of investigation with emphasis on the integration of HBV-DNA sequences in the hepatocyte genome. Integration was first observed in HCC cell lines [6,11,23], subsequently in HCC tissue samples [20,33], and later on in hepatocytes of chronic hepatitis B patients without HCC [4,5,13–15,19,32,35].

However, chronic hepatitis B patients with active viral replication usually have episomal HBV-DNA in their liver cells without detectable integration.

It has been speculated that disappearance of HBeAG in patients with episomal HBV-DNA is accompanied by negative selection of hepatocytes with at random integration. Some of the hepatocytes form a clonal population, all with the same integration pattern [34]. Spontaneous disappearance of HBeAg with regression of liver disease, however, occurs at a lower frequency than that induced by treatment and may therefore be accomplished by a different mechanism [18,21,27,40].

Previously, the guidelines in the selection of patients for antiviral therapy have not been clearly defined, nor has a detailed effort been made to correlate serological and histological findings in this selection process.

In this study we describe the HBV-DNA state in liver biopsies and sera from histologically classified groups of hepatitis B patients with the objective of helping to select candidates for antiviral therapy.

Patients and Methods

Patients were grouped according to standard histologic criteria [8] (Table 1).

Liver tissue

Liver tissue samples were obtained by percutaneous liver biopsy. One part of the tissue samples was immediately snap-frozen and stored in liquid nitrogen until

analysis. Another part was fixed in 10% buffered formaldehyde for routine histological analysis. HBsAg and HBcAg in liver tissue was demonstrated by immunofluorescence technique on slides from snap-frozen liver tissue using anti-HBs (Behringwerke, Frankfurt, F.R.G.) and anti-HBc antibodies (Organon, Oss, The Netherlands). The adjacent part of the snap-frozen samples was used for HBV-DNA analysis.

Serology

The HBV serologic markers HBsAg, HBeAg, anti-HBe and anti-HBc antibodies were determined by radioimmunoassays (Abbott Laboratories, IL, U.S.A.) in sera taken at the time of biopsy. Anti-delta agent antibodies were determined by ELISA (Abbott).

Detection of cellular HBV-DNA

Cellular DNA was isolated from 5–10 mm^3 tissue material. The tissue was suspended in 300 μl TNE (TNE = 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5) and its structure was disrupted with a glass rod. The resulting suspension was incubated overnight at 37°C after addition of proteinase K (Merck, Darmstadt, F.R.G.), final concentration 0.5 mg/ml, sodium dodecyl-sulphate (SDS) (1% w/v) and EDTA, final concentration 10 mM. The mixture was carefully extracted twice with phenol/chloroform, twice with chloroform, and precipitated with ethanol. The precipitate was washed once with 70% ethanol:water and solubilized overnight at 4°C in 50 μl TE buffer (TE = 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). From the DNA (usually about 20 μg) 10 μl was digested with *Eco*RI and another 10 μl with *Hin*dIII, both in restriction buffer according to the supplier (Boehringer, Mannheim, F.R.G.). The 2 samples as well as 5 μl undigested DNA were applied to a 0.8% agarose gel (Seaplaque, Marine Colloids Div., Rockland, U.S.A.). Control samples consisted of 5 μg PLC/PRF/5 cell line DNA. *Hin*dIII-digested bacteriophage lambda DNA was used as a molecular weight marker. The gel was electrophoresed in TRIS/acetate buffer (40 mM Tris-acetate, pH 8.2, 20 mM sodium-acetate, 2 mM EDTA) at 1 V/cm overnight. Thereupon the gel was blotted to nitrocellulose membrane according to Southern [37]. The nitrocellulose membrane was pre-hybridized in a solution containing 3 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 10% dextran sulphate (Pharmacia, Uppsala, Sweden), 0.1% SDS, 5 × Denhardt solution [9], and 100 μg/ml denatured and sheared herring sperm DNA in a plastic box for 1 h. Hybridization was done overnight in an identical solution containing ^{32}P labelled by nick translation HBV-DNA with a specific activity of 5.10^8 cpm/μg with an end concentration of 1.5 ng/ml. The HBV-DNA was the insert of the plasmid pCP10. This plasmid was kindly donated by Dr. C. Bréchot, Institut Pasteur, Paris, France. The 3.2 kb *Eco*RI HBV fragment was separated from the vector by gel electrophoresis and recovered from the gel by electro-elution. This was done twice to obtain a highly purified probe. The specificity of the probe was verified by hybridizing it to about 1 ng of pCP10

and pBR322 DNA, both cut by *Eco*RI, gel separated and blotted to nitrocellulose paper. The probe hybridized with the 3.2 kb HBV-DNA band, but not with the 4.3 kb pBR322 band.

Post-hybridizational washes were for 2 × 20 min each in 3 × SSC, 1 × SSC, and 0.3 × SSC, and 0.1% SDS at 65°C. Autoradiography was for 1–4 days at −70°C with Kodak XAR 5 film with the use of intensifying screens (Cronex Lightning-plus, Dupont, Dreieich, F.R.G.). HBV-DNA on gel was denoted as free or integrated. Free or episomal HBV-DNA was defined in our case as low-molecular weight hybridizing DNA (≤ 3.2 kb), detected simultaneously after *Eco*RI and *Hin*dIII digestion, with a smear pattern at 2.0–2.8 kb and < 1.35 kb, indicating active viral replication. Integration is defined as the appearance of discrete hybridizing bands, greater than the HBV genome length (3.2 kb), after digestion with *Eco*RI and/or *Hin*dIII and hybridization of the undigested high molecular weight DNA. When episomal HBV-DNA is present, weak bands of high molecular DNA indicating integrated sequences can only be seen after long exposure of the autoradiograph against a background of diffuse smear due to the presence of free HBV-DNA. It was estimated that about 0.1 copy/genome of integrated HBV-DNA can be detected when about 5 μg of total DNA is used for digestion.

Detection of HBV-DNA in serum

300 μl serum was incubated with 100 μl extraction buffer (0.1 M sodium acetate, 10 mM EDTA, 2% SDS, 10 mg/ml pronase E, 1.0 mg/ml denatured herring sperm DNA) for 2 h at 65°C. The mixture was once extracted with phenol: chloroform:isoamylalcohol (50:49:1) and once with chloroform:isoamylalcohol (24:1). 50 μl of the watery phase was denatured with 10 μl 2 M NaOH for 10 min at room temperature and neutralized with 10 μl 2 M acetic acid and 100 μl 3 M sodium acetate, pH 4.8. Denatured DNA samples were spotted on nitrocellulose mem-

TABLE 1

Characteristiscs of patients investigated for the presence of HBV-DNA homologous sequences in liver biopsy and serum.

Histology		No.	Sex m/f	Mean age	Range	Origin (*) m/f
'Normal liver'	HBsAg+	1	1/0	41		
	HBsAg−	2	2/0	65	59−71	
Chronic persistent hepatitis	HBsAg+	7	5/2	27	23−28	2/0
	HBsAg−	3	2/1	41	41−42	
Chronic active hepatitis	HBsAg+	19	17/2	34	18−59	1/0
Cirrhosis	HBsAg+	11	11/0	39	17−68	7/0
	HBsAg−	16	9/7	59	18−74	

*Non-Caucasian, from Surinam, Mediterranean countries, or Far East.

TABLE 2

HBV-DNA homologous sequences in liver biopsies and serum from CAH patients.

CAH patients	HBV-DNA in liver		HBV-DNA in serum	HBcAg in nuclei from the liver	Ground glass cells
	Integrated	free			
HBeAg-positive					
Patients 1–16[a,b]	–	+	+	+	sporadic
Patient 17	–	+	–	+	sporadic
HBeAg-negative[c]					
Patient 18	–	+	–	–	numerous
Patient 19	+	–	–	–	sporadic

[a] One patient had anti-delta agent antibodies in serum.
[b] One patient originated from Surinam.
[c] Patient 18: anti-HBe-antibody-positive; patient 19: anti-HBe-antibody-negative.

branes (Schleicher and Schuell, BA85 0.45 μm) in a 96-well BRL HybriDot manifold. Prior to application to the manifold, the membranes were soaked in distilled water. After the denatured samples were spotted, all wells were washed twice with 6 × SSC. Samples passed the membranes within 2 min. After disassembling, the manifold membranes were soaked in 6 × SSC for 5 min at room temperature, air-dried and baked at 80°C for 2 h. Hybridization was performed as described above. All serum DNA samples were compared to a standard serial dilution of cloned HBV-DNA (pCP10) covering the range 0.5–1000 pg.

Results

Patient categories

Table 1 shows the distribution of the 38 HBsAg-positive and 21 HBsAg-negative patients among histologic subgroups. In the control groups special emphasis

TABLE 3

HBV-DNA homologous sequences in liver biopsies and serum from CPH patients.

CPH patients (No.)	HBV-DNA in liver		HBV-DNA in serum	HBeAg	Anti-HBe-antibody	HBcAg in nuclei from the liver	Ground glass cells
	Integrated	free					
20	+	–	–	–	+	–	sporadic
21[a]	+	+	+	–	+	–	–
22	+	–	–	–	+	–	–
23	+	–	–	–	+	–	–
24	+	–	–	–	+	–	numerous
25	–	+	+	+	–	–	numerous
26	–	–	–	–	+	–	–

[a] Non-Caucasian.

was laid on HBsAg-negative cirrhosis patients, since nodular regeneration may favour detection of HBV-DNA integration in patients with a silent HBV infection [3,24].

HBV-DNA characteristics in hepatitis B patients

Table 2 shows data on HBV-DNA in CAH patients. Episomal HBV-DNA was found in liver biopsies of all 17 CAH patients positive for HBeAg. In none of these patients integrated sequences were observed. HBcAg-positive cells varied from 5–50% (mean 34%) in semi-quantitative determinations. Ground glass cells were found sporadically. Serum HBV-DNA was found in 16 out of the 17 patients.

HBV-DNA was also found in the liver of the 2 HBeAg-negative CAH patients (patients 18 and 19). These patients lacked nuclear HBcAg and serum HBV-DNA. Patient 18 had episomal HBV-DNA; patient 19 had integrated HBV-DNA sequences in the absence of episomal HBV-DNA: HBeAg and HBV-DNA had disappeared from the serum 6 months prior to the biopsy and HBV-DNA sequences in liver had not been tested prior to seroconversion.

A second group of patients (Table 3) were affected by CPH. Integrated HBV-DNA sequences were found in 5 out of the 7 cases (70%). The 2 patients with episomal HBV-DNA both had HBV-DNA in serum. HBeAg-positive nuclei were absent in all cases. Numerous 'ground-glass' cells were present in 2 cases. The only HBeAg-positive patient in this group lacked integrated HBV-DNA sequences.

In 7 out of 11 (64%) hepatitis B patients with cirrhosis we found integrated HBV-DNA sequences (Table 4). In 4 cases this was accompanied by episomal HBV-DNA as well as serum HBV-DNA. A positive relationship was found between oc-

TABLE 4

HBV-DNA homologous sequences in liver biopsies and serum from chronic hepatitis B patients with cirrhosis.

Patient No.	HBV-DNA in liver		HBV-DNA in serum	HBeAg	Anti-HBe-antibody	HBcAg in nuclei from the liver	Ground glass cells
	Integrated	free					
27[a]	–	+	+	+	–	+	sporadic
28	–	+	+	+	–	+	sporadic
29	+	+	+	+	–	+	sporadic
30[a]	+	+	+	+	–	+	numerous
31[a]	+	+	+	–	–	+	numerous
32[a]	+	+	+	–	+	+	numerous
33	+	–	–	–	–	+	numerous
34[a,b]	+	–	–	–	+	–	sporadic
35	+	–	–	–	–	–	sporadic
36[a]	–	–	–	–	–	+	numerous
37[a,b]	–	–	–	–	–	–	not detected

[a] Non-Caucasian.

[b] Anti-delta-agent positive in serum.

currence of HBcAg in the nuclei, episomal HBV-DNA, and HBV-DNA in serum (6 out of 7: 86%). HBeAg was found in 4 out of these 7 cases. Numerous 'ground glass' cells were seen in 5 patients and occasionally in 5 others.

One 'normal' carrier (patient 38, serum anti-HBe positive) was investigated for the presence of HBV-DNA in liver biopsy and serum. Both samples were HBV-DNA negative. Patient 38 lacked HBcAg-positive nuclei but numerous 'ground-glass' cells were present.

Control patients

To confirm the relationship between HBV infection and the presence of HBV-DNA homologous sequences, whether in serum or in liver biopsy, we examined histological material from HBsAg-negative patients with 'normal' livers (n=2), CPH (n=3) and cirrhosis (n=16) (Table 5). Integrated HBV-DNA sequences were detected in only one (cirrhotic) liver biopsy obtained from a patient with a history of hepatitis B (serum: HBsAg-negative, anti-HBs-negative, anti-HBc-positive). HBV-DNA in serum was undetectable in all cases.

Delta infection

The presence of delta agent infection was a rare occurrence in our patient population. One CAH patient (HBeAg-positive) with abundant episomal HBV-DNA in liver and HBV-DNA in serum but without integrated sequences in the liver sample possessed detectable anti-delta agent antibody in serum. In addition, 2 hepatitis B patients with cirrhosis (HBeAg-negative) had anti-delta agent antibodies in serum but lacked any marker of active hepatitis B viral replication (episomal HBV-DNA, serum HBV-DNA, HBcAg in the nuclei).

TABLE 5

HBV parameters in liver and serum of chronic hepatitis patients negative for HBsAg by immunofluorescence.

	HBV-DNA sequences		Serum markers		
	in liver	in serum	HBsAg	HBeAg	Anti-HBc-antibodies
'Normal' liver					
Patients 39, 40	–	–	–	–	–
CPH					
Patients 41–43	–	–	–	NT	–
Cirrhosis					
Patient 44	+[a]	–	–	NT	+[b]
Patients 45–59	–	–	–	NT	–

NT = not tested.

[a] Integrated.

[b] Anti-HBs-negative.

Discussion

In this study we have characterized the HBV-DNA state in liver and serum of potential candidates for antiviral therapy. In the subgroup of HBeAg-positive CAH patients we could not detect integrated sequences in liver biopsy material. The presence of HBV-DNA in serum corresponded well with the detection of episomal HBV-DNA in liver cells (16/17=94%).

However, the presence of integrated sequences in these cases, although unlikely, cannot be excluded for several reasons. Thus, the presence of high levels of episomal HBV-DNA may interfere with the detection of integrated HBV-DNA sequences. It should be mentioned, though, that in several cases simultaneous presence of episomal and integrated HBV-DNA was observed.

Secondly, integration may be below the detection limit as illustrated by the results obtained in the case of patients 26, 36 and 37. Although HBsAg was detected by immunofluorescence in liver biopsy material, and HBsAg was also detected in serum, HBV-DNA was not found. The presence of HBcAg in nuclei and numerous 'ground-glass' cells in the biopsy sample of patient 36, together with the absence of markers of active viral replication, like serum HBV-DNA or HBeAg, suggests intracellular accumulation of viral components without particle production. Suppression of HBV infection by delta agent [28] is exemplified by patient 37.

Thirdly, biopsy samples may show sampling errors. Although histological evaluation was adjacent to those areas sampled for DNA-assay, differences may occur particularly in cirrhotic patients with known variability between certain areas (nodi).

Finally, integration may occur at random and clonal outgrowth may not have taken place. Conceivably, in CAH the continuous destruction of HBV-infected cells overrides the outgrowth of virus-carrying cell clones with or without integrated sequences. Integration at random may be an intermediate state for CAH patients who develop cirrhosis.

In HBsAg-positive patients with cirrhosis we found active replication without integration (patients 27 and 28), active replication with integration (patients 29 and 30), integration with low grade replication after disappearance of HBeAg (patients 31 and 32), and integration with loss of virus production (patients 33, 34 and 35).

The existence of an intermediate state between virus production and solid integration is suggested by the absence of anti-HBe antibody in 5 out of the 7 HBeAg-negative cases among cirrhotic patients (Table 4), in contrast to the presence of anti-HBe in 6 HBeAg-negative persistent hepatitis B cases, with integration in 5 of these (Table 3).

Integrated sequences were also observed in 1 patient with CAH without HBeAg or anti-HBe in serum, shortly after the disappearance of HBeAg. Later on this patient showed anti-HBe. From 3 other CAH patients with episomal HBV-DNA and serum HBV-DNA, subsequent biopsies were taken shortly after spontaneous seroconversion from HBeAg to anti-HBe. In the latter biopsies, no hybridization was found in liver or serum (unpublished results).

In HBsAg-negative patients ('normal liver', CPH and cirrhosis patients) only 1 case of integration was observed. The patient showed anti-HBc antibodies as a serum marker of contact with the HBV. This result is in accordance with the observations of others [3,24]. Integrated HBV-DNA sequences were found in HBsAg-negative, anti-HBc-positive and/or anti-HBs-positive patients in the absence of serum HBV-DNA.

A simple concept of chronicity in hepatitis B patients adopted by several authors [16,34,35] postulates two states, i.e. the so-called permissive and the non-permissive state. The first is characterized by active viral replication with positive serum markers (HBeAg, DNA polymerase and HBV-DNA), liver HBV-DNA in episomal form, and liver cell destruction. The second state is characterized by the presence of anti-HBe antibody, the absence of HBV-DNA in serum, inactive liver disease, and integrated HBV-DNA.

According to Chu et al. [7], the chronic carrier state starts with an immune tolerance (phase I) to HBV (histology = CPH or non-specific reactive hepatitis (NSRH)) with either HBeAg and high levels of HBV-DNA in serum or anti-HBe without HBV-DNA in serum. This state is followed by immune clearance (phase II) (histology = CAH or chronic lobular hepatitis) with seroconversion to anti-HBe antibody for those with HBeAg in serum and medium levels of HBV-DNA in serum. A prolonged immunoclearance phase leads to cirrhosis. The last phase is characterized by residual integrated HBV (histology = CPH or NSRH), presence in serum of anti-HBe antibody, and absence of HBV-DNA. This description of sequential events in chronicity was solely based on HBV-DNA quantities in serum and age of the patients.

According to our experience and that of others [5,14,32,35], the evolution of the HBV-DNA state in chronic hepatitis B patients is more complicated. Active replication (episomal HBV-DNA in liver and serum HBV-DNA) was seen in the presence of integrated sequences (patients 21, 29–32) and also in the presence of anti-HBe antibody in serum (patients 18 and 32, and [2]). Numerous 'ground-glass' cells were seen in biopsies with nuclear HBcAg (patients 30–33, 36).

Our opinion about the natural history of hepatitis B infections will influence the selection for antiviral therapy. In our patient population, CAH patients will be the first candidates for antiviral therapy, based on the absence of integration and the presence of an inflammatory reaction with destruction of hepatocytes. In 3 cases of spontaneous seroconversion reported here, episomal HBV-DNA could no longer be detected in serum and liver, nor were integrated sequences detectable. However, the chronic carrier state was not terminated. From some latent state, HBsAg production still continued but without a host reaction with cell destruction.

Recent experiences suggest that antiviral therapy induces seroconversion of HBeAg [30] as well as HBsAg. In liver biopsies from chronic hepatitis B patients after seroconversion from HBsAg to anti-HBs antibody we could not detect episomal HBV-DNA nor integrated HBV-DNA [R. de Man, submitted for publication]. These patients should be considered as cured.

We consider patients simultaneously positive for episomal HBV-DNA and integrated HBV-DNA (i.e. part of our cirrhotic patients) as additional candidates

for antiviral therapy, although the hepatocytes with integrated HBV-DNA sequences are probably no longer sensitive to antiviral agents which inhibit active viral replication. These patients may benefit from antiviral therapy for 2 reasons. On the one hand therapy may reduce the clinical manifestation of the disease, on the other the regression of active replication and spread of the virus to progeny cells or neighbouring cells may restrict the chance of fatal integration; i.e. integration at a position in the cellular genome ultimately leading to HCC.

In conclusion, candidates for antiviral therapy may be selected according to symptomatology, histologic criteria, active viral replication with HBV-DNA in serum or to the qualitative state of liver cell HBV-DNA. The results presented here clearly show that information about the HBV-DNA state in liver cells is important in optimalizing the homogeneity of the study group in the case of clinical trials with antiviral agents and in evaluating changes in the episomal and integrated HBV-DNA state as a consequence of the therapy, since our knowledge of antiviral drug-induced changes is limited [12,44].

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