Distribution of HBcAg in hepatitis B detected by immunoperoxidase staining with three different preparations of anti-HBc antibodies

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SUMMARY To evaluate the role of the expression of hepatitis B core antigen (HBcAg) in liver cell damage the immunoperoxidase staining pattern of cryostat liver biopsy specimens from 16 chronic carriers of hepatitis B surface antigen (HBsAg) was investigated using three different kinds of anti-HBc antibodies. Polyclonal antibody prepared from recombinant HBcAg seemed to be more sensitive in detecting HBcAg than did monoclonal antibody from the same antigen. The topographical distribution of HBcAg detected by these two antibodies was similar, showing a close correlation to the histological activity of disease. Furthermore, the predominant localisation of cytoplasmic HBcAg usually reflected an active and severe ongoing hepatitis. On the other hand, monoclonal antibody prepared from purified Dane particles resulted in the prominent cytoplasmic staining for HBcAg regardless of histological severity of the hepatitis. The quantitative expression and topographical distribution of HBcAg depended on the type of anti-HBc antibodies used.

Three different antigens have been identified in the sera of persons infected with hepatitis B virus: surface antigen, core antigen, and e antigen. Hepatitis B core antigen (HBcAg) exists on the core of Dane particles, and hepatitis B e antigen (HBeAg) is a protein separate from the other particulate antigens. The presence of HBeAg in the serum correlates with HBcAg as an antigenic marker of Dane particles¹ and a high reactivity for HBsAg-associated DNA polymerase which is contained in the core of Dane particles.^{2,3}

The presence of HBcAg in infected liver tissue is usually assumed to indicate ongoing virus replication.⁴⁻⁸ Furthermore, HBcAg has been proposed as a possible immunological target for T cell mediated hepatocyte injury in chronic hepatitis B virus infection in experiments using autologous hepatocytes.⁹ Several investigators have attempted to correlate the distribution of HBcAg in the hepatocytes with the extent of liver inflammatory activity, but the results remained inconclusive.⁴⁻⁸ HBcAg was principally seen within hepatocyte nuclei. A more recent study showed that cytoplasmic and membrane associated HBcAg was found to be more widespread

than suspected when an improved immunochemical method was used.¹⁰ Our previous study also showed that HBcAg was found mainly in the cytoplasm of hepatocytes in patients with type B chronic liver disease.¹¹

These findings led us to consider that the difference of antibodies used to determine the localisation of HBcAg might have been responsible for the disparate results. The method used might also be an important factor in influencing the sensitivity of detection. We conducted the present study with three different antibodies to HBcAg to evaluate the localisation of HBcAg in livers chronically infected with hepatitis B virus by staining cryostat sections with immunoperoxidase.

Material and methods

Sixteen patients with chronic hepatitis B virus infection were studied. Ten were seropositive for HBeAg and five were seropositive for anti-HBe. Histological diagnoses were made according to the criteria of an international group.¹² Of the 16 cases, 10 had chronic active hepatitis, four had chronic persistent hepatitis, and two had non-specific reactive hepatitis. Details of the patients studied are summarised in table 1.

Serum HBsAg, anti-HBs, anti-HBc, HBeAg and

Distribution of HBcAg in hepatitis B detected by immunoperoxidase staining

Table 1 Clinical data and distribution of HBcAg in livers of 16 chronic HBsAg carriers

Case no	Age (years)	Sex	Histological diagnosis	Serum HBcAg/anti-HBe	DNA polymerase (cpm)	Intrahepatic distribution of HBcAg					
						096D		BA8		Hyb3105	
						Nucleus of hepatocyte	Cytoplasm of hepatocyte	Nucleus of hepatocyte	Cytoplasm of hepatocyte	Nucleus of hepatocyte	Cytoplasm of hepatocyte
1	26	M	Non-specific reactive hepatitis	+/-	16613	2	2	1	2	1	2
2	19	F	Non-specific reactive hepatitis	+/-	1302	2	3	1	2	0	2
3	50	M	Chronic persistent hepatitis	+/-	9469	4	3	4	2	0	2
4	42	F	Chronic active hepatitis	+/-	13	1	3	1	2	0	2
5	36	M	Chronic active hepatitis	+/-	3280	1	2	0	2	0	2
6	32	F	Chronic active hepatitis	+/-	2183	0	3	0	2	0	2
7	26	M	Chronic active hepatitis	+/-	593	0	2	0	1	0	1
8	30	M	Chronic active hepatitis	+/-	529	1	2	0	0	0	0
9	20	F	Chronic active hepatitis	+/-	2	1	1	0	0	0	0
10	36	M	Chronic active hepatitis	+/-	270	0	1	0	0	0	0
11	24	F	Chronic active hepatitis	+/-	30	0	2	0	0	0	0
12	32	M	Chronic persistent hepatitis	-/+	263	2	3	0	3	0	2
13	50	F	Chronic persistent hepatitis	-/+	0	0	2	0	0	0	0
14	25	M	Chronic persistent hepatitis	-/+	3	0	0	0	0	0	0
15	26	M	Chronic active hepatitis	-/+	617	1	1	0	0	0	0
16	38	M	Chronic active hepatitis	-/+	0	0	0	0	0	0	0

Scale of 0 to 4 corresponding to positivity in 0%, 1-10%, 11-30%, 31-50%, > 50% of total hepatocytes examined.

096D = Lot No of polyclonal anti-HBc antibody to recombinant HBcAg, BA8 = monoclonal antibody (clone BA8) to recombinant HBcAg, Hyb 3105 = monoclonal antibody (clone 3105) to core of Dane particle.

anti-HBe were assayed by commercially available radioimmunoassay kits (Abbott Laboratories, Chicago, Illinois).

Three kinds of antibodies to HBcAg were used as first antibody for the immunoperoxidase procedure. First, rabbit polyclonal whole serum to recombinant HBcAg (Lot No 096D) was purchased from Dako Corporation (Santa Barbara, California) and diluted to 1/200. HBcAg as immunogen was purified from lysates of *Escherichia coli* clones containing the viral core DNA, according to the method of Stahl *et al.*¹³ Second monoclonal antibody (produced by clone BA8) to recombinant HBcAg (purchased from Biogen SA, Geneva, Switzerland) was kindly provided by Green Cross Corporation (Osaka, Japan)¹⁴ and used at a concentration of 100 µg/ml. Thirdly, monoclonal antibody (produced by cell line 3105) to HBcAg, in which core particles were prepared from Dane particles purified from sera of asymptomatic carriers of HBsAg,¹⁵ was kindly provided by Dr M Imai at Jichi Medical School (Tochigi-Ken, Japan) and used at a concentration of 20 µg/ml. All dilutions were done by adding 2% bovine serum albumin (BSA) to phosphate buffered saline (PBS) to reduce unwanted background staining.

Liver biopsy samples obtained from all patients were divided into two parts. One part was fixed in 10% formalin for routine histological examination. The other portion was fixed in a periodate-lysine-paraformaldehyde (PLP) solution for immunohistochemistry.¹⁶ Cryostat consecutive sections (6µm) of the fixed liver samples were pretreated with hydrogen peroxidase and methanol to inactivate endogenous tissue

peroxidase. The sections were incubated with three kinds of monoclonal and polyclonal antibodies to HBcAg as first antibody at 4°C for 24 hours. After washing in PBS they were treated with normal goat serum for 10 minutes and followed by incubation with horseradish-peroxidase-conjugated goat F(ab')₂ anti-mouse IgG diluted 1/80 or goat anti-rabbit IgG diluted 1/80 (TAGO Corporation, Burlingame, California) (second antibody), which had been absorbed to remove cross reactivity to human serum proteins at 4°C for 12 hours. After washing in PBS they were treated with diaminobenzidine solution containing hydrogen peroxidase for 10 minutes, counterstained with methyl green, dehydrated, and mounted. All reagents were previously absorbed against normal human liver. No staining was seen using negative control reagents such as normal mouse or rabbit serum in place of anti-HBc antibodies as the first antibody. There was no staining when polyclonal and monoclonal anti-HBc used here were preincubated with rHBcAg¹³ or purified core particles.¹⁵

Each slide was coded and read by two independent observers on a light microscope. The expression of HBcAg was scored on a 0 to 4 scale corresponding to positivity in 0%, 1-10%, 11-30%, 31-50%, > 50% of total hepatocytes examined.

The difference in the number of hepatocytes positive for HBcAg between two groups of patients was compared using the Wilcoxon rank sum test.

Results

HBcAg in liver cells from 16 patients was found in 14

Table 2 Association between serological patterns (HBcAg/anti-HBe and DNA polymerase) and intrahepatic distribution of HBcAg

Serum patterns or histological diagnosis	No of cases	Intrahepatic distribution of HBcAg (positive No)					
		096D		BA8		Hyb 3105	
		Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
HBcAg Positive	11	7	11	4	7	2	7
Negative	5	2	3	0	1	0	1
DNA polymerase Positive	11	7	11	3	7	2	7
Negative	5	2	3	1	1	0	1
Non-specific reactive hepatitis	2	2	2	2	2	1	2
Chronic persistent hepatitis	4	2	3	1	2	1	2
Chronic active hepatitis	10	5	9	1	4	0	4

096D = Lot No of polyclonal anti-HBc antibody to recombinant HBcAg, BA8 = monoclonal antibody (clone BA8) to recombinant HBcAg, Hyb 3105 = monoclonal antibody (clone 3105) to core of Dane particle.

subjects by 096D, eight by BA8, and eight by Hyb 3105 anti-HBc antibody, respectively. Of five patients with negative serum DNA-P values, HBcAg was detected in three by 096D, in one by BA8, and in one by Hyb 3105, while of 11 patients with positive serum DNA-P activities, HBcAg was present in all by 096D, seven by BA8, and seven by Hyb 3105 antibody, respectively (table 2).

When 096D was used, HBcAg was almost equally detected in both the nuclei and cytoplasm in patients with non-specific reactive hepatitis and chronic persistent hepatitis; cytoplasmic expression of HBcAg was more prominent in patients with chronic active hepatitis (tables 1 and 2). Similar results were obtained for HBcAg distribution by BA8, although the sensitivity to detect HBcAg was lower in BA8 than in 096D. On the other hand, Hyb 3105 gave more prominent cytoplasmic staining of HBcAg even in patients with non-specific reactive hepatitis and chronic persistent hepatitis, and the sensitivity of Hyb 3105 for detecting HBcAg was similar to that of BA8.

As shown in the figure, distinct patterns of HBcAg distribution and different numbers of HBcAg positive hepatocytes were seen when different anti-HBc antibodies (096D and Hyb 3105) were used for the frozen sections of the liver from an HBcAg positive patient with chronic persistent hepatitis (case 3).

Discussion

The results of this study have confirmed previous observations by others that there are various patterns of HBcAg distribution in the hepatocytes of patients with chronic hepatitis B virus infection.⁴⁻¹⁰ The important finding of this study is that the quantitative expression and topographical distribution of HBcAg depend on the anti-HBc antibodies used, although they are also closely related to the histological activity and to the antibody status of HBcAg/anti-HBe of the

patients. 096D and BA8 antibodies are derived from the same immunising antigen, rHBcAg of Biogen SA, Switzerland.¹³ 096D is a polyclonal antibody raised in rabbits and BA8 is a monoclonal antibody. These two antibodies gave very similar staining for the topographical distribution of HBcAg, but 096D was more sensitive in detecting HBcAg than BA8. This finding suggests that the affinity of these antibodies to the corresponding epitopes may be different; polyclonal antibody could recognise plural antigenic determinants and then react more strongly with the corresponding antigen. The immunising antigen against Hyb 3105 antibody was core particles which were prepared from Dane particles.¹⁵ Therefore Hyb 3105 may react with an epitope of HBcAg which is distinct from the epitope reacted with BA8. Thus when Hyb 3105 was used, cytoplasmic staining for HBcAg always predominated regardless of histological activity in the liver, unlike the results obtained with 096D or BA8.

Even when studies were performed with the same anti-HBc antibody (Dako corporation) and on the similar category of patients, some investigators noted that HBcAg was distributed mainly in the nuclei, while others observed prominent expression of cytoplasmic HBcAg.^{8,10,17,18} Gowans *et al* showed that of the two immunofluorescence techniques for detecting HBcAg in frozen sections, the indirect method was more sensitive than the direct one.¹⁰ They also indicated that the indirect reaction detected HBcAg in the cytoplasm of a population of cells previously thought to be negative for HBcAg and in the cytoplasm of some cells which were thought to contain only nuclear HBcAg. We fixed liver biopsy samples in a PLP solution for immunohistochemistry. Yamada *et al* reported that PLP fixative effectively preserved the tissue structure and the antigenicity of HBsAg and HBcAg.¹⁹ Our present results were consistent with those of Gowans *et al*.¹⁰

The predominant localisation of cytoplasmic

Figure Comparison of same field in two sequential frozen sections from liver (case 3) stained for HBcAg by immunoperoxidase procedure. (a) HBcAg was distributed both in the nuclei and cytoplasm of many hepatocytes when polyclonal anti-HBc antibody (096D) prepared from recombinant HBcAg was used. (b) HBcAg was detectable in the cytoplasm of many hepatocytes, but nuclear staining was seen only in a few hepatocytes when monoclonal anti-HBc antibody (Hyb 3105) prepared from Dane particles was used.

HBcAg usually reflects an active and severe ongoing hepatitis.^{8,18} It remains unclear, however, whether the changes in expression and distribution of HBcAg in liver are the cause or the effect of the worsening of the chronic liver disease. In any case future studies of the clinical importance of hepatic HBcAg as an indicator of virus replication and as a possible target for immune cell damage should clearly distinguish nuclear from cytoplasmic sites of HBcAg. A method (including appropriate anti-HBc antibody) capable of detecting low concentrations of cytoplasmic HBcAg should be used.

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