Hepatocyte expression of HBcAg and serum HBeAg in hepatitis B: Comparison of polyclonal and monoclonal antibodies during a trial of interferon

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Abstract

The distribution and quantitative expression of HBcAg in relation to serum HBeAg and liver histology before and after a trial of interferon in 50 patients with chronic type B hepatitis were evaluated using polyclonal and monoclonal antibodies. In general, both antisera showed a similar pattern in terms of the distribution of HBcAg, with predominant localisation of HBcAg in the cytoplasm in HBeAg positive patients with chronic active hepatitis. Semiquantitative analysis showed, however, that there was a higher degree of cytoplasmic expression of HBcAg with polyclonal than with monoclonal anti-HBc. Some of the HBeAg positive patients with only a focal expression of HBcAg in the cytoplasm by polyclonal anti-HBc showed no expression of HBcAg with monoclonal anti-HBc. The expression of HBcAg with polyclonal anti-HBc correlated better with the histological features of chronic active hepatitis or the persistence of serum HBeAg on follow up, suggesting that it did not result from non-specific or false positive staining. All of the HBeAg negative patients with minimal histological changes or inactive cirrhosis were HBcAg negative with both antisera.

In conclusion, though both polyclonal and monoclonal antibodies produced a quite similar distribution of HBcAg in patients with chronic type B hepatitis, polyclonal antibody seemed to be more sensitive in detecting HBcAg in the cytoplasm than did monoclonal anti-HBc, and the expression of HBcAg with polyclonal anti-HBc correlated better with the clinical and histological outcome.

In chronic hepatitis B virus (HBV) infection, the presence of hepatitis B core antigen (HBcAg) in infected liver detected by immunohistochemical techniques is usually assumed to indicate ongoing virus replication.¹⁻⁶ HBcAg has been typically seen in hepatocyte nuclei by direct immunofluorescence using fluorescein conjugated human anti-HBc.^{1,2,5,6} Cytoplasmic HBcAg has also been reported using indirect immunofluorescence or immunoperoxidase with human anti-

HBc, but the clinical importance of the different cellular locations remained unclear.^{3,4} In a recent study reported by Gowans and Burrell⁷ widespread expression of cytoplasmic and membrane associated HBcAg was shown by an improved immunochemical method using rabbit anti-HBc. Since then, several investigators have shown that the predominant cytoplasmic localisation of HBcAg in chronic HBV infection usually reflects an active and severe ongoing hepatitis.⁸⁻¹¹ The specificity, however, of the cytoplasmic expression of HBcAg shown in these studies using polyclonal anti-HBc required further investigation. Monoclonal antibodies against HBcAg have recently become available for tissue staining.¹²⁻¹⁴ Studies by Kakumu *et al* showed that monoclonal anti-HBc seemed to be less sensitive in detecting HBcAg in liver than did rabbit polyclonal anti-HBc.¹⁴ These findings also argued against the specificity of the cytoplasmic staining of HBcAg using polyclonal anti-HBc.

As the expression of HBcAg in liver in chronic HBV infection has both diagnostic and prognostic implications, the evaluation of antisera for usefulness in immunohistochemistry is of great importance. In this study intrahepatic expression of HBcAg was investigated in patients with chronic type B hepatitis before and after a trial of interferon. The quantitative expression and distribution of HBcAg in liver were correlated with clinical outcome in terms of serum HBeAg and histological activity using a commonly used polyclonal anti-HBc and a commercially available monoclonal anti-HBc, respectively.

Methods

Paired liver biopsy specimens before and 18 months after a trial of interferon (a 12 week course of human lymphoblastoid interferon $\alpha 2$; Wellferon, $4-6 \times 10^6$ IU/m² intramuscularly three times a week) were taken from 50 Chinese men with chronic type B hepatitis. Their ages ranged from 19 to 56 years. All of them had been seropositive for HBsAg and HBeAg for more than six months and had histological features of chronic active hepatitis before the start of the trial. Patients were not positive for antibody against hepatitis delta virus (anti-HDV). None admitted intravenous drug abuse, nor had any ever received antiviral or immunosuppressive

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treatment. Serum HBsAg, HBeAg, anti-HBe and anti-HDV were assayed using commercially available radioimmunoassay kits (Ausria-II, HBe-RIA, and anti-delta, Abbott Laboratories, North Chicago, Illinois, USA).

Liver biopsy specimens were obtained by percutaneous needle biopsy under local anaesthesia with a Menghini needle. Fragments of specimens were snap-frozen in isopentane cooled in 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. Consecutive cryostat sections were stained for HBcAg by indirect immunofluorescence using rabbit polyclonal and murine monoclonal antibodies, respectively. The rabbit polyclonal anti-HBc was purchased from Dako Corporation (Santa Barbara, California). The staining of HBcAg by indirect immunofluorescence on frozen sections using rabbit anti-HBc was performed according to the method described by Gowans and Burrell,⁷ as reported elsewhere.⁸ The monoclonal antibody (produced by cell line 3105) to HBcAg, in which core particles were prepared from Dane particles purified from asymptomatic carriers of HBsAg,¹⁵ was purchased from the Institute of Immunology, Tokyo, Japan, and used at a concentration of 20 $\mu\text{g}/\text{ml}$. Cryostat sections (5 μm) were dried overnight at room temperature and fixed in acetone at 4°C for 10 minutes, followed by extensive washing with phosphate-buffered saline (PBS) before staining. The sections were incubated with monoclonal anti-HBc as primary antibody at room temperature for 60 minutes. After washing in PBS they were incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories, West Grove, Pennsylvania) diluted 1 in 50 for 60 minutes. All reagents were previously absorbed against normal human liver. Each slide was coded and read by two independent observers on a Zeiss fluorescence microscope (Carl Zeiss, Inc, Thornwood, New York) without knowledge of the clinical and laboratory diagnosis and the antisera used.

The expression of HBcAg was scored semiquantitatively on a 0 to 4 scale corresponding to positivity in 0%, 1–10%, 11–25%, 26–50% and more than 50% of the total hepatocytes examined.

Statistical analyses were conducted using the χ^2 test with Yates' correction, Fisher's exact test, McNemar's test, and the Wilcoxon signed-rank test, where appropriate.

Results

The correlation between serum HBeAg and histological diagnosis before and after the trial of interferon are summarised in table 1. Twenty eight (56%) patients remained persistently HBeAg positive 18 months after the trial. Of these, 17 had histological features of chronic active hepatitis and the other 11 had only minimal histological changes. Twenty two (44%) patients cleared HBeAg from their serum, including 20 with anti-HBe seroconversion. Of these, only one case still had histological features of chronic active hepatitis and the other 21 had only minimal histological changes or inactive cirrhosis.

BEFORE THE TRIAL

Of the 50 HBeAg positive patients with chronic active hepatitis, all were HBcAg positive with polyclonal anti-HBc (one pure nuclear staining, 26 mixed nuclear and cytoplasmic staining, and 23 pure cytoplasmic staining). Forty six (92%) cases were HBcAg positive with monoclonal anti-HBc (one pure nuclear staining, 24 mixed nuclear and cytoplasmic staining, and 21 pure cytoplasmic staining). The expression of HBcAg showed a good correlation with these two antisera (McNemar's test, $p > 0.05$). Semiquantitative analysis, however, showed that while nuclear expression of HBcAg showed no significant difference with these two antisera, the polyclonal antibody gave a significantly higher degree of cytoplasmic HBcAg expression than did the monoclonal anti-HBc (Wilcoxon signed rank test, $p < 0.001$) (table 2). Four patients with focal expression of HBcAg in the cytoplasm (less than 10% of cells) detected by using polyclonal antibody were HBcAg negative with monoclonal antibody.

AFTER THE TRIAL

HBcAg was positive in 21 (42%) cases with polyclonal anti-HBc (seven mixed nuclear and cytoplasmic staining, 14 pure cytoplasmic staining) and in 17 (34%) cases with monoclonal anti-HBc (six mixed nuclear and cytoplasmic staining, 11 pure cytoplasmic staining). The expression of HBcAg showed a good correlation with these two antisera (McNemar's test, $p > 0.05$). Polyclonal anti-HBc, however, also showed a significantly higher degree of cytoplasmic expression of HBcAg than did monoclonal anti-HBc (Wilcoxon signed rank test, $p < 0.01$); the nuclear expression of HBcAg showed no significant difference (table 3).

In HBeAg positive patients the expression of HBcAg correlated significantly with the liver histology with polyclonal anti-HBc (16 of 17 with chronic active hepatitis compared with four of 11 with minimal histological changes; $p < 0.01$), as well as with monoclonal anti-HBc (14 of 17 with chronic active hepatitis com-

Table 1 Intrahepatic expression of HBcAg before and after interferon: correlation with serum HBeAg and histological results

Category	HBcAg expression (No/%)	
	Polyclonal	Monoclonal
Before trial		
HBeAg positive chronic active hepatitis (n = 50)	50 (100)	46 (92.0)
After trial		
HBeAg positive chronic active hepatitis (n = 17)	16 (94.1)*	14 (82.4)*
HBeAg positive minimal histological change (n = 11)	4 (36.3)*	2 (18.2)*
HBeAg negative chronic active hepatitis (n = 1)	1 (100)	1 (100)
HBeAg negative minimal histological change (n = 18)	0	0
HBeAg negative inactive cirrhosis (n = 3)	0	0
Subtotal (n = 50)	21 (42)	17 (34)

* $p < 0.01$, χ^2 with Yates' correction.

Table 2 HBcAg expression before interferon: semiquantitative comparison of polyclonal and monoclonal antibodies

Cellular location	Nuclear					Cytoplasmic				
	0	1+	2+	3+	4+*	0	1+	2+	3+	4+*
0†	23	2	0	0	0	1	4	0	0	0
1+	0	13	0	0	0	0	3	2	0	0
2+	0	0	7	0	0	0	0	19	7	0
3+	0	0	0	4	0	0	0	0	12	0
4+	0	0	0	0	1	0	0	0	0	2

Scale of 0 to 4+ corresponding to positivity in 0%, 1–10%, 11–25%, 26–50%, >50% of total hepatocytes examined.

*Polyclonal antibody, †monoclonal antibody.

pared with two of 11 with minimal histological changes; $p < 0.01$) (table 1). Four HBeAg positive patients (two with chronic active hepatitis and two with minimal histological changes) who were positive for HBcAg in the cytoplasm in less than 10% of cells with polyclonal anti-HBc were negative for HBcAg with monoclonal antibodies. Of the 11 HBeAg positive patients with minimal histological changes, six subsequently seroconverted to anti-HBe within six months on follow up and the other five were persistently HBeAg positive. The expression of HBcAg with polyclonal antibody correlated inversely with subsequent HBeAg/anti-HBe seroconversion ($p < 0.05$), while that with monoclonal antibody did not (table 4).

Only one HBeAg negative patient whose histological results still showed chronic active hepatitis was HBcAg positive, and the others with only minimal histological changes or inactive cirrhosis were HBcAg negative with both polyclonal and monoclonal antibody.

Discussion

Since Gowans and Burrell described a more widespread cytoplasmic expression of HBcAg in the infected liver than previously suspected,⁷ several investigators including ourselves have not only confirmed these findings, but have also shown a significant correlation between the predominant localisation of HBcAg in the hepatocyte cytoplasm and active or severe ongoing hepatitis in chronic type B hepatitis.^{8–11} The recent availability of monoclonal antibody for staining tissue HBcAg has enabled us to evaluate further the specificity of cytoplasmic HBcAg expression in chronic HBV infection. In this study extensive cytoplasmic expression of HBcAg in liver, though quantitatively less than with polyclonal antibody, has also been shown with the monoclonal

Table 3 HBcAg expression after interferon: semiquantitative comparison of polyclonal and monoclonal antibodies

Cellular location	Nuclear					Cytoplasmic				
	0	1+	2+	3+	4+*	0	1+	2+	3+	4+*
0†	43	1	0	0	0	29	4	0	0	0
1+	0	4	0	0	0	0	3	2	0	0
2+	0	0	2	0	0	0	0	5	2	1
3+	0	0	0	0	0	0	0	0	2	0
4+	0	0	0	0	0	0	0	0	0	2

Scale of 0 to 4+ corresponding to positivity in 0%, 1–10%, 11–25%, 26–50%, >50% of total hepatocytes examined.

*Polyclonal antibody, †monoclonal antibody.

Table 4 Correlation of HBcAg expression and subsequent HBeAg seroconversion in HBeAg positive patients with minimal histological changes

HBcAg expression	HBeAg seroconversion		p value*
	Yes	No	
Polyclonal antibody			
Positive	0	4	0.03
Negative	6	1	
Monoclonal antibody			
Positive	0	2	0.36
Negative	6	3	

*Statistical analysis by Fisher's exact test.

antibody in HBeAg positive patients with chronic active hepatitis (table 2). These findings seemed to confirm further the clinical importance of cytoplasmic expression of HBcAg in chronic HBV infection, and add support to our previous postulation that hepatocytes with cytoplasmic or membranous HBcAg are possible targets for immune hepatocytolysis in chronic type B hepatitis.⁸

Furthermore, the results of our study showed that, though both polyclonal and monoclonal antibodies used gave a quite similar distribution of HBcAg in chronic type B hepatitis, on semiquantitative analysis the polyclonal antibody seemed to be more sensitive in detecting HBcAg in the cytoplasm than did the monoclonal antibody (tables 2 and 3). Meanwhile, some of the HBeAg positive patients in whom the expression of HBcAg with polyclonal antibody was only focal in the cytoplasm were indeed negative for HBcAg by the monoclonal antibody. The expression of HBcAg detected only with polyclonal antibody raises the question of its specificity. Further analysis of these cases showed that most of them (six out of eight, table 1) had histological features of chronic active hepatitis, and the other two had only minimal histological changes. Notably, in these HBeAg positive patients with minimal histological changes the expression of HBcAg with polyclonal antibody correlated inversely with subsequent HBeAg/anti-HBe seroconversion (table 4). Similar findings have been reported in which the HBeAg positive patients without HBV-DNA in serum soon clear HBeAg from their serum with anti-HBe seroconversion on follow up,¹⁶ which agrees with the previous suggestion that during the natural history of chronic HBV infection the loss of serum HBV-DNA or liver HBcAg precedes HBeAg or anti-HBe seroconversion.¹⁷ The expression of HBcAg with polyclonal antibody seems to be of greater diagnostic and prognostic importance than that with monoclonal antibody in chronic HBV infection. Further studies to correlate the cytoplasmic expression of HBcAg with serum HBV-DNA using spot hybridisation or the polymerase chain reaction are required.

The reason for the more extensive expression of HBcAg in the cytoplasm with polyclonal antibody than with monoclonal antibody remains unclear. Theoretically, the polyclonal antibody might recognise plural antigenic determinants and then react more strongly with the corresponding antigen. It has also

been shown that the rabbit polyclonal antibody from Dako Corporation used in the present study contained both anti-HBc and anti-HBe activity.¹⁸ Several studies with monoclonal anti-HBc and anti-HBe have also shown a relatively predominant distribution of HBcAg in the nuclei and HBeAg in the cytoplasm.^{12,13} The polyclonal antibody used here can probably recognise antigenic components derived from both HBcAg and HBeAg in the liver, and the latter, which is predominantly distributed in the hepatocyte cytoplasm, may be missed with monoclonal anti-HBc. This interesting issue needs to be elucidated by double staining with polyclonal anti-HBc and monoclonal anti-HBc or anti-HBe.

In conclusion, both polyclonal and monoclonal antisera showed a quite similar pattern in the distribution of HBcAg in liver, with a predominant localisation of HBcAg in the cytoplasm in HBeAg positive patients with chronic active hepatitis. Semiquantitative analysis, however, showed that polyclonal anti-HBc seemed to be more sensitive in detecting HBcAg in the cytoplasm than monoclonal anti-HBc. The presence of HBcAg in liver with polyclonal anti-HBc correlated better with the histological features of chronic active hepatitis and the persistence of HBeAg in serum, suggesting that the cytoplasmic expression of HBcAg with polyclonal anti-HBc does not result from non-specific or false positive staining.

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