Immunohistological study of intrahepatic expression of hepatitis B core and E antigens in chronic type B hepatitis

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Abstract

Aims: **To study the intrahepatic expression of hepatitis B virus (HBV) nucleocapsid antigen; and to determine the differential distribution of hepatitis B core and E antigens in chronic hepatitis B.**

Methods: **Hepatocyte expression of HBV nucleocupsid antigen was studied using rabbit anti-HBc, directed against both HBcAg and HBeAg; differential distribution of HBcAg and HBeAg was studied using murine monoclonal anti-HBc and anti-HBe in 120 patients with chronic hepatitis B.**

Results: **HBV nucleocapsid antigen was detected in 14 of 16 (87·5%) HBeAg seropositive patients with chronic persistent hepatitis (CPH), and in 54 of 64 (84·4%) HBeAg seropositive patients with chronic active hepatitis (CAH). Nuclear expression of nucleocapsid antigen was more prevalent in patients with CPH than in those with CAH; this was reversed in terms of exclusive cytoplasmic expression of nucleocapsid antigen ($p < 0·05$). Of 45 patients with nucleocapsid antigen in the nucleus, samples from 44 (97·8%) and 17 (37·8%) stained positively with monoclonal anti-HBc and anti-HBe, respectively. Of 65 patients with cytoplasmic nucleocapsid antigen, samples from 61 (93·8%) and 57 (87·7%) stained positively with monoclonal anti-HBc and anti-HBe, respectively.**

Conclusions: **HBV nucleocapsid antigen is more prevalent in HBeAg positive patients with CPH than in those with CAH. Cellular expression of HBcAg and HBeAg in the cytoplasm is more or less the same; in the nucleus HBcAg exceeds HBeAg expression.**

Hepatitis B virus (HBV) is a 42 nm particle (Dane particle) associated with at least three different structural antigens: hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), and hepatitis Be antigen (HBeAg). HBsAg is present on a 7 nm lipoprotein coat that envelopes HBV, while the core particle (nucleocapsid) of HBV contains two closely related antigens, HBcAg and HBeAg.[1] The isolated core particles of HBV contain HBcAg but not HBeAg.[2 3] However, treatment of core particles with detergent and reducing agent results in the apparent conversion of HBcAg into the closely related HBeAg.[2 3]

Intrahepatic distribution of HBsAg and HBcAg has long been studied in chronic HBV infection. HBsAg is expressed on the surface of hepatocytes during active HBV replication, regardless of histological activity, it is expressed exclusively in the hepatocyte cytoplasm when there is no active HBV replication.[4-7] HBcAg is detectable in hepatocyte nucleus or cytoplasm, and the presence of HBcAg in hepatocytes usually indicates active HBV replication.[4-7] Interestingly, in chronic HBV infection the topographical distribution of HBcAg in the liver correlates significantly with levels of HBV viraemia[8] as well as with biochemical and histological activities.[6 9] Notably, intrahepatic expression of HBcAg in most of the previous reports was studied using polyclonal anti-HBc, which might react with HBcAg and HBeAg.[10 11] Recently, monoclonal anti-HBc and anti-HBe have become available for tissue staining. Intrahepatic distribution of HBcAg and HBeAg has been studied in patients with chronic HBV infection, but the results still remain controversial.[8 12-14]

Methods

One hundred and twenty 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. Eighty patients were seropositive for HBeAg and the other 40 were seropositive for antibody against HBeAg (anti-HBe). All were negative for antibody against hepatitis δ virus (anti-HDV). None admitted intravenous drug abuse, nor had they every received antiviral or immunosuppressive treatment. The histological diagnosis of chronic hepatitis was made according to standard criteria.[15] 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, Chicago, Illinois).

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

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Table 1 Mean 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	16	26 (8)	16:0	46 (10)	58 (16)
HBeAg positive CAH	64	30 (9)	62:2	96 (44)	210 (90)
Anti-HBe positive CAH	15	34 (5)	15:0	90 (62)	202 (93)
Anti-HBe positive MHC	25	34 (7)	25:0	26 (10)	34 (15)

HBeAg: hepatitis Be antigen; anti-HBe: antibody against HBeAg; CPH: chronic persistent hepatitis; CAH: chronic active hepatitis; MHC: minimal histological changes.

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 HBV nucleocapsid antigen was studied by indirect immunofluorescence using rabbit anti-HBc (Dako Corporation, Santa Barbara, California), followed by fluorescein isothiocyanate (FITC)-labelled swine anti-rabbit immunoglobulin G, as reported before.[6] Intrahepatic expression of HBcAg and HBeAg was studied by indirect immunofluorescence using murine monoclonal anti-HBc and anti-HBe, respectively, followed by FITC conjugated rabbit anti-mouse immunoglobulin G (Jackson Immuno Research Laboratories, West Grove, Pennsylvania). Monoclonal anti-HBc and anti-HBe, which were produced by cell lines 3105 and 905, respectively, were obtained from Institute of Immunology, Tokyo, Japan.[16] The expression of viral antigens in liver was semi-quantitatively scored according to the proportion of positive cells on a 0 to 4+ scale, corresponding to positivity in 0%, 1–10%, 11–25%, 25–50% and more than 50% of hepatocytes examined.

The statistical analyses were performed using the χ^2 test with Yates's correction, McNemar's test or Wilcoxon rank sum test, where appropriate.

Results

The optimal dilution for staining HBV nucleocapsid antigen with polyclonal anti-HBc was 1 in 50, as reported previously.[6] The optimal concentration for staining of HBV nucleocapsid antigen with monoclonal anti-HBc and monoclonal anti-HBe resectively, was 40 μg/ml. HBcAg and HBeAg could be detected in the hepatocyte nuclei or cytoplasm, but the staining of HBeAg was weaker than that of

Table 2 Intrahepatic distribution of HBV nucleocapsid antigen in chronic hepatitis B virus infection

Autisera	HBeAg positive CPH (n = 16)	HBeAg positive CAH (n = 64)	Anti-HBe positive CAH (n = 15)
Polyclonal anti-HBc	14	54	7
Nuclear	1	2	0
Nuclear and cytoplasmic	12	30	0
Cytoplasmic	1	22	7
Monoclonal anti-HBc	13	51	6
Nuclear	1	2	0
Nuclear and cytoplasmic	12	29	0
Cytoplasmic	0	20	6
Monoclonal anti-HBe	11	48	6
Nuclear	0	1	0
Nuclear and cytoplasmic	6	10	0
Cytoplasmic	5	37	6

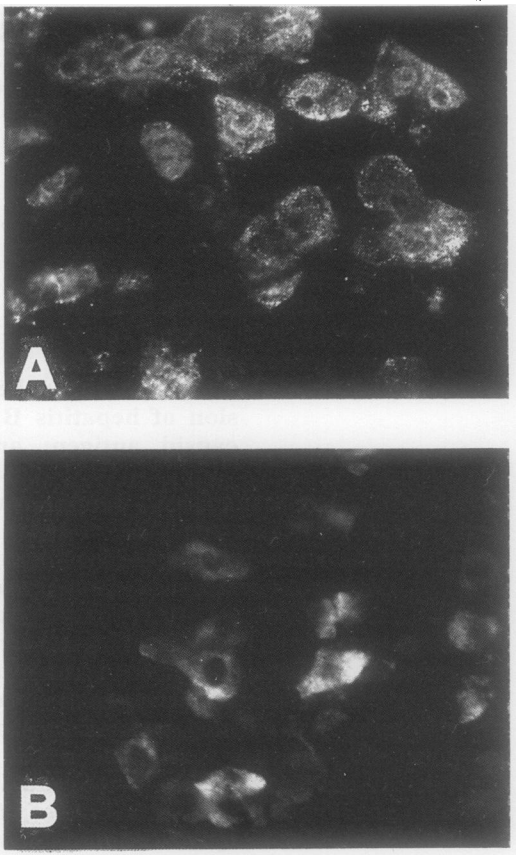

Figure 1 Indirect immunofluorescence of HBcAg on frozen section of liver specimen using monoclonal anti-HBc, showing a mixed nuclear and cytoplasmic staining (A), and an exclusive cytoplasmic staining (B).

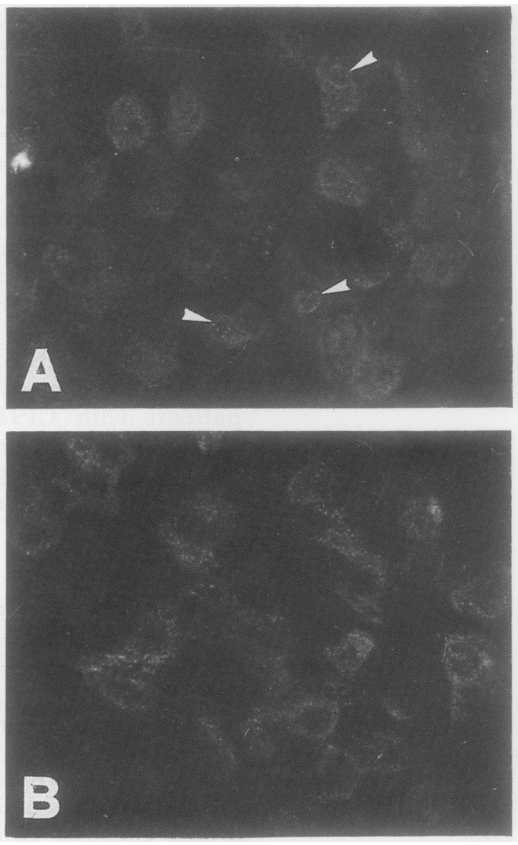

Figure 2 Indirect immunofluorescence of HBeAg on frozen section of liver specimen using monoclonal anti-HBe, showing a mixed nuclear (arrows) and cytoplasmic staining (A), and an exclusive cytoplasmic staining (B). The staining of HBeAg is weaker than that of HBcAg, as shown in figure 1.

HBcAg (figs 1 and 2). In three experiments preincubation with polyclonal anti-HBc resulted in partial or complete abolition of staining reaction by monoclonal anti-HBc or anti-HBe, and vice versa.

The expression and distribution of HBV nucleocapsid antigen in liver using different antisera are listed in table 2. Samples from 14 (87·5%), 13 (81·3%), and 11 (68·8%) of 16 HBeAg seropositive patients with chronic persistent hepatitis (CPH), 54 (84·4%), 51 (79·7%), and 48 (75%) of 64 HBeAg seropositive patients with chronic active hepatitis (CAH), and seven (46·7%), six (40%), and six (40%) of 15 anti-HBe positive patients with chronic active hepatitis (CAH) stained positively with polyclonal anti-HBc, monoclonal anti-HBc, and monoclonal anti-HBe, respectively. None of the 25 anti-HBe positive patients with minimal histological changes had detectable HBV nucleocapsid antigen in liver using any of these antisera. Table 2 shows that the HBeAg positive patients with CPH had a significantly higher prevalence of HBV nucleocapsid antigen expression in the nucleus compared with the HBeAg positive patients with CAH using polyclonal anti-HBc (13/14 *v* 32/54; $p < 0·05$), monoclonal anti-HBc (13/13 *v* 31/51; $p < 0·05$), or monoclonal anti-HBe (6/11 *v* 11/37; $p < 0·05$). In contrast, exclusive cytoplasmic expression of HBV nucleocapsid antigen was significantly more common in the latter than in the former.

Table 3 lists the prevalence of HBV nucleocapsid antigen expression in liver using different antisera in 80 HBeAg positive patients. HBV nucleocapsid antigen expression in the nucleus was significantly more prevalent using polyclonal anti-HBc or monoclonal anti-HBc than using monoclonal anti-HBe ($p < 0·001$, respectively; McNemar's test), while polyclonal anti-HBc and monoclonal anti-HBc stained the hepatocyte nucleus without any significant difference. The prevalence of HBV nucleocapsid antigen expression in the cytoplasm using monoclonal anti-HBc showed no significant difference compared with that using polyclonal anti-HBc or monoclonal anti-HBe, while polyclonal anti-HBc stained hepatocyte cytoplasm significantly more frequently than monoclonal anti-HBe ($p < 0·05$; McNemar's test). Table 4 lists the semiquantitative expression of HBV nucleocapsid antigen in liver using different antisera in 80 HBeAg positive patients. A significantly higher extent of HBV nucleocapsid antigen expression in the nucleus was noted using monoclonal anti-HBc than anti-HBe. The extent of cytoplasmic expression of HBV nucleocapsid antigen was higher using monoclonal anti-HBc than monoclonal anti-HBe, but the difference was not significant.

Seven (46·7%) of 15 tissue samples from anti-HBe positive patients with CAH expressed HBV nucleocapsid antigen in the cytoplasm using polyclonal anti-HBc. Of these, six stained positively with both monoclonal anti-HBc and monoclonal anti-HBe, and exclusively in the cytoplasm; the other one stained negatively with either antisera.

Discussion

Several studies on intrahepatic expression of HBcAg and HBeAg in chronic HBV infection have been reported recently, but the results still remained controversial. Studies by Yamada *et al*[14] and Naoumou *et al*[13] showed that HBcAg and HBeAg generally had a similar cellular expression. However, Mondelli *et al* reported that while the expression of HBcAg and HBeAg in the hepatocyte nucleus was largely coincident, cytoplasmic expression of HBeAg exceeded that of HBcAg.[12] By contrast, studies

Table 3 Prevalence of HBV nucleocapsid antigen expression in liver tissue in HBeAg positive patients with chronic hepatitis B

		Nucleus Polyclonal anti-HBc		*Cytoplasm Polyclonal anti-HBc*	
Monoclonal anti-HBc	*Monoclonal anti-HBe*	*Positive (n = 45)*	*Negative (n = 35)*	*Positive (n = 65)*	*Negative (n = 15)*
Positive	Positive	17	0	57	0
Positive	Negative	27	0	4	0
Negative	Positive	0	0	0	0
Negative	Negative	1	35	4	15

Table 4 Semiquantitative expression of HBV nucleocapsid antigen in liver in HBeAg positive patients with chronic hepatitis B

	Nucleus					*Cytoplasm*				
Antisera	*0*	*1+*	*2+*	*3+*	*4+*	*0*	*1+*	*2+*	*3+*	*4+*
Polyclonal anti-HBc	35	27	10	6	2^{ab}	15	11	17	26	11^{de}
Monoclonal anti-HBc	36	27	11	4	2^{ac}	19	9	26	17	9^{df}
Monoclonal anti-HBe	63	12	4	1	0^{bc}	23	15	27	10	5^{ef}

Scale of 0 to 4+ corresponding to positivity 0%, 1–10%, 11–25%, 26–50%, > 50% of total hepatocytes examined
[a]d $p > 0·05$, [bc] $p < 0·001$, [e] $p < 0·005$.

by Ballare *et al* showed that there was a prevalent expression of HBcAg in the nuclei, while the prevalence of HBcAg and HBeAg expression in the cytoplasm showed little or no difference.[8] In our study the distribution and quantitative expression of HBV nucleocapsid antigen in liver tissue were evaluated using polyclonal anti-HBc, and the differential distribution of HBcAg and HBeAg was evaluated using monoclonal anti-HBc and monoclonal anti-HBe. The specificity of the monoclonal anti-HBc and anti-HBe has been described.[16] The finding that the staining reaction of monoclonal anti-HBc or anti-HBe was partially or completely blocked by preincubation of polyclonal anti-HBc, and vice versa, was consistent with the suggestion that the polyclonal anti-HBc used in the present study contained both anti-HBc and anti-HBe.[10 11] The staining of HBeAg was weaker than that of HBcAg, which was also in keeping with the observation by others.[11 14] As shown in tables 3 and 4, our data indicated that the HBV nucleocapsid antigen in the hepatocyte nucleus contained HBcAg predominantly, while in the hepatocyte cytoplasm HBcAg and HBeAg were usually coexpressed more or less equally. These findings seem to agree with the observations of Ballare *et al*,[8] but contrast with the observations by Yamada *et al*,[14] Naoumou *et al*,[13] and Mondelli *et al*.[12] The discrepancy between each study might have been due to the difference in the choice of antisera, tissue manipulation, staining protocols or patient populations.

The mechanisms for regulating the differential distribution of HBcAg and HBeAg in hepatocytes in chronic HBV infection remain unclear. Based on studies of immunoelectron microscopy, it has been suggested that the nucleocapsid protein of HBV is produced on the ribosomes in the cytoplasm of hepatocytes, and that it then migrates into the nucleus as building material from which core particles are assembled. The assembled core particles then move from the nucleus to the cytoplasmic matrix and become covered with HBsAg positive endoplasmic reticulum membranes by budding into the cisternae of the endoplasmic reticulum, thus forming the complete Dane particles.[17-20] As the nucleocapsid protein of HBV expresses both HBcAg and HBeAg determinants[2 3] and the core particles display HBcAg and mask the HBeAg determinant,[2 3] the findings of the preferential expression of HBcAg in the hepatocyte nucleus and the coexpression of HBcAg and HBeAg in the cytoplasm in our study (tables 3 and 4) might suggest that the nucleocapsid protein dominates in the cytoplasm, while the assembled core particles dominate in the nucleus. This would substantiate the suggestion that HBV nucleocapsid protein is produced on the ribosomes in the cytoplasm, while core particles are assembled in the nucleus.

Another important finding of our study is that nuclear expression of HBcAg and HBeAg is prevalent in patients with CPH, while exclusive cytoplasmic expression of HBcAg and HBeAg is prevalent in patients with CAH (table 2). Previous studies by Yamada *et al*[14] and Ballare *et al*[8] have suggested that nuclear expression of HBcAg and HBeAg is associated with high concentrations of HBV DNA in serum compared with cytoplasmic expression of HBcAg and HBeAg. The prevalent expression of HBcAg and HBeAg in the hepatocyte nucleus in HBeAg positive patients with low inflammatory activity in the liver (table 2), might therefore indicate a high level of HBV replication, and thus suggests an immune tolerance to HBV in these patients, as we have suggested before.[21] Furthermore, the target viral antigen for T cell medicated immune lysis of hepatocytes in chronic HBV infection has been suggested to be the HBV nucleocapsid antigen rather than envelope protein,[22 23] and hepatocyte with cytoplasmic expression of HBV nucleocapsid antigen has been suggested as a possible target for immune hepatocytolysis.[6] The findings of a prevalent exclusive cytoplasmic expression of HBcAg and HBeAg in patients with CAH seem to agree with the suggestion that HBcAg or HBeAg might have a role as a possible target viral antigen for T cell mediated lysis of hepatocytes in chronic HBV infection.[23 24]

Finally, some of the HBeAg seronegative patients with CAH had demonstrable HBcAg and HBeAg in the liver, indicating active HBV replication. It has been shown that the HBsAg positive patients with active HBV replication but without HBeAg in their serum had a point mutation of pre-core region of HBV genome which prevented the secretion of HBeAg into the circulation by the liver.[25] Of interest is that both HBcAg and HBeAg localised exclusively in the hepatocyte cytoplasm in these patients (table 2). Studies of HBV nucleocapsid protein expression in culture cell lines by Ou *et al* have shown that the pre-core protein of HBV could be transported into the nucleus after signal peptide cleavage, while core protein remained exclusively in the cytoplasm.[26] It has been suggested that a point mutation of the pre-core region of HBV genome might result in synthesis and expression of core protein instead of pre-core protein, and that the core protein would therefore remain exclusively in the cytoplasm.[27] The findings of exclusive cytoplasmic expression of HBcAg and HBeAg in HBsAg positive patients without HBeAg in serum, as shown in our study, seem to agree with the postulation of a point mutation of the pre-core region of the HBV genome in these patients, which prevented secretion of HBeAg as well as transportation of nucleocapsid protein from cytoplasm into nucleus.

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