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Differential effect of chronic hepatitis D virus infection on intrahepatic expression of hepatitis B viral antigen

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Abstract

Aims: **To determine how chronic hepatitis D virus (HDV) infection affects intrahepatic hepatitis B virus (HBV) antigen expression.**

Methods: **Ninety eight liver biopsy specimens from 68 patients seropositive for total antibody to HDV were studied by immunohistochemistry, and the amount of HBV antigens was also quantified by radioimmunoassay in 12 patients and compared with 30 patients with chronic HBV infection.**

Results: **Forty nine of the 68 patients were positive for intrahepatic HDV antigen and only five were positive for HBV core antigen (HBcAg). HBV surface antigen (HBsAg) was present in 55 (80·9%) patients and was always cytoplasmic in distribution. Hepatic pre-S_1 and pre-S_2 expressions paralleled that of HBsAg, and were detected in 53 (77·9%) and 54 (79·4%) patients, respectively. There was no relation between the intrahepatic expression of HDV antigen and HBsAg/pre-S_1/pre-S_2. Follow up biopsy specimens in 25 patients showed either static or deteriorating histology while intrahepatic HDV antigen remained the same or fell. The patients with intrahepatic expression of HBcAg had either absent or noticeably decreased expression of HBcAg in their follow up biopsy specimens (median two years). In contrast, HBsAg/pre-S_1/pre-S_2 were the same or increased ($p < 0·0001$). Quantification of intrahepatic HBsAg in patients with chronic HDV infection (0·61 pg/hepatocyte, range: 0·05–1·08, n = 12) showed no difference with patients with chronic HBV infection alone (0·64 pg/hepatocyte, range: 0·02–1·02, n = 30, p = NS).**

Conclusion: **These data indicate that chronic HDV infection suppresses intrahepatic expression of HBcAg but not HBsAg and pre-S antigens, suggesting a differential effect of chronic HDV infection on HBV gene expression.**

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Hepatitis D virus (HDV) is a defective virus requiring the helper function of hepatitis B virus (HBV) for its replication.[1 2] HBV surface antigens (HBsAg) in HBV infected cells is used by HDV, which apparently has no envelope protein of its own.[1 2] Even though HBsAg is important for chronic HBV carriage, acute HDV infection was found, paradoxically, to suppress serum HBsAg titre in humans and chimpanzees.[2 3] The effects of chronic HDV infection on intraheptic expression of HBsAg in humans are less clear.

It is now known that the protein envelope of the complete HBV virion contains pre-S_1 and pre-S_2 peptides in addition to HBsAg.[4 5] Bonino *et al* showed that the HBV capsid of HDV virus contains all three HBV surface antigens (HBsAg and the pre-S antigens) but its composition resembles more closely that of the 22 nm serum HBsAg particles than that of complete HBV.[6] The purpose of the present study was, therefore, to determine the expression of intrahepatic HBV antigens in chronic HDV infection and to correlate these findings with intrahepatic expression of HDV antigen, a marker of HDV replication, and liver disease activity.

Methods

All patients seropositive for HBsAg seen at the Institute of Liver Studies of King's College Hospital between 1983 and 1989 were routinely tested for total antibody to HDV.[7] Antibody to human immunodeficiency virus (HIV) was tested in all patients with risk factors. Among the 80 patients seropositive for total antibody to HDV, 68 patients with 98 liver biopsy/hepatectomy specimens were included in this study (table 1). Twelve patients were excluded, either because a liver biopsy was not performed (n = 10), or seropositive for HIV markers (n = 2). Thirty consecutive patients with chronic HBV infection alone seen during

Table 1 Clinical, biochemical, and histological data of patients studied

Number of patients	68	
Male:female	60:8	
Median age (range)	37	(21–71)
Serum biochemistry	*Mean*	*(range)*
SGOT (IU/l)	114	(14–440)
Bilirubin (μmol/l)	53	(6–411)
Albumin (g/l)	33	(20–49)
Alkaline phosphatase (IU/l)	114	(46–569)
Liver histology (No of biopsy specimens)		
Non-specific reactive change	1	
Chronic persistent hepatitis	11	
Chronic active hepatitis	23	
Active cirrhosis (with hepatocellular carcinoma)	63	(5)

Table 2 Clinical, serological, and histological data of patients with quantification of their intrahepatic HBsAg expression

	Chronic HDV infection	*Chronic HBV infection alone*
Number of patients	12	30
Male:female	11:1	26:4
Serum HBsAg (median)	1:3200	1:12 800
(range)	1:8–12 800	1:64–214 800
Serum HBeAg positive (number)	0	19
Serum HBV-DNA positive (number)	0	13
Liver histology		
Non-specific reactive change	0	5
Chronic persistent hepatitis	1	11
Chronic active hepatitis	1	8
Active cirrhosis	10	5
Inactive cirrhosis	0	1

1989–1990 were also studied for comparative purposes (table 2).

SEROLOGY

Serum was tested for HBsAg, HBV e antigen (HBeAg), and antibody to HBeAg (anti-HBe) by radioimmunoassay (Abbott Diagnostics, Maidenhead, England). Quantification of serum HBsAg was by serial dilution using reverse passive haemagglutination (Wellcome Diagnostics, Dartford, England). Enzyme immunoassays were used to detect total antibody to HDV and HIV (Abbott). Serum HBV DNA was determined using a quantitative dot-blot technique.[8]

HISTOLOGICAL ASSESSMENT

Histological diagnosis was established by one of us (BCP) using internationally accepted criteria.[9] Three histological features were semiquantitatively assessed and recorded in a 0–3+ scale: lobular inflammation (1+ – mild, 2+ – moderate, 3+ – severe); portal tract inflammation (1+ – sparse, 2+ – moderate, 3+ – dense); and piece-meal necrosis (1+ – patchy, 2+ – =50% of the limiting plates affected, 3+ – most limiting plates affected).[7] When these were added together, a total histological activity index was derived (0–9+). The extent of fibrosis was also graded from 0 to 4+ (1+ – portal, 2+ – portoseptal, 3+ – bridging, 4+ – cirrhosis).

DETECTION OF HDV ANTIGEN AND HBV ANTIGENS IN LIVER TISSUE

Immunohistochemical demonstration of intrahepatic HDV and HBV antigens were performed on 5 μm consecutive formalin fixed paraffin wax embedded liver sections prepared to maximise HBV antigen detection according to previous recommendations.[10 11] The method of detection of HDV antigen in liver tissue has been detailed previously.[7]

For the detection of HBV antigens, the dewaxed liver sections were first blocked by 5% rabbit serum (for HBsAg/pre-S_1/pre-S_2) or swine serum (for HBcAg) in TRIS-buffered saline (TBS) at room temperature for 15 minutes. First layer antibody (HBsAg: mouse monoclonal antibody D2H5, developed by Drs Tedder and Ferns, London, dilution 1 in 5; pre-S_1: monoclonal mouse antibody MA18/7, donated by Professor WH Gerlich and Dr KH Heermann, Gottingen, Germany, dilution 1 in 100; pre-S_2: mouse monoclonal antibody 5535, kind gift of Professor A Alberti, Padova, Italy, dilution 1 in 50; HBcAg: rabbit polyclonal antibody, Dako, UK, dilution 1 in 200) in 1% appropriate serum in TBS were applied for 30 minutes. The monoclonal antibodies to pre-S_1 and pre-S_2 were chosen according to a recent study which showed that these antibodies were the most sensitive for detecting the pre-S antigens.[12] After washing in TBS, appropriate second layer antibody conjugated to alkaline phosphatase was applied (HBsAg/pre-S_1 pre-S_2: rabbit anti-mouse, HBcAg: swine anti-rabbit, dilution 1 in 30) for 30 minutes at room temperature. The alkaline phosphatase reaction was developed using napthol and Fast Red TR salt (Sigma, Dorset, England) as described. The sections were then washed, counterstained with haematoxylin, and mounted in glycerol in TBS (1:1).

The expression of HDV antigen and HBV antigens was scored independently by two of us (JYNL, BCP) on a 0–4+ scale, corresponding to positivity in 0%, 1–5%, 5–30%, 30–60%, and >60% of hepatocytes examined.

QUANTIFICATION OF INTRAHEPATIC HBSAG

The amount of intrahepatic HBsAg was quantified by radioimmunoassay in 12 patients with chronic HDV infection and compared with 30 patients with chronic HBV infection (table 2) according to a previously described method.[13] Hepatocytes were isolated from liver biopsy specimens by the method described by Trevisan *et al*[14] and 200 000 washed hepatocytes were resuspended in 3 ml of RPMI 1640. Previous evaluation by phase-contrast microscopy had shown that the hepatocytes isolated by this method retained an intact plasma membrane and should have retained the HBV antigens.[13]

The cell suspensions were sonicated over ice at 10 amplitude microns, 10 seconds per spell, for three spells (Soniprep 150, 23 kHz, MSE, Sussex, England) to release intracellular HBsAg. Sonication according to this method did not denature the HBsAg.[13] The cell sonicates were stored at −20°C until assayed in batches using commercially available radioimmunoassays for HBsAg (Abbott). The proportion of HBsAg containing cells in these preparations was determined using immunocytochemical staining as described above. By dividing the amount of HBsAg measured by radioimmunoassay by the number of hepatocytes positive for HBsAg, the amount of HBsAg per hepatocyte was determined.

The results were analysed on a microcomputer (Dell, USA) using the SPSS-PC programme (SPSS Inc, Chicago, Illinois, USA). The χ^2 test, Student's *t*-test, Mann-Whitney test of Spearman's rank correlation test were used, as appropriate.

Results

HDV antigen, HBcAg, HBsAg, pre-S_1 and pre-S_2 were detected in varying frequencies in the biopsy specimens (table 3). HDV antigens

Table 3 Intrahepatic expression of HDV antigen and HBV antigens

Antigen	No (%) of biopsy specimens (n = 98)	No (%) of patients (n = 68)
HDV antigen	75 (76·5)	49 (72·1)
HBV antigens:		
HDV antigen positive	(n = 75)	(n = 49)
HBcAg	5 (6·7)	3 (6·1)
HBsAg	63 (84·0)	39 (79·6)
Pre-S_1	62 (82·7)	38 (77·6)
Pre-S_2	62 (82·7)	38 (77·6)
HDV antigen negative	(n = 23)	(n = 19)
HBcAg	2 (8·7)	1 (5·3)
HBsAg	22 (95·7)	16 (84·2)
Pre-S_1	21 (91·3)	15 (79·0)
Pre-S_2	22 (95·7)	16 (84·2)

were all nuclear in distribution and in only one patient; cytoplasmic HDV antigen was also present.

INTRAHEPATIC EXPRESSION OF HBCAG/SERUM HBV REPLICATION MARKERS

Four patients were positive for intrahepatic HBcAg (median 3+, range 1+ − 4+) and all were nuclear in distribution. Three had a repeated biopsy specimen taken in a median of two years (one to three years) and two showed a decrease in HBcAg (4+ → 1+, 3+ → 1+). These patients also lost serum HBV DNA within three years and three seroconverted from HBeAg to anti-HBe in a median of two years (range one to four years).

SERUM HBSAG TITRE

Compared with patients with chronic HBV infection (median HBsAg titre 1:12 800, range 1:64–214 800, n = 30), patients with chronic HDV infection had a lower serum HBsAg titre (median 1:3200, range 1:4–51 200, n = 68; $p < 0·01$). Patients with chronic HDV infection related to cirrhosis had a lower serum titre of HBsAg compared with those who were not cirrhotic (median 1:128 (n = 63) *v* 1:12 800 (n = 25); $p < 0·05$). When the patients with chronic HBV and HDV infection were stratified according to analysis of liver histology, there was no difference in serum HBsAg titre between patients with chronic HBV infection alone and patients with chronic HDV infection in each histological group. The lower serum titre of HBsAg in chronic HDV infection was probably related to the higher proportion of patients who had liver cirrhosis.

Patients with positive intrahepatic HDV antigen (n = 49) had a higher serum titre of HBsAg (median 1:12 800, range 1:32–51 200) compared with those negative for intrahepatic HDV antigen (median 1:1600, range 1:4–6400, n = 19; $p < 0·05$).

INTRAHEPATIC EXPRESSION OF HBSAG AND PRE-S ANTIGENS

Intrahepatic HBsAg was detected in the liver biopsy specimens of most patients (table 3) and the distribution was nearly always diffuse cytoplasmic, with three patients showing a membraneous pattern of HBsAg on top of the predominantly cytoplasmic HBsAg. Two of these three patients were positive for intrahepatic HBcAg and seropositive for both HBeAg and HBV DNA. The median expression of HBsAg was 1+ (range 0–4+), but a strong diffuse positive reaction was seen in seven patients (>3+, 10·3%), six of whom had histological evidence of cirrhosis. Pre-S_1 and pre-S_2 were detected in the liver in nearly all biopsy specimens positive for HBsAg ($p < 0·0001$). Immunohistochemical examination in serial liver sections showed that the distribution of the pre-S antigens was the same as HBsAg with most hepatocytes expressing HBsAg also express both pre-S antigens.

There was no correlation between intrahepatic expression of HDV antigen and HBsAg

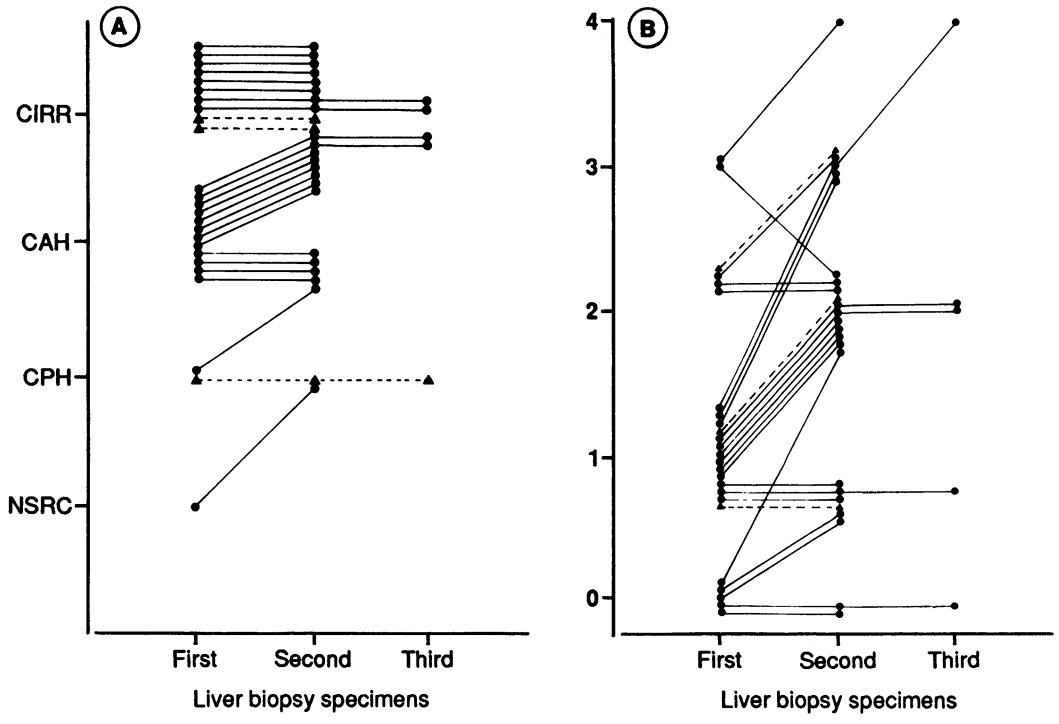

Figure 1 Changes in (A) liver histology and (B) intrahepatic expression HBsAg in the 25 patients with follow up biopsy specimens. Solid circles and solid lines represent those with positive intrahepatic HDV antigen on presentation; solid triangles and dotted lines represented those with no detectable intrahepatic HDV antigen in the first liver biopsy specimen.

or pre-S antigens. Immunohistochemical staining in serial liver biopsy specimens showed that hepatocytes expressing HDV antigen were frequently negative or weakly positive for HBsAg and pre-S antigens. However, occasional hepatocytes positive for HDV antigen were also strongly positive for HBsAg and pre-S antigens.

No correlation exists between serum titre of HBsAg and intrahepatic expression of HBsAg, pre-S_1 and pre-S_2, although patients with strong diffuse intrahepatic expression of HBsAg and pre-S antigens (n = 7) tended to have a lower titre of serum HBsAg (p = 0·0677). Intrahepatic expression of HBsAg or pre-S antigens had no association with either histological activity index or any of its individual factors (lobular inflammation, piece-meal necrosis, portal inflammation). Subset analysis of biopsy specimens positive (n = 75) or negative (n = 23) for intrahepatic HDV antigen also showed no correlation between intrahepatic expression of HBV surface antigens and histological activities. There was no correlation between the expression of intrahepatic HBsAg or pre-S antigens and the extent of fibrosis.

In relation to patients with chronic HBV infection alone, there was no difference in the intrahepatic expression of HBsAg and the pre-S antigens as determined by immunohistochemistry in different histological groups (according to the international accepted criteria listed in table 1) between patients with chronic HBV (n = 30) and chronic HDV infection (n = 68). Quantification of the amount of intrahepatic HBsAg per positive hepatocyte (table 2) showed no difference between patients with chronic HBV infection (median 0·64 pg/hepatocyte, range 0·02–1·02, n = 30) and chronic HDV infection (median 0·71, range 0·05–1·08, n = 12; p = NS).

In 25 patients (three negative for intrahepatic HDV antigen) with follow up biopsy specimens at a median of two years (range one to five years), liver histology either remained static or deteriorated while intrahepatic expression of HDV antigen remained the same or fell. In contrast, these follow up biopsy specimens showed either the same (n = 10) or increased intrahepatic HBsAg expression (figure).

Discussion

Our observation that intrahepatic HBcAg expression was suppressed in chronic HDV infection agrees with the findings of previous studies,[15–17] indicating viral interference or a suppressive effect of HDV on HBV replication and the expression of HBV nucleocapsid antigens. In contrast, intrahepatic expression of HBsAg was detected in most patients with chronic HDV infection in a pattern similar to chronic HBV infection with comparable histological changes, suggesting that the expression of intrahepatic HBsAg was not suppressed by chronic HDV infection.

The close expression pattern between pre-S_2 and HBsAg agrees with the current view of the molecular biology of HBV findings that pre-S_2 was translated, together with HBsAg, from either the 2·1 or 2·4 kilobase (with pre-S_1) mRNA.[5 18] The detection of pre-S_1 in most cells positive for HBsAg indicates that the 2·4 kilobase mRNA was also translated in these cells. This relatively unaffected expression of HBV surface antigens contrasts sharply with the suppression of HBV nucleocapsid antigen expression which is translated from the 3·5 kilobase mRNA,[18] indicating a differential effect of chronic HDV infection on HBV gene expression. This is ecologically advantageous to HDV as this may avoid the competitive use of HBV surface antigens by HBV to form its capsid.

Of interest is that some hepatocytes staining positively for HDV antigen were negative for HBsAg and the pre-S antigens. Previous studies in patients with chronic HBV infection have shown that hepatocytes positive for HBcAg may be negative for HBsAg and that this is related to the ability of the hepatocytes to secrete or export the HBsAg as determined in a primary hepatocyte culture system.[13 19 20] A satisfactory system to study the export of HBsAg in hepatocytes infected with HDV has not been established but HDV replication, like HBV, may also affect the export of HBsAg.

Early studies suggested that the liver damage in chronic HDV infection was related to a direct cytopathic effect of the virus. The expression of intrahepatic HBV surface antigens increased with time in contrast to HDV antigen which fell with a deterioration in liver histology.[7] Whether cellular accumulation of HBsAg and pre-S antigens has a role in the pathogenesis of liver damage/dysfunction in chronic HDV infection is not known. In transgenic mice models accumulation of intracellular HBV surface antigens was directly cytotoxic to hepatocytes.[21] These hepatocytes become ballooned, hydropic, eosinophilic, displayed the characteristic features of ground-glass cells, and underwent coagulative necrosis with time.[21] We have also recently reported a massive accumulation of intracellular HBV antigens (both HBV surface and nucleocapsid antigens) in the liver grafts of some transplant recipients with chronic HBV infection. This is associated with severe graft dysfunction but relatively mild inflammatory activities, suggesting that accumulation of HBV antigens in hepatocytes may lead to cellular dysfunction.[22 23]

Recently, several reports have indicated that an immune mediated mechanism may also be involved.[7 24 25] The absence of an association between the expression of HBV surface antigens and histological inflammatory activity shown in the present study indicates that HBV surface antigens are unlikely to be immune targets in chronic HDV infection.

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