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Liver and biliary

Contribution of low level HBV replication to continuing inflammatory activity in patients with anti-HBe positive chronic hepatitis B virus infection

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SUMMARY The relationship between the histological diagnosis and serological and tissue markers of HBV replication in 41 Greek and 29 British patients with chronic HBV infection were studied. All the nine Greek and 13 British patients who were HBeAg positive had HBV-DNA in serum and HBcAg expression in the hepatocytes. The majority (73%) of these patients had active liver disease. Forty seven per cent of the Greek and 19% of the British patients who were anti-HBe positive continued to display HBcAg in the liver with or without HBV-DNA detected in serum. All but three of these patients had persistently active liver disease. Continuing inflammatory activity in the liver, however, was also found in 31% of anti-HBe positive patients who had no evidence of HBV replication. In these patients, other factors such as delta agent, NANB viruses, alcohol abuse or an autoimmune reaction initiated by HBV may be contributory.

There are two phases of chronic HBV infection. In the earlier phase, the patients are HBeAg positive. They have active viral replication as evidenced by the presence of HBV-DNA in serum[1 2] and hepatitis B core antigen (HBcAg) in the liver.[3] These patients usually have active disease because of continuous immunological attack on hepatocytes which bear viral antigens.[4–6] After a period of time, usually several years, viral replication ceases and the patients seroconvert from HBeAg to anti-HBe.[7 8] During this latter phase, the continuous production of HBsAg protein (22 nm spheres) is maintained by hepatocytes containing integrated viral DNA.[9–12] In most patients, during this anti-HBe positive phase, there is a reduction in the inflammatory activity of the liver disease.[4 7 8] In some patients, however, the liver disease remains active. Recent reports have shown that a significant proportion of patients who are anti-HBe positive may have persistent viral replication with HBV-DNA in serum and/or HBcAg in the liver.[2 13 14] Such phenomena appear to be more common in patients of Mediterranean and Oriental origin and may account for continuing activity of liver disease in these patients[15] (in preparation). Alternatively, persistently active liver disease may be unrelated to continuing HBV replication and other factors such as coexistent delta virus infection, alcohol abuse or autoimmune reaction may be contributory.

In this study, we have attempted to correlate the activity of liver disease with the level of HBV replication. We studied the relationship between the histological diagnosis and HBeAg/anti-HBe status and HBV-DNA in serum and the presence of HBcAg and delta antigen (δ Ag) in the liver in 41 Greek and 29 British patients with chronic HBV infection.

Methods

PATIENTS

Liver biopsies and serum samples from 32 Greek patients, referred to Hippokration General Hospital, Athens, and nine Greek and 29 British patients at The Royal Free Hospital, London, who had been HBsAg positive for more than one year, were studied. These patients were referred because of symptomatic liver disease or concern about infectivity. In some patients studied at the Royal Free Hospital, serial specimens were available.

Liver biopsy specimens were divided into two parts. One was embedded in OCT compound and

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immediately frozen in cold isopentane and the other was fixed in 10% formalin and processed for histological examination. Cryostat sections were cut from the frozen biopsies and examined for HBs, HBc, and delta antigens (δ Ag) by direct immunofluorescence. Details of the methods have been described previously.[16 17] The specificity of HBs, HBc and δ Ag fluorescence was checked by absorption and blocking tests. All antisera used were also tested for non-specific staining in several control liver specimens obtained from HBsAg negative individuals. The amount of HBcAg in the liver was quantified by determining the percentage of hepatocyte nuclei positive for this antigen.

All serum samples were taken on the same day as or within 10 days of the liver biopsy, and were kept at −20°C up to the time of serological testing. HBsAg, HBeAg and anti-HBe were determined by radioimmunoassay (Abbott Laboratories). Sera were analysed for HBV-DNA sequences by molecular hybridisation using a ^{32}P-labelled HBV-DNA probe in the hybridot technique. This method can detect quantities of HBV-DNA as little as 0·05 pg.

Results

The serum HBeAg/anti-HBe status in relation to markers of HBV replication (serum HBV-DNA and hepatocyte display of HBcAg) in the 41 Greek and 29 British patients studied are shown in Tables 1 and 2.

FORTY ONE GREEK PATIENTS

Nine were HBeAg positive, 30 were anti-HBe positive and the remaining two were negative for both HBeAg and anti-HBe.

All the nine patients who were HBeAg positive had evidence of active viral replication with HBV-DNA in serum and HBcAg in the liver. The majority (six), had chronic active hepatitis. The remaining patients had chronic persistent hepatitis (two) and active cirrhosis (one).

Among the 30 anti-HBe positive patients (Table 3), 14 (47%) continued to display HBcAg in the hepatocytes, and eight of these also had HBV-DNA in the serum. Seventeen of these 30 patients (57%) had active liver disease: chronic active hepatitis (16) and active cirrhosis (one). In 11 there was evidence of persistent HBV replication. Intrahepatic HBcAg was also detected in one of these 11 patients. In the

Table 1 *Correlation of serum HBeAg/anti-HBe status to serum HBV-DNA and HBcAg in liver in 41 Greek patients with chronic HBV infection*

	HBcAg+ve		*HBcAg−ve*	
	HBV-DNA+	*HBV-DNA−*	*HBV-DNA+*	*HBV-DNA−*
HBeAg+/anti-HBe− (9)	9	0	0	0
HBeAg−/anti-HBe+ (30)	8	6	0	16
HBeAg−/anti-HBe− (2)	0	0	0	2
All groups (41)	17	6	0	18

Table 2 *Correlation of serum HBeAg/anti-HBe status to serum HBV-DNA and HBcAg in liver in 29 British patients with chronic HBV infection*

	HBcAg+ve		*HBcAg−ve*	
	HBV-DNA+	*HBV-DNA−*	*HBV-DNA+*	*HBV-DNA−*
HBeAg+/anti-HBe− (13)	13	0	0	0
HBeAg−/anti-HBe+ (16)	0	2	1	13
All groups (29)	13	2	1	13

Table 3 *Serological and tissue markers of HBV replication in relation to histological diagnosis in 30 anti-HBe positive Greek patients*

Patient no	*Serum HBV-DNA*	*HBcAg in liver*	*Histology*
1	−	+	Normal
2	−	−	,,
3	−	−	,,
4	−	++	CPH†
5	−	−	,,
6	−	−	,,
7	−	−	,,
8	+	+++	CAH‡
9	+	+	,,
10	+	+++	,,
11	+	+++	,,
12	+	++	,,
13	+	++	,,
14	+	++	,,
15*	−	++	,,
16	−	++	,,
17	−	+	,,
18	−	−	,,
19	−	−	,,
20	−	−	,,
21	−	−	,,
22	−	−	,,
23	−	−	,,
24	+	+	Cirrhosis (active)
25	−	−	,, (inactive)
26	−	−	,, (inactive)
27*	−	+++	HCC§
28	−	−	,,
29	−	−	,,
30	−	−	,,

* Presence of intrahepatic δ Ag. † Chronic persistent hepatitis. ‡ Chronic active hepatitis. § Hepatocellular carcinoma.

remaining six patients, however, continuing activity of the liver disease could not be accounted for by active HBV replication or delta infection. The 13 anti-HBe positive patients with inactive liver lesions had normal liver histology (three), chronic persistent hepatitis (four), inactive cirrhosis (two) or hepatocellular carcinoma (four). Three of these had HBcAg expression in the hepatocytes but serum HBV-DNA was not detectable.

Both patients who were HBeAg and anti-HBe negative were also negative for HBcAg in liver and HBV-DNA in serum. One had normal liver on biopsy and the other had chronic active hepatitis.

TWENTY NINE BRITISH PATIENTS

Thirteen were HBeAg and the remaining 16 anti-HBe positive.

As with the Greek patients, all the 13 HBeAg positive patients displayed HBcAg in the hepatocytes and HBV-DNA in their serum. The majority (nine), had chronic active hepatitis. The remaining four had chronic persistent hepatitis.

In contrast with the Greek patients, only three of the 16 anti-HBe positive patients had persistent HBV replication (Table 4). Two of these three patients (numbers 8 and 9) had seroconverted from HBeAg to anti-HBe three and five months before biopsy. The remaining patient (no 13) had HBc and δ Ag detected in the hepatocytes but was negative for HBV-DNA in serum. Six of the 16 anti-HBe positive patients (38%) continued to have active liver disease: chronic active hepatitis (five) and active cirrhosis (one). Three had evidence of HBV replication. Coexistent delta infection was also present in one of these three and another patient (no 10) in whom HBV replication was not detectable but no apparent cause was identified in the remaining two patients. The other 10 patients had inactive liver disease, either normal liver histology (three), chronic persistent hepatitis (four), or inactive cirrhosis (three). None of them showed any evidence of continuing HBV replication.

Table 4 *Serological and tissue markers of HBV replication in relation to histological diagnosis in 16 anti-HBe positive British patients*

Patient no	*Serum HBV-DNA*	*HBcAg in liver*	*Histology*
1	−	−	Normal
2	−	−	,,
3	−	−	,,
4	−	−	CPH†
5	−	−	,,
6	−	−	,,
7	−	−	,,
8	+	−	CAH‡
9	−	+	,,
10*	−	−	,,
11	−	−	,,
12	−	−	,,
13*	−	+	Cirrhosis (active)
14	−	−	,, (inactive)
15	−	−	,, (inactive)
16	−	−	,, (inactive)

* Presence of intrahepatic δ Ag. † Chronic persistent hepatitis.
‡ Chronic active hepatitis.

DELTA INFECTION

Intrahepatic δ Ag was detected in two Greek and two British patients. All four patients were anti-HBe positive, but three had HBcAg in their liver tissue. The histological diagnoses included chronic active hepatitis (two), active cirrhosis (one) and hepatocellular carcinoma (one).

Discussion

The HBeAg/anti-HBe status of a patient has been regarded as an indicator of the level of HBV replication and hence of relative infectivity.[18 19] With the availability of the techniques of molecular hybridisation to detect HBV-DNA in serum, it has now been confirmed that HBV may continue to replicate after seroconversion from HBeAg to anti-HBe.[2 13 14] HBc antigen display in the liver may also reflect ongoing HBV replication in that it is usually present when HBV-DNA is present in the serum.[20]

In this study, we confirm that the presence of HBeAg is associated with evidence of active viral replication in both Greek and British patients. In addition, 47% of anti-HBe positive Greek patients exhibited HBcAg in the liver with or without detectable HBV-DNA in the serum. In contrast, only three (19%) of 15 anti-HBe positive British patients, had evidence of continuing HBV replication. In two of these, the liver biopsies had been taken within six months of HBeAg/anti-HBe seroconversion, and in the remaining patient there was coexistent delta infection. In the Greek patients serial specimens were not available so that the relationship of a particular serological pattern to HBe antigen/antibody seroconversion could not be determined. These patients were, however, similar to the British patients in that they had been referred to a hepatology service with symptomatic liver disease.

The explanation for the difference in the prevalence of low level HBV replication in the two populations of anti-HBe positive patients is not clear. Recent evidence suggests that clearance of hepatocytes supporting HBV replication is dependent on an immune response to HBc

antigen[3 21] whereas clearance of HBe antigen is related to an independent immune response to the determinants on this soluble protein. It is possible that both of these responses are independently genetically determined and in Greek patients the immune response to and the clearance of HBe antigen is more efficient than in British patients. Alternatively, the cell mediated response to HBc antigen displayed on the surface of hepatocytes supporting HBV replication may be less effective in Greek than British patients. The restricted number of epitopes displayed on HBe antigen – two identified by murine monoclonal antibodies[22] – make it likely that very few immune response genes control the clearance of this antigen and therefore large differences may occur between different populations. Elimination of HBV infected hepatocytes probably depends on the recognition of viral antigens in association with HLA class 1 proteins by cytotoxic T lymphocytes.[23] It is possible that certain HLA types present in the Greek population may not associate as well as others with the HBV antigens leading to relative failure of immune lysis of infected hepatocytes.[24] This has been reported in influenza.[25] Thus, there are reasons to suggest that genetic factors may determine the difference between the Greek and British patients. Other factors may also be important. Unlike the British patients, most of the Greek patients were not studied serially and it remains possible that some of those who continued to replicate HBV during the anti-HBe phase, may have been biopsied near to seroconversion from HBeAg to anti-HBe, as was the case with the British patients. Finally, the time of infection may also determine the efficiency of the various components of the immune system. It is likely that the Greek patients are infected early in life, whereas the British patients acquire the infection in adulthood.

It has been suggested that inflammatory liver disease is associated with viral replication.[4] Although the majority (68%) of HBeAg positive patients had active liver lesions on biopsy, 57% of Greek and 38% of British patients who were anti-HBe positive also had chronic active hepatitis or active cirrhosis. Fourteen of these 23 anti-HBe positive patients with active inflammatory liver disease also had persistent HBV replication showed by the presence of HBcAg in the liver or HBV-DNA in serum. The remaining nine patients, however, continued to have active liver disease in the absence of detectable HBV replication. Delta infection accounted for persistent inflammatory activity in only one of these patients. While it is possible that HBV replication continues below the levels of detection, other factors should be considered. Coexistent NANB hepatitis or alcohol abuse may contribute to the active liver disease in some of these patients. An alternative explanation could be a perpetuation of an autoimmune reaction against liver membrane antigens, initiated during and against liver membrane antigens, and persisting after cessation of HBV replication.[4] In support of this, liver membrane antibodies have been detected in some patients with chronic HBV infection.[26] Thus, although low level HBV replication, identified by the presence of HBV-DNA in serum or HBc antigen in the liver, may explain the continuing inflammatory activity in some anti-HBe positive patients, there is a large proportion in whom this is not the case and other factors may be contributory.

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References

1 Weller IVD, Fowler MJF, Monjardino J *et al.* The detection of HBV-DNA in serum by molecular hybridisation: a more sensitive method for detection of complete HBV particles. *J Med Virol* 1982; **9:** 273–80.
2 Bonino F, Hoyer B, Nelson J *et al.* Hepatitis B virus DNA in the sera of HBsAg carriers: a marker of active hepatitis B virus replication in the liver. *Hepatology* 1981; **1:** 386–91.
3 Gudat F, Bianchi L, Sonnabend W, Theil G, Aenishaenslin W, Stalder GA. Pattern of core and surface expression in liver tissue reflects state of specific immune response in hepatitis B. *Lab Invest* 1975; **32:** 1–5.
4 Thomas HC, Montano L, Goodall A, deKoning R, Oladapo J, Wiedmann KH. Immunological mechanisms in chronic hepatitis B infection. *Hepatology* 1982; **2:** 116S–21S.
5 Eddleston ALWF, Williams R. Inadequate antibody response to HBV or suppressor T cell defect in development of active chronic hepatitis. *Lancet* 1974; **2:** 1543–5.
6 Dudley FJ, Fox RA, Sherlock S. Cellular immunity and hepatitis-associated, Australia antigen liver disease. *Lancet* 1972; **1:** 723–6.
7 Realdi G, Alberti A, Ruzzi M *et al.* Seroconversion from HBe antigen to anti-HBe in chronic HBV infection. *Gastroenterology* 1980; **79:** 195–9.
8 Hoofnagle J, Dusheiko GM, Seeff LB. Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. *Ann Intern Med* 1981; **94:** 744–8.
9 Hirschman SZ. Integration enzyme hypothesis for replication of hepatitis B virus. *Lancet* 1975; **2:** 436–8.
10 Fowler MJF, Monjardino J, Weller IVD, Lok ASF, Thomas HC. An analysis of the molecular state of HBV-DNA in the liver and serum of patients with

chronic hepatitis or primary liver cell carcinoma and the effects of therapy with adenine arabinoside monophosphate. *Gut* 1984; **25:** 611–8.

11 Brechot C, Hadchovel M, Scotto J *et al.* Detection of hepatitis B virus DNA in liver and serum: a direct appraisal of the chronic carrier state. *Lancet* 1981; **2:** 765–7.

12 Shafritz DA, Shouval D, Shermon HI *et al.* Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. *N Engl J Med* 1981; **305:** 1067–73.

13 Scotto J, Hadchovel M, Hery C, Yvart J, Tiollais P, Brechot C. Detection of hepatitis B virus DNA in serum by a simple spot hybridisation technique: comparison with results for other viral markers. *Hepatology* 1983; **3:** 279–84.

14 Lieberman HM, LaBrecque DR, Kew MC, Hadziyannis SJ, Shafritz DA. Detection of hepatitis B virus DNA directly in human serum by a simplified molecular hybridisation test: comparison to HBeAg/anti-HBe status in HBsAg carriers. *Hepatology* 1983; **3:** 285–91.

15 Sherlock S, Thomas HC. Hepatitis B virus infection: the impact of molecular biology. *Hepatology* 1983; **3:** 455–6.

16 Hadziyannis S, Gerber MA, Vissoulis C, Popper H. Cytoplasmic hepatitis B antigen in 'ground glass' hepatocytes of carriers. *Arch Pathol* 1973; **96:** 327–35.

17 Montano L, Miescher GC, Goodall AH, Janossy G, Thomas HC. Hepatitis B virus and HLA antigen display in the liver during chronic hepatitis B virus infection. *Hepatology* 1982; **2:** 557–61.

18 Okada K, Kamiyama I, Inomata M *et al.* E antigen and anti-e in the plasma of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. *N Engl J Med* 1976; **294:** 746–9.

19 Alter JH, Seeff LB, Kaplan PM *et al.* Type B hepatitis: The infectivity of blood positive for HBe antigen and DNA polymerase after accidental needlestick exposure. *N Engl J Med* 1976; **295:** 909–13.

20 Hadziyannis SJ, Lieberman HM, Karvountzes GG, Shafritz DA. Analysis of liver disease, nuclear HBcAg, viral replication, and hepatitis B virus DNA in liver and serum of HBeAg versus Anti-HBe positive carriers of hepatitis B virus. *Hepatology* 1983; **3:** 656–62.

21 Eddleston ALWF, Mondelli M, Mieli-Vergani G, Williams R. Lymphocyte cytotoxicity to autologous hepatocytes in chronic hepatitis B virus infection. *Hepatology* 1982 suppl; **2:** 122–7S.

22 Takahashi K, Machida A, Funatsu G *et al.* Immunochemical structure of hepatitis B e antigen in the serum. *J Immunol* 1983; **130:** 2903–7.

23 Doherty PC, Zinkernagel RM. A biological role for major histocompatibility antigens. *Lancet* 1975; **1:** 1406–9.

24 Thomas HC, Lok ASF. The immunopathology of autoimmune and hepatitis B virus induced chronic hepatitis. *Sem Liver Dis* 1984; **4:** 36–46.

25 McMichael A. HLA restriction on human cytotoxic T lymphocyte specific for influenza virus. Poor recognition of virus associated with HLA AZ. *J Exp Med* 1978; **148:** 1458–67.

26 Wiedmann KH, Bartholomew TC, Brown DJC, Thomas HC. Liver membrane antibodies detected by immunoradiometric assay in acute and chronic virus induced and autoimmune liver disease. *Hepatology* 1984; **4:** 199–204.