Detection, by three techniques, of hepatitis B surface antigen (HBsAg) and determination of HBsAg and anti-HBs titres in patients with chronic liver disease

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SUMMARY The sensitivities of three techniques used to detect serum hepatitis B surface antigen (HBsAg) were compared in 411 patients with various types of chronic liver disease. Counterimmuno-electrophoresis proved an unreliable test. Two haemagglutination techniques were slightly less sensitive than radioimmunoassay but were more rapidly performed. Less sensitive techniques were particularly unreliable in active liver disease where HBsAg titres were low. HBsAg was detected in patients with chronic persistent hepatitis, alcoholic liver disease, chronic active liver disease with or without cirrhosis, and primary liver cell carcinoma. Forty-six of the 68 (68%) HBsAg positive subjects were males coming from outside the United Kingdom. The HBsAg titres in 13 subjects with chronic persistent hepatitis were significantly higher ($P < 0.001$) than those in 43 subjects with chronic active liver disease. Corticosteroid therapy did not alter the HBsAg titre significantly. None of the 28 HBsAg positive subjects studied serially for up to two years cleared HBsAg from the serum. Anti-HBs was examined by passive haemagglutination and found in 35 subjects, 26 of whom had no evidence of liver disease, 80% came from abroad. Anti-HBs was believed to be of epidemiological rather than of pathological importance.

The prevalence of serum HBsAg in association with different forms of chronic liver disease has varied from study to study related to the method used but also the geographical origin of the subjects investigated (Fox *et al.*, 1969; Hadziyannis *et al.*, 1970; Prince *et al.*, 1970; Hollinger *et al.*, 1971; Bianchi *et al.*, 1972; Cooksley *et al.*, 1972; Doniach *et al.*, 1972; Ling and Overby, 1972; Peterson *et al.*, 1973; Reed *et al.*, 1973; Reesink *et al.*, 1973; Chrystie *et al.*, 1974; Vandervelde *et al.*, 1974). The present study concerns HBsAg and anti-HBs in patients with chronic liver disease both from the United Kingdom and elsewhere. Three sensitive methods, two haemagglutination and a radioimmunoassay and the less sensitive standard counterimmuno-electrophoresis

(CIEP) techniques were compared. The amount of circulating HBsAg may vary with the underlying hepatic disease (Dudley *et al.*, 1971), and serum HBsAg titres in positive patients were measured. Finally to obtain further evidence of exposure to the hepatitis B virus serum anti-HBs was examined using a passive haemagglutination method.

Methods

All serum samples were tested for the presence of HBsAg by four techniques.

CIEP using commercial 'ad' anti-HBs was used (Gocke and Howe, 1970), with reference 'ad' sera being employed as a control.

The reversed haemagglutination test (Hepanosticon-Organon Laboratories) which employed sheep anti-HBs was used on undiluted sera (Schuurs and Kacaki, 1974). An absorption procedure on all positive results was carried out, using sheep red cells coated with anti-sheep globulin.

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The turkey cell haemagglutination (Hepatest-Burroughs Wellcome) screening test, using turkey red cells coated with horse anti-HBs and test sera diluted at 1:8, was the second haemagglutination method applied (Cayzer *et al.*, 1974).

All sera were also tested by radioimmunoassay (RIA) (Ausria I—Abbott Laboratories) for HBsAg, the procedure being carried out according to the manufacturers' instructions (Ling and Overby, 1972).

All the sera found to be HBsAg positive by any of the techniques employed were later titred by doubling dilutions to a maximum of 1:8192 using the Hepatest method, except when the sera were positive only by RIA.

The passive haemagglutination method, as described by Vyas and Shulman, was used, with some modifications, to detect serum anti-HBs (Vyas and Shulman, 1970).

All the sera which gave a positive result for HBsAg by only one of the methods used were checked a second time. Also all sera found to be borderline positives by RIA were repeated with careful washing.

The Wilcoxon rank sum test was used to compare the results of one diagnostic group with another.

PATIENTS STUDIED

Four hundred and sixty-eight subjects were studied. All had been referred to the Liver Clinic at the Royal Free Hospital, London, but, in some, liver disease was not confirmed. The final diagnosis was based on clinical, biochemical, and immunological studies and, in the majority, liver biopsy sections were studied. The sex, country of origin, and any current treatment with immunosuppressive drugs was noted.

The patients suffered from chronic persistent hepatitis (19), alcoholic liver disease (53), primary biliary cirrhosis (58), primary liver cell carcinoma (15), miscellaneous hepatic tumours (12), biliary tract disease (47), cryptogenic cirrhosis and chronic active hepatitis (131). The miscellaneous liver disease group included primary haemochromatosis (six), Budd-Chiari syndrome (six), Wilson's disease (six) and chronic drug-related liver disease (three) and α_1 anti-trypsin deficiency (five). Other diagnoses were Gilbert's syndrome (22), and patients without proven liver disease (57).

Results

COMPARISON OF METHODS (Table 1)

One hundred and seventeen of the 604 sera collected were HBsAg positive by RIA and 116 of these were positive by the Hepatest. The Hepanosticon

Table 1 Comparison of HBsAg results using four different techniques: counterimmunoelectrophoresis, reversed haemagglutination, turkey cell haemagglutination, and radioimmunoassay in subjects with a variety of chronic liver diseases and also some with no liver disease

Diagnosis	HBsAg results by different techniques (nos.)				
	Total no.	CIEP	RHA	HA	RIA
Chronic persistent hepatitis	23	23	23	23	23
Alcoholic liver disease	6	6		6	6
Primary liver cell carcinoma	5	3	5	5	5
Cryptogenic cirrhosis and chronic active liver disease	79	44	76	78	79
No liver disease	4	4	4	4	4
Totals	117	80	114	116	117

test detected 114 of these HBsAg positive sera but CIEP detected only 80. Only titres of HBsAg exceeding 1:256 could be detected by CIEP. Titres less than 1:256 were often found in subjects with active liver disease and primary liver cell carcinoma and hence the results for CIEP testing were unreliable, particularly in these subjects.

CHRONIC PERSISTENT HEPATITIS

Thirteen of the 19 subjects were HBsAg positive by all four methods and in titres exceeding 1 in 512 (Fig. 1). Serial samples were taken from seven of these 19 subjects; five were positive initially and on subsequent testing; two were initially HBsAg negative and remained so. Five of the 13 HBsAg positive subjects were from the United Kingdom and four were male. All the remaining eight HBsAg positive subjects were of Mediterranean origin and seven were male. In another subject, anti-HBs was detected in a titre of more than 1 in 2048; two years later, she had been found to be HBsAg positive when screened for blood donation (Table 3). In none of the 19 was HBsAg and anti-HBs found simultaneously.

ALCOHOLIC LIVER DISEASE

In three of the 53 subjects, HBsAg was detected by all four methods in a titre of more than 1 in 512 (Fig. 1). Of six subjects tested serially, one was initially HBsAg positive and remained so and the other five remained HBsAg negative. None of the HBsAg positive subjects had received a blood transfusion; all were males from the United Kingdom.

Four patients had anti-HBs, one in a titre of more than 1 in 2048 and three in titres of less than 1 in 64

Fig. 1 Details of serum HBsAg in subjects with chronic liver disease. HBsAg titres in patients with chronic persistent hepatitis were significantly higher ($P > 0.01$) than in subjects with chronic active liver disease, with and without cirrhosis. HBsAg +ve by ● RIA, RPA and CIEP, ○ RIA and RHA. ■ RIA, CPH V CALD $P < 0.01$ (Wilcoxon's).

(Table 3). All were from the UK, one had had a previous blood transfusion, and three were male.

In none were both HBsAg and anti-HBs detected simultaneously.

PRIMARY LIVER CELL CARCINOMA

Five of 15 patients were found to be HBsAg positive; in three HBsAg was detected by all four methods, the titre being greater than 1 in 256 but less than 1 in 2048. In the other two, HBsAg was detected only by the three sensitive techniques, the titres being 1 in 128 and 1 in 64 respectively. Four of the five positive patients came from outside the UK; four were male. One other patient, from Iraq, had serum anti-HBs in a titre of 1 in 64 (Table 3).

In none could HBsAg and anti-HBs be detected at the same time.

CRYPTOGENIC CIRRHOSIS AND CHRONIC ACTIVE LIVER DISEASE WITHOUT CIRRHOSIS

Forty-three of the 131 patients had a positive HBsAg. Thirty-three of these were male and four were females from outside the UK (Table 2). None of the 32 females from the UK was HBsAg positive but six of the 26 males from the UK were HBsAg positive (Table 4).

Twenty-two patients who were initially HBsAg positive were studied serially (58 samples). The mean time interval between serial tests was 7.5 months (range one week to two years). All remained positive on serial testing.

Forty-four of the 79 sera were positive by all four methods. Thirty-two were positive only by the three sensitive techniques. Two sera were positive only by the Hepatest and RIA and one serum was positive only by RIA (Table 1).

In eight HBsAg positive patients, serum samples were obtained before and after starting prednisolone therapy (10–20 mg daily). The HBsAg titre did not alter significantly (Fig. 2). The other 14 HBsAg positive patients studied serially were already taking corticosteroids, but, regardless of any change in their liver function or in treatment, the HBsAg titres did not alter markedly.

The HBsAg titres in the positive sera ranged from 1 in 8 to the maximum dilution made, 1:8192 (Fig. 1).

Seventy samples were taken serially from 19 patients initially HBsAg negative and none was subsequently found to be positive.

Sera from 10 of the 131 subjects contained only anti-HBs; in six the titre exceeded 1 in 64 (Table 3). Retrospective analysis of 13 samples from four of these subjects, taken seven months to two years earlier, showed antibody to have been present then and in similar titre. Nine of these 10 patients were from outside the UK.

In three of the 43 HBsAg positive patients, anti-HBsAg could be detected simultaneously on more than one occasion. In two the antigen and antibody titres remained stable over a period of one year. In the third, before treatment, the antibody titre was 1 in 64 and the antigen titre 1 in 32, but after

Table 2 Serum HBsAg and anti-HBs results in 468 patients with wide variety of chronic liver disease*

Diagnosis	Results of HBsAg and anti-HBs testing (nos.)				UK	Male
	Patients	Sera	HBsAg +ve	Anti-HBs +ve		
Chronic persistent hepatitis	19	28	13	1	8	15
Alcoholic liver disease	53	61	3	4	43	38
Primary biliary cirrhosis	58	74	0	0	47	7
Primary liver cell carcinoma	15	15	5	1	8	11
Liver tumours (other)	12	14	0	0	7	5
Biliary tract disease	47	51	0	2	32	14
Cryptogenic cirrhosis and chronic active liver disease	131	218	43	13	58	82
Chronic liver disease (miscellaneous)	54	63	0	4	32	33
Gilberts syndrome	22	23	0	1	20	19
No liver disease	57	57	4	9	33	34
Totals	468	604	68	35	288	258

*Serum HBsAg found only in subjects with chronic persistent hepatitis, chronic active liver disease, with and without cirrhosis, primary liver cell carcinoma, alcoholic liver disease, and in some subjects with no obvious liver disease. Serum anti-HBs found in association with nearly all forms of chronic liver disease studied and in patients with no obvious liver disease.

Table 3 Details of serum anti-HBs findings

Total no.	Diagnosis	Anti-HBs findings			
		United Kingdom		Outside UK	
		Low titre <1:64	High titre >1:64	Low titre <1:64	High titre >1:64
19	Chronic persistent hepatitis	0	1	0	0
53	Alcoholic liver disease	3	1	0	0
15	Primary liver cell carcinoma	0	0	0	1
47	Biliary tract disease	0	0	2	0
131	Cryptogenic cirrhosis and chronic active liver disease	0	1	7	5
54	Chronic liver disease (miscellaneous)	0	0	2	2
22	Gilberts syndrome	0	0	1	0
57	No liver disease	0	1	6	2
	Totals	3	4	18	10

Not all subjects found to be positive had evidence of liver disease. Twenty-eight of 35 (80%) anti-HBs positive subjects came from overseas.

Table 4 Details of serum HBsAg findings in subjects with chronic active liver disease with and without cirrhosis*

Chronic active liver disease (with and without cirrhosis)									
United Kingdom					Outside UK				
	Total no.	HBsAg +ve	HBsAg -ve		Total no.	HBsAg +ve	HBsAg -ve		
		(no.)	(%)	(no.)		(no.)	(%)	(no.)	(%)
Males	26	6	23	20	56	33	58	23	42
Females	32	0	0	32	17	4	23	13	77

*No female from the United Kingdom was HBsAg positive. Six of 26 (23%) of males from the UK and 33/56 (58%) of males from overseas were HBsAg +ve. Four of 17 (23%) of females from overseas were HBsAg positive.

starting immunosuppressive therapy over 18 months, the antibody titre fell to 1 in 8 and the antigen titre rose to 1 in 512.

MISCELLANEOUS HEPATOBILIARY DISEASE

No patient with primary biliary cirrhosis, other biliary tract diseases, miscellaneous liver tumours, Gilbert's syndrome, or miscellaneous chronic liver

diseases was found to be HBsAg positive by any technique.

Serum anti-HBs was detected in two subjects with biliary tract disease, one with congenital hyperbilirubinaemia, two with primary idiopathic haemochromatosis, one with hydatid cysts in the liver, and one subject with a thrombosed portal vein. All these seven subjects were from outside the

Fig. 2 Serum HBsAg titres in eight subjects with chronic active liver disease, studied before and after treatment with corticosteroids. There was no significant change in the titres.

United Kingdom, one had received several blood transfusions, none gave a history of past hepatitis. In all but two anti-HBs titres were less than 1 in 64 (Table 3).

NO LIVER DISEASE

Four of the 57 subjects were found to be HBsAg positive by all four methods. In one the titre was 1 in 4092 and in the other three was 1 in 8192 (Table 2). All were male, two were from outside the UK. Although it is unproven, these four subjects may have been asymptomatic HBsAg carriers.

Nine of the 57 subjects had serum anti-HBs, six in a titre of less than 1 in 64, eight came from outside the UK, five were males (Table 3).

Discussion

As previously reported RIA was found to be the most sensitive technique for the detection of HBsAg (Reed *et al.*, 1973). However, all but one of the 117 sera found to be HBsAg positive by the RIA were HBsAg positive by the Hepatest. The results from Hepatest are available within 20 minutes and the method may be performed on single samples without the necessity for batches so that test material is not wasted. Hepatest, although slightly less sensitive is, therefore, more practical than RIA for clinical use. The other haemagglutination test (Hepanosticon) appeared to be less sensitive than the Hepatest.

The two sera found HBsAg positive by RIA and Hepatest but negative by Hepanosticon and CIEP had low titres of HBsAg (less than 1 in 16). As expected a serum with HBsAg titres less than 1 in 256 could not be detected using CIEP (Chrystie *et al.*, 1974). In subjects with chronic liver disease and primary liver cancer titres were low and the presence of HBsAg was often missed using CIEP. Although, because of the prozone phenomenon, false negative results with CIEP and Hepanosticon have been noted by Vandervelde (Vandervelde *et al.*, 1974) and ourselves, this was not seen in the present study.

The presence of HBsAg in patients with chronic active liver disease varied according to the geographical origin of the subjects studied. Whereas in Australia, and in the United Kingdom, the majority of chronic active liver disease is HBsAg negative (Fox *et al.*, 1969; Cooksley *et al.*, 1972), in Italy figures as high as 50% have been recorded (Bianchi *et al.*, 1972). We found chronic active liver disease with positive HBsAg to be much commoner in those coming from outside the United Kingdom—37 of 43 in fact, usually in those from Mediterranean countries. The predominance of males with HBsAg positive chronic liver disease was confirmed both in those from the United Kingdom and elsewhere. In fact not one female with chronic active liver disease and positive HBsAg test came from the United Kingdom. Both country of origin and male sex seem relevant features in the development of this disease.

None of the 28 HBsAg positive subjects followed serially for up to two years cleared HBsAg from the serum. Resolution of chronic hepatitis is said to be associated with clearance of HBsAg (Gentilini *et al.*, 1972); our subjects may not have been followed for sufficiently long for clearance to have occurred, but in none did liver function tests return to normal. Corticosteroid therapy had little effect on the HBsAg titres but the activity of the disease process does not seem to have been greatly changed by the therapy. However, an inverse correlation existed between the HBsAg titre and the activity of liver disease, in that those with chronic persistent hepatitis had significantly higher HBsAg titres than those with the more severe chronic active liver disease.

Although HBsAg was found in the serum of three alcoholics this is probably irrelevant to the pathogenesis. The association is rare and the HBsAg titre is very high, similar to that found with chronic persistent hepatitis and in asymptomatic carriers. Moreover, all three subjects were male homosexuals and such persons have a high rate of exposure to hepatitis B antigen. It is suggested that the positivity is a coincidental finding in those who are alcoholics.

The development of a carrier state in such patients might be due to depression of cell-mediated immunity, although if this were so some subjects with primary biliary cirrhosis should also become HBsAg carriers as they have depressed delayed hypersensitivity reactions in the later stages of the disease and are commonly exposed to blood from transfusions (Fox *et al.*, 1970). However, none of the 58 subjects with primary biliary cirrhosis was, in fact, HBsAg positive.

Serum anti-HBs appears to be of environmental importance as an indication of previous exposure to the hepatitis B virus. It was detected in seven subjects with a wide variety of unrelated hepatic disorders and in nine with no apparent liver disease. Fifteen of these 16 positive subjects originated from outside the United Kingdom where a high antibody prevalence rate is expected. Anti-HBs in subjects with chronic persistent and chronic active hepatitis and in primary liver cell carcinoma was again more often seen in those from abroad.

Patients with chronic active liver disease found to have detectable anti-HBs as well as HBsAg did not differ from those in whom only HBsAg could be detected.

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