PRESENCE OF A HEPATOMA-LIVER ANTIGEN IN THE SERA OF PATIENTS WITH PRIMARY HEPATOCELLULAR CARCINOMA

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SUMMARY

A Hepatoma-Liver Antigen (H-L Ag) was detected in the sera of 26 out of 51 (51%) patients with primary hepatocellular carcinoma (PHC) ($p < 0.001$). H-L Ag is a new antigen, not previously described in PHC patients. It was present in hepatoma tissue, sera of PHC patients, foetal tissue and also normal liver tissue.

It was not detectible in normal adult sera nor did it react immunologically with alphafoetoprotein or Hepatitis B surface antigen. It was even detectible in PHC patients who had no detectible elevation of alphafoetoprotein. The presence of the H-L antigen was not related to the extent of the tumour mass. HL Ag may be an altered component of normal liver or a foetal liver component present in normal liver tissues, such as foetal fibrinogen.

INTRODUCTION

Primary hepatocellular carcinoma (PHC) is a common cancer cause of death in Asia and the Pacific region. Early detection followed by either effective resection and/or effective chemotherapy offer the best chance for survival. Detecting high circulating levels of alphafoetoprotein (AFP) though reliable in most PHC patients has certain practical limitations e.g. seronegative AFP (by radioimmunoassay) are seen in about 14% of Singapore patients (Oon et al, 1976). AFP is a product of not only hepatoma cells (Ishizuka et al, 1976) but also of regenerating liver nodules (Karvounzis and Redeker, 1974). False positive AFP are also sometimes found in patients with chronic hepatitis, (15%) acute fulminating hepatic necrosis, Hepatitis B infections and in normal pregnancy. These elevations are usually in the range of 20-500 ng/ml. To distinguish this false positive group, it is thereore necessary to study other tumour markers in PHC patients. Price and Baldwin (1974) demonstrated the presence of a hepatoma-specific antigen in aminoazo dye induced rat hepatoma. Indirect evidence of a possible human hepatoma antigen was also shown by the inhibition of the leucocytes from PHC patients in the leucocyte inhibition test (Halliday et al, 1974).

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This report is to describe the presence of a "Hepatoma-Liver antigen(s)" (H-L Ag) in the sera of patients with histology proven — PHC.

PATIENTS

The sera of 51 patients with histology confirmed PHC were screened repeatedly. Similarly, the sera of 23 patients with clinical cirrhosis (who had normal levels of alphafoetoprotein by radioimmunoassay i.e. below 16 ng/ml) and 26 patients with acute hepatitis with normal AFP levels (both Hepatitis B and non B) were examined.

The sera of 56 normal adults, aged between 18 years to 65 years were screened concurrently. None of these individuals had any clinical stigmata of liver disease and their alphafoetoprotein levels were normal by radioimmunoassay.

MATERIALS AND METHODS

(A) **Preparation of soluble extracts from normal human liver and human hepatoma tissue**
Fresh autopsy material was used. Normal liver tissue was obtained from fatal road traffic accidents. Hepatoma tissue was obtained from patients, with previously histologically proven PHC. Such tissue was washed free of blood and finely minced. Chilled 3M Potassium chloride was added at 4 ml per gram wet weight of tissue, which was homogenised. The homogenate was stirred in the cold for 4 hours. It was then centrifuged at 20,000 g for 30 mins. after which the pellet was discarded. The soluble supernatant was dialysed against 0.01 M phosphate buffered saline at pH 7.3. The extracts were finally concentrated by ultrafiltration and the protein content of the extracts were determined by the method of Lowry et al (1951). It was then stored at -20°C.

(B) **Preparation of Rabbit anti-human hepatoma serum (RAHH)**
Rabbits were immunised subcutaneously with 5 mg of the tumour liver extract prepared above in complete Freund's adjuvant (Behringwerke AG Marburg and Radiochemical Laboratory, Frankfurt/M, West Germany). Repeat injections were given in incomplete Freund's adjuvant at weekly intervals. Animals were bled one week after the final injection and the antiserum was exhaustively absorbed with pooled normal human serum, and AFP.

(C) **Immunodiffusion and Immunoelectrophoresis**
Immunodiffusion was carried out according to the method of Ouchterlony et al (1953). Immunoelectrophoretic analyses were performed according to Scheidegger et al (1955) on microscope slides coated with 1% agarose in 0.025 M veronal buffer pH 8.6. Following development of precipitin bands in the agarose plates, the plates were washed in 0.15 M NaCl, followed by distilled water and then dried and stained for protein with amido black 10 B. (Sigma Chemical Co., St. Louis, Missouri, USA).

(D) Alphafoetoprotein levels were measured by radioimmunoassay (Dainabot). In 100 normal adult so tested the range was between 0-16 ng per ml. (mean 6 ng/ml).
Hepatitis B surface Antigen HBsAg (per kindness Dr Chan So Har, W.H.O. Immunology & Training Centre, Singapore) was measured by counterimmunoelectrophoresis (CIE) and immunodiffusion.

(E) Foetal tissue studied were 3M KCl extracts from 16 weeks aborted human gestational foetuses. The tissues analysed were liver, kidney, heart, stomach and lung.

RESULTS

(A) **Reactivity against Hepatoma Extracts, normal liver and foetal tissue extracts and sera of Hepatoma patients**
The absorbed RAHH reacted against the extracts of human hepatoma cells, normal human liver various human foetal tissue extracts, and the sera of 26 out of 51 patients with PHC in immunodiffusion plates (see Fig. 1 and 2). Following absorption with hepatoma or normal liver cells, this antiserum showed no further reaction against hepatoma sera, normal liver, hepatoma liver or extract foetal tissue. Immunoelectrophoretic analysis of liver extract and hepatocarcinoma sera showed that this antigen has an electrophoretic mobility of a rapidly migrating β_2 — globulin (Fig. 3).

(B) **Reactivity against Hepatitis B surface antigen and alphafoetoprotein**
The RAHH serum showed no cross reactivity against Hepatitis B surface antigen, or against alphafoetoprotein when tested by counterimmunoelectrophoresis. Only 6 patients in the hepatitis group were positive for HBsAg.

(C) **Reactivity in Hepatoma patients (see Table 1 and 2)**
Repeated tests on the sera of 51 patients showed the presence of the H-L antigen in 26 patients. H-L antigen was detectible in many patients with advanced disease but not in those who were in complete clinical remission. False negative results were present regardless

of the extent of the disease. The presence of this HL antigen did not correlate with the degree of hyperbilirubinaemia or the clinical severity of the disease. All 6 of the 51 PHC patients with seronegative AFP levels had detectible H-L antigen in their sera i.e. 100% positive detection for seronegative AFP patients. (See Table 1).

(D) **Reactivity in Cirrhosis and Hepatitis (see Fig. 4)**
4 out of 23 patients with cirrhosis and 5 out of 26 acute hepatitis patients had detectible H-L antigen in their sera. Of the 4 patients with cirrhosis, one patient, a male, aged 57 had a level of AFP of 2,750 ng per ml detected 2 months after the finding of H-L antigen. At necropsy, liver cell carcinoma was seen. However, in a second cirrhosis patient, no obvious tumour was seen at necropsy.

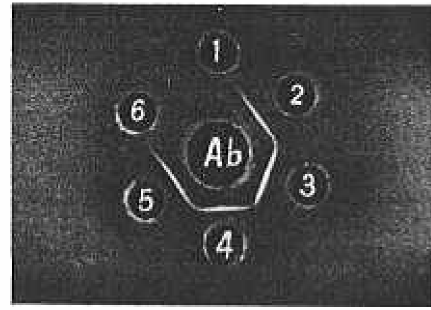
DISCUSSION

Our results show the presence of a H-L antigen in the sera of patients with PHC. This finding has never been described in man. Over 50% of hepatoma patients had detectible H-L antigen(s) in their sera. False negative results were also seen in 49% of patients, but this may reflect the insensitivity of the present testing system. This antigen(s) was present in normal liver, and foetal tissue such as foetal liver, heart, kidney, and stomach. Following absorption with normal human liver, the RAHH showed no further reaction against hepatoma liver extracts, the sera of PHC patients, and different foetal tissue extract suggesting that the HL Ag had a common antigenicity with some component of normal and foetal tissues.

H-L antigen was not found in the sera of healthy normal adults. It showed no immunological cross reactivity with alphafoetoprotein or with HBsAg. H-L antigen was also present in the sera of 4/23 (17%) with cirrhosis, 5/26 (19%) with acute hepatitis and 4 other patients with chronic aggressive hepatitis. HBsAg was positive in one of the 4 patients. This patient had histological evidence of massive liver necrosis (See Table 3). However, 6 other patients with chronic persistent hepatitis had no detectible H-L antigen in their sera.

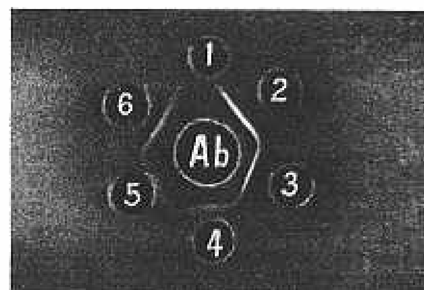
Indirect evidence of a possible human hepatoma specific antigen in the sera of patients was shown by the inhibition of leucocyte adherence inhibition test by the serum of hepatoma patients (Halliday et al, 1974).

Smither and Iverson (1973), and Suguwara and Smith (1976) described a liver specific antigen (F-antigen) which was found in the sera of patients with hepatic necrosis, metastatic cancers, viral hepatitis and infrequently in hepatoma (10%). The



CENTRAL WELL CONTAINS ABSORBED RABBIT ANTI-HUMAN HEPATOMA ANTISERUM

Figure 1: Double diffusion — showing precipitation lines between rabbit anti-human hepatoma antiserum (central well) and normal liver extract, hepatoma liver extract, hepatoma sera of three different patients.
Peripheral wells contain:—
1. Normal human serum.
2. 3M KCl extraction of normal human liver.
3. 3M KCl extraction of hepatoma liver.
4. Hepatoma patient with negative AFP (O ng/ml).
5. Hepatoma patient with positive AFP (186,000 ng/ml).
6. Hepatoma patient with positive AFP (180 ng/ml).



CENTRAL WELL CONTAINS ABSORBED RABBIT ANTI-HUMAN HEPATOMA ANTISERUM

1. Normal Human Serum
2. Human Hepatoma Liver Extract
3. Human Foetal Liver Extract
4. Human Foetal Heart Extract
5. Human Foetal Lung Extract
6. Human Foetal Kidney Extract

Figure 2: Double diffusion — showing precipitation lines between rabbit antihuman hepatoma (central well) and hepatoma extract, foetal liver, foetal heart, foetal lung and foetal kidneys.

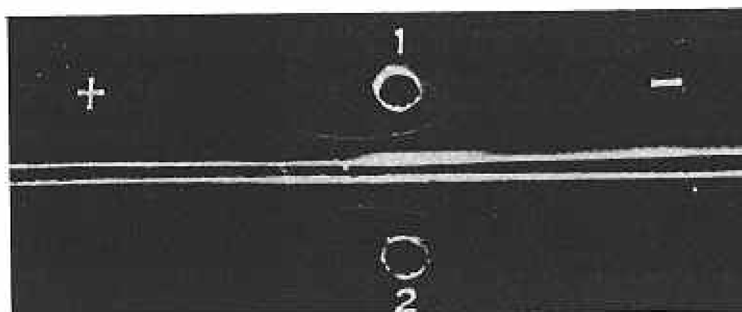


Figure 3: Immunoelectrophoretic pattern of:-
1. 3M KCl extraction of hepatoma liver and
2. serum from a hepatoma patient against absorbed rabbit anti-human hepatoma antiserum (RAHH).

TABLE 1 Relationship between the levels of serum AFP, Bilirubin and SGPT and the presence of H-L antigen in patients with hepatocellular carcinoma

HEPATOMA PATIENTS			H-L Ag	AFP ng/ml (0-16 ng/ml)	SGPT 5-40 IU/L	SERUM BILIRUBIN mg/100 ml
1.	L.H.P.	M/68	Pos.	0	30	0.9
2.	T.T.K.	M/76	Pos.	0	37	1.7
3.	T.W.K.	F/42	Pos.	2	68	2.0
4.	C.K.C.	M/63	Pos.	3	23	0.9
5.	L.L.N.	F/59	Pos.	6	36	2.8
6.	T.K.C.	M/55	Pos.	15	45	1.4
7.	L.K.T.	M/55	Pos.	36	148	2.9
8.	C.C.S.	M/61	Pos.	174	78	1.9
9.	A.K.	M/66	Pos.	190	32	2.6
10.	H.S.H.	M/55	Pos.	400	40	2.5
11.	L.C.M.	M/72	Pos.	520	38	0.7
12.	W.N.M.	M/73	Pos.	3,800	80	0.7
13.	P.S.	M/65	Pos.	5,000	144	23.5
14.	N.V.Q.	M/46	Pos.	5,250	80	0.5
15.	N.P.W.	M/53	Pos.	12,300	76	0.9
16.	L.K.	M/69	Pos.	18,600	31	3.7
17.	T.C.H.	M/51	Pos.	30,000	124	17.1
18.	T.G.T.	M/55	Pos.	32,000	88	0.9
19.	K.K.	M/63	Pos.	32,000	38	0.7
20.	N.H.L.	F/45	Pos.	35,000	20	0.5
21.	T.J.C.	M/65	Pos.	64,000	70	0.9
22.	C.T.E.	F/47	Pos.	64,000	58	7.4
23.	C.E.T.	F/28	Pos.	64,000	26	0.9
24.	L.A.S.	M/28	Pos.	80,000	43	0.9
25.	Y.N.C.	M/48	Pos.	80,000	44	1.4
26.	M.H.	M/71	Pos.	90,000	60	6.4

TABLE 2 Relationship between levels of serum AFP, Bilirubin, SGPT and the presence of H-L antigen in the serum

HEPATOMA PATIENTS			H-L Ag	AFP levels (Normal 16 ng/ml)	SGPT IU/L	Bilirubin mg/DL
27.	K.B.H.	F/23 (clinical remission)	Neg.	3	28	0.5
28.	L.T.T.	M/57	Neg.	40	26	0.9
29.	S.K.T.	M/52	Neg.	45	60	2.6
30.	T.C.H.	M/48	Neg.	80	>200	11.5
31.	C.F.S.	M/78	Neg.	100	125	2.4
32.	O.L.D.	M/54	Neg.	310	68	2.3
33.	C.A.Y.	M/75	Neg.	400	40	1.9
34.	T.C.P.	M/44	Neg.	500	58	3.0
35.	S.T.G.	M/31	Neg.	600	50	2.9
36.	T.H.M.	M/58	Neg.	600	64	0.8
37.	T.K.Y.	M/60	Neg.	600	40	0.7
38.	L.Y.L.	M/60	Neg.	1,600	66	2.8
39.	L.F.	M/57	Neg.	2,250	20	0.4
40.	J.K.	M/69	Neg.	2,580	68	1.5
41.	C.Y.P.	M/57	Neg.	5,200	84	5.5
42.	Y.T.S.	M/64	Neg.	10,400	164	1.6
43.	L.B.B.	M/65	Neg.	10,400	30	0.5
44.	L.Y.P.	M/57	Neg.	10,600	50	12.0
45.	N.P.W.	M/52	Neg.	12,300	76	0.9
46.	T.G.K.	M/70	Neg.	16,000	42	0.5
47.	K.L.C.	F/28	Neg.	40,500	30	0.5
48.	A.J.B.F.	M/56	Neg.	64,000	>200	16.5
49.	L.P.H.	M/44	Neg.	96,000	136	4.0
50.	Y.B.K.	M/44	Neg.	128,000	51	5.7
51.	L.G.K.	F/17	Neg.	>160,000	26	0.9

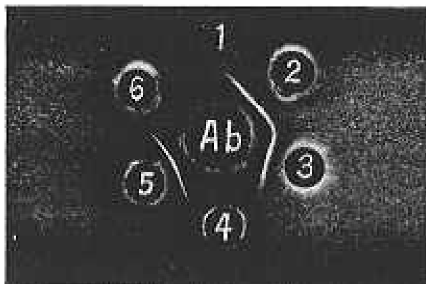


Figure 4: Double diffusion — showing precipitation lines between rabbit antihuman hepatoma antiserum and hepatoma extract, the sera of two patients with cirrhosis and two patients with acute hepatitis.
Peripheral wells contain:-
1. Normal human serum
2. 3M KCl extraction of hepatoma.
3. Serum of Cirrhosis patient (T.E. M/44).
4. Serum of Cirrhosis patient (L.K.E.M./44).
5. Serum of acute hepatitis (T.J.K. M/28) — (HBsAg Neg.).
6. Serum of Chronic active hepatitis (T.J.H. M/29) — (HBsAg Pos.)

F-antigen prepared by Smith and Iverson was a mouse anti-mouse liver extract which showed immunological cross reactivity with other species including man.

Our absorbed rabbit anti human hepatoma sera showed a high detection rate for PHC (51%) suggesting that the H-L antigen is probably different from the F-antigen because the latter is not found in other tissues. By using an insensitive immunodiffusion technique so far, the presence of the H-L antigen appears to be as sensitive a marker for PHC as AFP. Where it was even more useful was the finding that H-L Ag was detectible even when the AFP levels were not elevated in biopsy positive PHC patients.

If the liver antigen is believed to be released into the circulation following liver cell necrosis which was suggested by the studies of Smith and Iverson, this does not seem to be shown in our study. Many patients with severe hepatic necrosis e.g. various forms of hepatitis with very high levels of SGOT and SGPT still had a small percentage of detectible HL Ag in their sera.

This preliminary data, thus suggest that H-L Ag may be an unusual component derived from normal liver cells, which is also present in foetal tissues. This antigen is also present in over 50% of the sera of PHC patients. Preliminary information shows the H-L antigen to be stable and its presence is effected by treating to 40°C. It is also unlikely to be a Liver Specific lipoprotein (Cochrane 1977, McFarlane, et al, 1977) which is more heat liable. If H-L Ag is a hepatic enzyme released by hepatoma cells, then the low HL-Ag detection rate in hepatic necrosis makes this hypothesis also unlikely.

Surprisingly, HL Ag was found in normal plasma and has immunological identity with fibrinogen in double diffusion plates. However, preliminary studies using 7.5% polyacrylamide disc gel electrophoresis show that the mobility of H-L antigen was different to pure human fibrinogen, suggesting that this antigen may be related to an abnormal fibrinogen such as foetal fibrinogen as described by Gralnick et al, (1978).

The further characterisation of HL Ag is a subject of a further report. If present studies confirm its specificity for PHC patients, then this antigen would be another useful additional tumour marker for PHC patients with seronegative AFP levels.

ACKNOWLEDGEMENTS

We thank the Shaw Foundation and the Singapore Turf Club for their generous support. S.L.Y. is funded by a Lee Foundation Liver Cancer Research Fellowship.

We also thank our clinical colleagues for referring patients for this study and to Drs. Chao Tze Cheng, Gilbert Chiang, T. Mathews, Jimmy Sng Ewe Hui, Chan So Har and staff of the W.H.O. Immunology and Training Centre, Singapore for technical advice and assistance.

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