Hepatitis B virus antigens in primary hepatic carcinoma: immunofluorescent techniques on fixed liver tissue

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SUMMARY The presence of hepatitis B surface (HBsAg) and core (HBcAg) antigens was investigated by immunofluorescence in specimens of liver tissue obtained at necropsy in 107 patients with primary hepatic carcinoma. HBsAg was detected in the cytoplasm of liver cells in 16 cases, and in eight of them the antigen was also found in malignant cells.

HBcAg, which was present in the nuclei of liver cells in eight cases, was detected in the nuclei of tumour cells in six of these and also in two other cases showing HBsAg, but not HBcAg, in the non-neoplastic tissue. Although most of the primary hepatic carcinomas studied were associated with cirrhotic changes in the non-neoplastic tissue, HBsAg and HBcAg were also detected in the absence of underlying cirrhosis.

Hepatitis B virus markers were demonstrated in non-neoplastic tissue, mainly in patients with a well-differentiated carcinoma, and only in these cases were they found also in the neoplastic tissue.

These results show that hepatitis B virus antigens, including HBcAg, can be detected in the neoplastic cells of well-differentiated carcinoma of the liver. Although these cells could have been infected after the malignant transformation, a direct oncogenic role of the virus cannot be excluded.

Serological markers of hepatitis B virus (HBV) infection (HBsAg, anti-HBs, anti-HBc) have been detected in patients with hepatocellular carcinoma, their incidence varying considerably in different geographical areas (Smith and Francis, 1972; Reed *et al.*, 1973; Blumberg *et al.*, 1975; Chainuvati *et al.*, 1975; Maupas *et al.*, 1975; Larouze *et al.*, 1976; Welsh *et al.*, 1976; Kubo *et al.*, 1977).

The HBsAg carrier state, with or without associated chronic liver disease, and hepatocellular carcinoma tend to occur in familial clusters in countries where both these conditions are common (Denison *et al.*, 1971; Ohbayashi *et al.*, 1972), suggesting a possible role of HBV in the aetiology of primary hepatic carcinoma. Direct evidence for HBV oncogenicity is, however, lacking.

The presence of HBV antigens (HBsAg and HBcAg) in the liver of chronic carriers of HBsAg has been extensively documented; in patients with HBV infection and primary hepatic carcinoma, HBsAg

has been frequently detected in non-neoplastic liver cells, while the presence of HBV antigens in neoplastic tissue remains controversial (Portmann *et al.*, 1976; Shikata, 1976). Nazarewicz *et al.* (1977) recently described HBsAg in the cytoplasm of cells of well-differentiated primary hepatic carcinoma; but HBcAg was not observed in neoplastic tissue by Hadziyannis *et al.* (1976).

In a retrospective study, we have now investigated by immunofluorescence the localisation of HBsAg and HBcAg in the livers of 107 cases of primary hepatic carcinoma.

Material and methods

A total of 107 necropsy cases of primary hepatic carcinoma (77 male and 30 female; age range 1-93 years) seen at the Istituto di Anatomia Patologica of the University of Padua and at the Servizio di Anatomia Patologica degli Ospedali Civili Riuniti in Venice from 1973 to 1977 were included in our study.

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Paraffin-embedded liver specimens were available from both neoplastic and non-neoplastic tissue, and serial sections were used for routine histological staining and immunofluorescent studies. Histological classification was based on the architectural structure of the tumour, according to Leevy and Tygstrup (1976). In addition, the tumours were graded according to their resemblance to original tissue, nuclear pleomorphism, and the number of mitoses, and were graded I to III, according to Hutt and Anthony (1973).

Seventeen patients with metastatic epithelial carcinoma of the liver were examined as controls.

IMMUNOFLUORESCENCE OF LIVER TISSUE

Paraffin sections, 5 μ thick, were mounted on clean slides without adhesive. Deparaffinisation was accomplished by sequential passage through xylene and graded alcohols, and the sections were washed in phosphate-buffered saline (PBS) pH 7.4. These sections were then used for the identification of HBcAg and HBsAg by means of direct and indirect immunofluorescent techniques respectively. Serial sections were prepared from each block of liver.

Indirect immunofluorescent techniques for HBsAg

The sections were incubated at 37°C for 45 minutes with rabbit anti-HBs (Behringwerke, Marburg) diluted 1:5, washed in PBS, and then treated for 30 minutes at 37°C with fluoresceinated goat anti-rabbit immunoglobulin (7s) (Meloy Lab. Dil. 1:8, F/P 4, Prot. C-12 mg/ml). The slides were then briefly rinsed in PBS and mounted in glycerol plus PBS. Immunofluorescent staining was examined with the Leitz Orthoplan Ploemak fluorescence microscope. Control tests consisted of specific

abolition of fluorescence by adsorption of the rabbit anti-HBs serum with a purified preparation of HBsAg containing a mixture of *ad* and *ay* subtypes, prepared from plasma samples of healthy carriers according to Gerin *et al.* (1971).

Direct immunofluorescent techniques for HBcAg

Anti-HBc human IgG was obtained from acute hepatitis B convalescent serum, which was anti-HBs negative by radioimmunoassay and anti-HBe negative by immunodiffusion. The IgG fraction was conjugated with fluorescein isothiocyanate (FITC), according to Arnold and Mayersbach (1972), and used at a final concentration of 0.5 mg protein/ml. This serum showed a strong nuclear fluorescence when tested on reference liver tissue rich in HBcAg.

Deparaffinised sections were incubated at 37°C for 30 minutes with the conjugated serum. Fluorescence was considered specific when blocked by unlabelled antiserum in the inhibition test. Moreover, positive results were confirmed with two reference anti-HBc FITC-labelled sera. All the sections with a positive reaction for HBsAg and/or HBcAg were then washed in PBS, stained with haematoxylin and eosin, and permanently mounted. Photographs were taken using Kodak Ektachrome daylight 200 ASA film before and after this last procedure.

Results

HISTOLOGICAL CLASSIFICATION (Table 1)

Thirty-six patients had a well-differentiated carcinoma grade I, 37 a moderately well-differentiated carcinoma grade II, while a poorly differentiated carcinoma grade III was present in 34 cases.

The trabecular and/or pseudoglandular patterns were observed most frequently in the grades I and II

Table 1 *Histological classification of primary hepatic carcinoma and underlying liver pathology*

No. of cases	Sex	Age (mean range) (years)	Histological classification		Underlying liver pathology		
			Grade	Morphology	NSRH	CAH	Cirrhosis
36	29 M	61 (1-88)	I Well differentiated	Trabecular	4	3	22 (3)*
				Pseudoglandular	0	1	3 (1)*
				Solid	0	0	2
	7 F	71 (53-93)		Clear cells	0	0	1
37	27 M	61 (42-76)	II Moderately differentiated	Trabecular	2	1	13 (3)*
				Pseudoglandular	1	1	14 (4)*
				Solid	0	0	2 (1)*
	10 F	60 (32-80)		Pleomorphic	0	0	3
34	21 M	62 (42-74)	III Poorly differentiated	Trabecular	0	0	1 (1)*
				Pseudoglandular	1	0	3
				Solid	0	0	2
	13 F	63 (19-79)		Pleomorphic	5	2	20 (2)*

NSRH = non-specific reactive hepatitis; CAH = chronic active hepatitis.

*Numbers of cases with cirrhosis and associated chronic active hepatitis are given in parentheses.

Table 2 Relationship between histological pattern of primary hepatic carcinoma, underlying liver disease, and HBsAg and HBcAg localisation in non-neoplastic and neoplastic liver

Patient Sex/Age	Histological pattern of PHC		Underlying liver disease	HBsAg localisation		HBcAg localisation	
	Grade	Cytoarchitectural structure		Non-neoplastic tissue	Neoplastic tissue	Non-neoplastic tissue	Neoplastic tissue
1 M 69	I	Trabecular	Cirrhosis	++	Negative	+	+
2 M 62	I	Trabecular	Cirrhosis	++	+	Negative	Negative
3 F 82	I	Pseudoglandular	Cirrhosis	++	+	Negative	Negative
4 M 47	I	Trabecular	Cirrhosis	+++	+++	+	+
5 M 66	I	Clear cells	Cirrhosis	++	Negative	Negative	+++
6 M 68	I	Trabecular	Cirrhosis	++	Negative	Negative	+++
7 M 63	II	Pseudoglandular	Cirrhosis	++	Negative	Negative	Negative
8 M 66	III	Pleomorphic	Cirrhosis	++	Negative	+	Negative
9 M 54	I	Pseudoglandular	CAH + cirrh	++	+	+	+
10 M 59	I	Trabecular	CAH + cirrh	+++	+	Negative	Negative
11 M 76	II	Pseudoglandular	CAH + cirrh	++	Negative	+	Negative
12 F 62	I	Pseudoglandular	CAH	++	+	Negative	Negative
13 M 52	I	Trabecular	CAH	++	++	+	++
14 M 62	I	Trabecular	CAH	++	Negative	++	+++
15 M 64	I	Trabecular	NSRH	+++	+	Negative	Negative
16 M 75	I	Trabecular	NSRH	+++	Negative	+	+

CAH = chronic active hepatitis; NSRH = non-specific reactive hepatitis.
+++ several cells; ++ few cells; + rare cells.

tumours, while the pleomorphic pattern was predominant in the poorly differentiated tumours. An underlying cirrhosis was present in 85 cases, and chronic active hepatitis was seen in eight. Features ascribed to only non-specific reactive hepatitis were observed in 13 cases.

DETECTION OF HBSAg BY IMMUNO-FLUORESCENCE (Table 2)

HBsAg was detected in the non-neoplastic tissue of

16 (14.9%) of the 107 cases of hepatocellular carcinoma (13 grade I, 2 grade II, 1 grade III). Cirrhosis was present in 11 of these 16 cases, and the remaining five showed underlying chronic active hepatitis (3 cases) or non-specific reactive hepatitis (2 cases).

HBsAg positive cells were scattered among cases with chronic active hepatitis or cirrhosis, while a sheet distribution was found in cases of non-specific reactive hepatitis. HBsAg was also present in the

Fig. 1 Hepatitis B surface antigen in cells of well-differentiated hepatocellular carcinoma (indirect immunofluorescence $\times 400$).

Fig. 2 (a) Cytoplasmic localisation of HBsAg in a large number of cells from well-differentiated primary hepatic carcinoma; (b) the same field stained by haematoxylin and eosin ($\times 280$).

cytoplasm of cancer cells in eight cases (all grade I). The number of HBsAg containing cells was lower in the neoplastic tissue compared to the normal tissue. Many positive neoplastic cells were arranged in clusters in one case (No. 4) (Fig. 1), while in the others, cells containing HBsAg were rare and were located at the periphery of the neoplastic tissue. After haematoxylin and eosin staining these cells were considered to be part of the carcinoma (Fig. 2). The brightness of the HBsAg positive cells in malignant tissue was uniformly weaker in comparison with the positively stained hepatocytes in the non-neoplastic area.

HBsAg specific fluorescence was never observed in connective tissue, bile ducts, proliferating ductular cells or vessels. Small dense round deposits of HBsAg positive material were occasionally detected in necrotic areas.

Immunofluorescent specificity was confirmed by blocking tests with HBsAg, prepared as previously described. FITC goat anti-rabbit immunoglobulins completely lost their staining ability on positive liver tissue when the first incubation was carried out with anti-HBs serum mixed with purified HBsAg.

DETECTION OF HBcAg BY IMMUNO-FLUORESCENCE

HBcAg in a granular pattern was detected in the nuclei of non-neoplastic cells in eight cases (6 grade I; 1 grade II; 1 grade III) with a focal distribution of positive hepatocytes. In six of these cases HBcAg was also found in scattered nuclei in the neoplastic

cells (Fig. 3). In two other cases, which were negative for HBcAg in non-neoplastic tissue, many cancer cells showed a positive nuclear reaction with anti-HBc serum (Fig. 4). The nuclear pattern of HBcAg in neoplastic tissue was similar to that observed in normal cells.

All positive results could be inhibited by unlabelled anti-HBc serum and were confirmed when reference anti-HBc sera were used.

Neither HBsAg nor HBcAg was detected in any of the metastatic epithelial carcinomas of the liver studied.

Discussion

This study represents, to our knowledge, the largest series yet reported of cases with primary hepatic carcinoma investigated for the presence of hepatitis B virus markers in the liver (Hadziyannis *et al.*, 1976; Portmann *et al.*, 1976; Shikata, 1976; Nazarewicz *et al.*, 1977).

Serum was not available in most cases, this study being retrospective, and therefore it was difficult to establish the exact incidence of HBV infection in our patients. It is likely that the subjects positive in the liver (15%) represent only those with a more active rate of virus replication.

We detected HBsAg in both neoplastic and non-neoplastic tissue not only in primary hepatic carcinoma with underlying cirrhosis but also in association with chronic active hepatitis or non-specific reactive hepatitis. The expression of surface

Fig. 3 Staining of intranuclear HBcAg in well-differentiated tumour cells (direct immunofluorescence $\times 260$).

and core antigens in non-neoplastic tissue often recalled the distribution pattern seen in chronic carriers of HBV infection (Gudat *et al.*, 1975). In primary hepatic carcinoma with underlying non-specific reactive hepatitis, many HBsAg positive cells were arranged in sheets while HBcAg was poorly represented; HBsAg positive hepatocytes were irregularly scattered throughout the lobules or arranged in a nodular form, with a variable number of positive nuclei for HBcAg in the cases with underlying cirrhosis or chronic active hepatitis.

HBsAg and HBcAg were distributed more irregu-

larly in the malignant areas than in non-neoplastic tissue. This difference in viral expression may reflect different metabolic properties of the neoplastic cells. Tumour cells contained different amounts of viral antigens, and virus proliferation usually occurred in the peripheral neoplastic cells rather than in the central tumour areas. These findings suggest that virus infection in neoplastic cells may be an expression of variable metabolic environment within uniform cell clones. Cytochemical properties different from those of normal hepatocytes could determine differences in virus infection and replication,

Fig. 4 (a) Nuclear localisation of HBcAg in a well-differentiated primary hepatic carcinoma, using an FITC-labelled anti-HBc; (b) the same field stained by haematoxylin and eosin ($\times 480$).

since the number of susceptible cells and their accessibility to a virus are related to their cooperation with its replicative cycle (Smith and Francis, 1972).

HBV markers were detected mainly in the liver of patients with a well-differentiated tumour, and in all these cases either HBsAg or HBcAg or both were found also in malignant cells.

The association we found between HBV infection and well-differentiated primary hepatic carcinoma is a surprising finding, and its interpretation is difficult. However, HBsAg was also detected in the liver of three patients with less well-differentiated tumour, and it was interesting to note that in these cases HBV antigens were never observed in the malignant cells. These observations suggest that HBV replicates in well-differentiated neoplastic cells, in agreement with the findings of Nazarewicz *et al.* (1977). We have now described the presence of HBcAg, which is related more to HBV replication than is HBsAg, in these cells. This is in agreement with the results of Watanabe *et al.* (1976), who found an active replication and proliferation of HBV in 'oval cell' (immature hepatocyte) cultures.

The higher susceptibility to HBV infection of well-differentiated primary hepatic carcinoma cells, as compared to poorly differentiated tumours, could support the hypothesis that highly differentiated hepatoma cells, showing minimal deviation from normal (Glazer *et al.*, 1974), are as susceptible as hepatocytes to HBV growth.

Our results revive the question whether virus has a direct oncogenic role in primary hepatic carcinoma associated with HBV infection. Moreover, the absence of an underlying cirrhosis in five cases of hepatocellular carcinoma suggests that the association with HBV infection cannot be explained simply by the occurrence of malignant changes in the cirrhotic nodules.

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