

Detection of Hepatitis B Surface Antigen in Fixed Tissues of Patients With Cirrhosis and Hepatoma

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Summary. HBsAg has been sought by light microscopy in liver specimens from patients with cirrhosis (79 cases) and hepatoma (99 cases). The study was carried out on fixed material using orcein staining, immunoperoxidase technique and indirect immunofluorescence. HBsAg was detected in the serum by radio-immunoassay (RIA) using Ausria II-125 in 38 patients with cirrhosis and in 36 with hepatoma. In the 38 seropositive cases of cirrhosis HBsAg-positive cells were observed in 31 (81.6%) by the orcein staining and in 32 (84.2%) by the peroxidase and immunofluorescence staining. Among the 36 seropositive patients with hepatoma, HBsAg was detected in the surrounding non-neoplastic part of the liver, cirrhotic or not, in 30 (83.3%) by orcein staining and in 34 (94.4%) by the immunoperoxidase method and immunofluorescence. Positive solitary-cells were seen occasionally in the tumor tissue in 16 cases using orcein, in 9 using peroxidase and in 7 by fluorescence, out of the 36 seropositive patients with hepatoma. The results of this study do not support the hypothesis of a direct oncogenic effect of HBsAg on the liver cells, since this antigen was detected mainly in the non-neoplastic part of the liver tissue and only occasionally in the tumor cells. Of the 63 cases of seronegative hepatoma, 3 showed some round orcein-positive inclusion bodies in the cytoplasm of the neoplastic and the non-neoplastic cells; these bodies were not stained by the two immunological methods.

Key words: Hepatitis B antigen – Hepatoma – Cirrhosis – Immunoperoxidase – Immunofluorescence.

Introduction

The role of hepatitis B virus in the aetiology of liver cirrhosis is now established. It is usual to classify a case of chronic carriage of hepatitis B surface antigen

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(HBsAg) with "cryptogenic" cirrhosis, as post-hepatic. In some countries the prevalence of hepatitis B markers in the serum and in the liver tissue of cirrhotics is high, while in others it has been found to be low (Buffet et al., 1977; Bogomoletz, 1976; Deodhar et al., 1975; Nayak and Sachdeva, 1975; Theodoropoulos et al., 1978; Turbitt et al., 1977; Zuckerman, 1975). These geographical differences are due to the different factors implicated in the aetiology of cirrhosis in each country. However, the relationship of hepatitis B virus to liver oncogenesis remains unclear (Peters et al., 1977). In some countries, the prevalence of HBsAg in the serum of cases of hepatocellular carcinoma was found to be high, while in others it was low (Theodoropoulos et al., 1974).

In this report we present the results of a study of HBsAg in the liver tissue of Greek patients with cirrhosis and hepatoma, and correlate our findings with the presence of this antigen in the serum.

Materials and Methods

HBsAg has been looked in formalin-fixed, paraffin-embedded liver specimens from patients with cirrhosis (79 cases) and hepatoma (99 cases). Specimens were taken by using the Menghini needle in 101 cases, after laparotomy in 49 and after autopsy in 28. In all cases of hepatoma neoplastic as well as non-neoplastic tissues were present in the specimen. HBsAg was detected in the serum by radioimmunoassay using Ausria II-125 (Abbott). HBsAg was found to be present in the serum in 38 patients with cirrhosis and in 36 with hepatoma.

The study of the HBsAg in the liver tissue was carried out using three techniques:—Orcein staining was performed according to the method of Shikata et al. (1974). — The immunoperoxidase method of Burns (1975) was applied to 6 µm paraffin sections of liver with modifications to diminish background and endogenous peroxidase staining. Rabbit anti-HBs antiserum was obtained from Behringwerke and the other antisera and the peroxidase-antiperoxidase complex from Dakopatts (Mercia Diagnostics). — Indirect fluorescence was carried out as described by Camilleri (1977). Fluoresceinated goat anti-rabbit immunoglobulin was obtained from Behringwerke. Fluorescence was studied using a Zeiss photomicroscope, equipped with an HBO-200 watt darkfield condenser, UG-I exciter filter and 47/65 barrier filter.

Results

The results of this study were as follows: In the 38 cases of seropositive cirrhosis HBsAg was detected in the cytoplasm of hepatocytes in 31 of cases (81.6%) by the orcein staining and in 32 (84.2%) by the peroxidase and the fluorescence methods. Among the 41 seronegative cirrhotics, none showed positive staining or fluorescence. The intensity and distribution of the staining varied widely in each case. In most cases there were substantial areas of liver tissue with large numbers of positive cells, and other areas which did not stain. In these sections there were scattered solitary positive cells (Table I). The appearance in a positively stained hepatocyte in the orcein stain was that of a homogenous dark brown deposit within the cytoplasm of the cell. Nuclei remained unstained (Fig. 1).

The appearance of a positive cell after peroxidase staining was of a finely granular brown pattern contrasting with the pale background of the surrounding negative cells. In some instances the staining reaction appeared to fill the entire

Table 1. Detection of HBsAg in paraffin sections of cirrhosis and hepatoma by orcein and immunoperoxidase stainings and indirect immunofluorescence

	No examined	Orcein (+)	Immuno- peroxidase (+)	Immuno- fluorescence (+)
<i>Seropositive cirrhosis</i>	38	31 (81.6%)	32 (84.2%)	32 (84.2%)
Menghini biopsy	20	15 (75%)	16 (80%)	16 (80%)
Surgical specimen	13	11 (84.6%)	11 (84.6%)	11 (84.6%)
Autopsy	5	5 (100%)	5 (100%)	5 (100%)
<i>Seronegative cirrhosis</i>	41	—	—	—
Menghini biopsy	28	—	—	—
Surgical specimen	6	—	—	—
Autopsy	7	—	—	—
<i>Seropositive hepatoma</i>				
non-neoplastic tissue:	36	30 (83.3%)	34 (94.4%)	34 (94.4%)
Menghini biopsy	11	6 (54.5%)	9 (81.8%)	9 (81.8%)
Surgical specimen	17	16 (94.1%)	17 (100%)	17 (100%)
Autopsy	8	8 (100%)	8 (100%)	8 (100%)
neoplastic tissue:	36	16 (44.4%) ^a	9 (25%) ^a	7 (19.4%) ^a
Menghini biopsy	11	3 (27.2%)	1 (9%)	1 (9%)
Surgical specimen	17	6 (35.2%)	4 (23.5%)	2 (11.7%)
Autopsy	8	7 (87.5%)	4 (50%)	4 (50%)
<i>Seronegative hepatome</i>	63	3 (round bodies)	—	—
Menghini biopsy	42	—	—	—
Surgical specimen	13	2	—	—
Autopsy	8	1	—	—

^a Solitary neoplastic cells (+)

cytoplasm while in others it was localised (Fig. 2). The fluorescence had a dense homogenous appearance and was strongly positive (Fig. 3).

Among the 36 seropositive patients with hepatoma, HBsAg was detected in the surrounding non-neoplastic part of the liver, cirrhotic or not, in 30 (83.3%) by orcein staining and in 34 (94.4%) by immunoperoxidase method and immunofluorescence. Positive solitary tumor-cells were occasionally seen in the tumor tissue of sixteen cases by orcein staining, in 9 by peroxidase and in 7 by fluorescence, out of the 36 seropositive patients with hepatoma (Fig. 5). In all the positive findings described above there was less intensity of staining in the tumor-cells.

Of the 63 cases of seronegative hepatoma, there were three in which some round orcein-positive inclusion bodies were observed in the cytoplasm of both the neoplastic and the non-neoplastic cells; these bodies were not stained by the two immunological methods (Fig. 4). Immunoperoxidase and fluorescence techniques gave negative results in all 63 cases of seronegative hepatoma (Table 1).

There was complete identity of the material stained positively by orcein and by the two immunostaining techniques, with the exception of the three cases of seronegative hepatoma mentioned above.

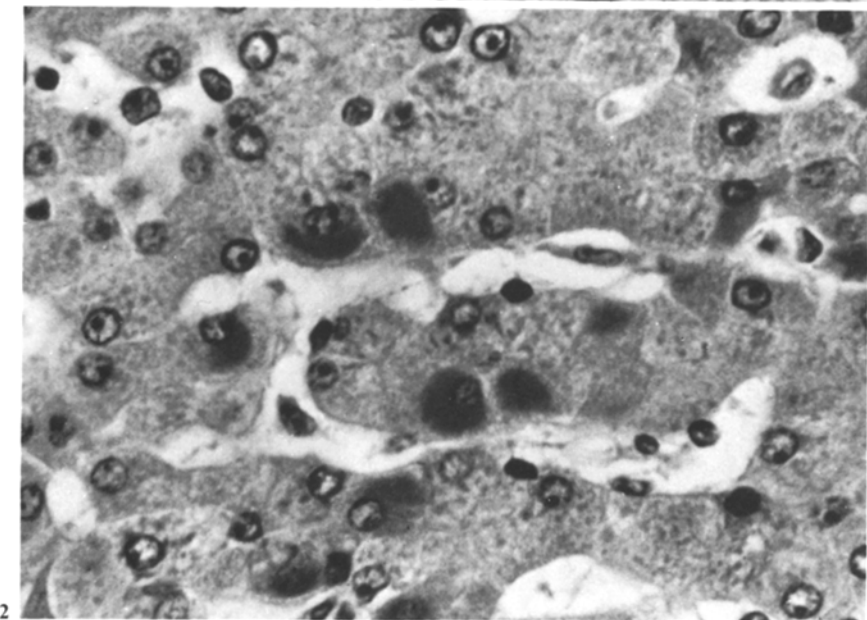
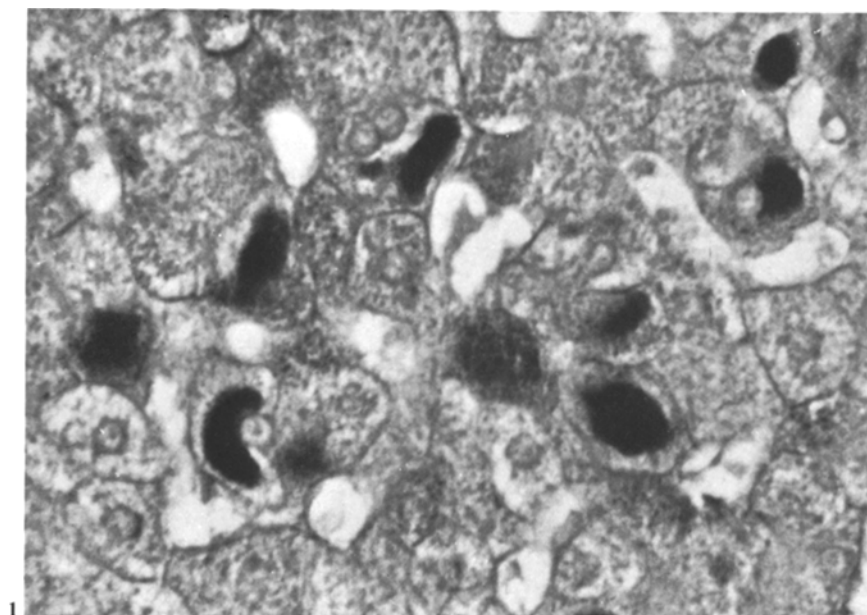


Fig. 1. Orcein-positive hepatocytes in cirrhotic liver. $\times 375$

Fig. 2. Immunoperoxidase-positive cells in cirrhotic liver. $\times 250$

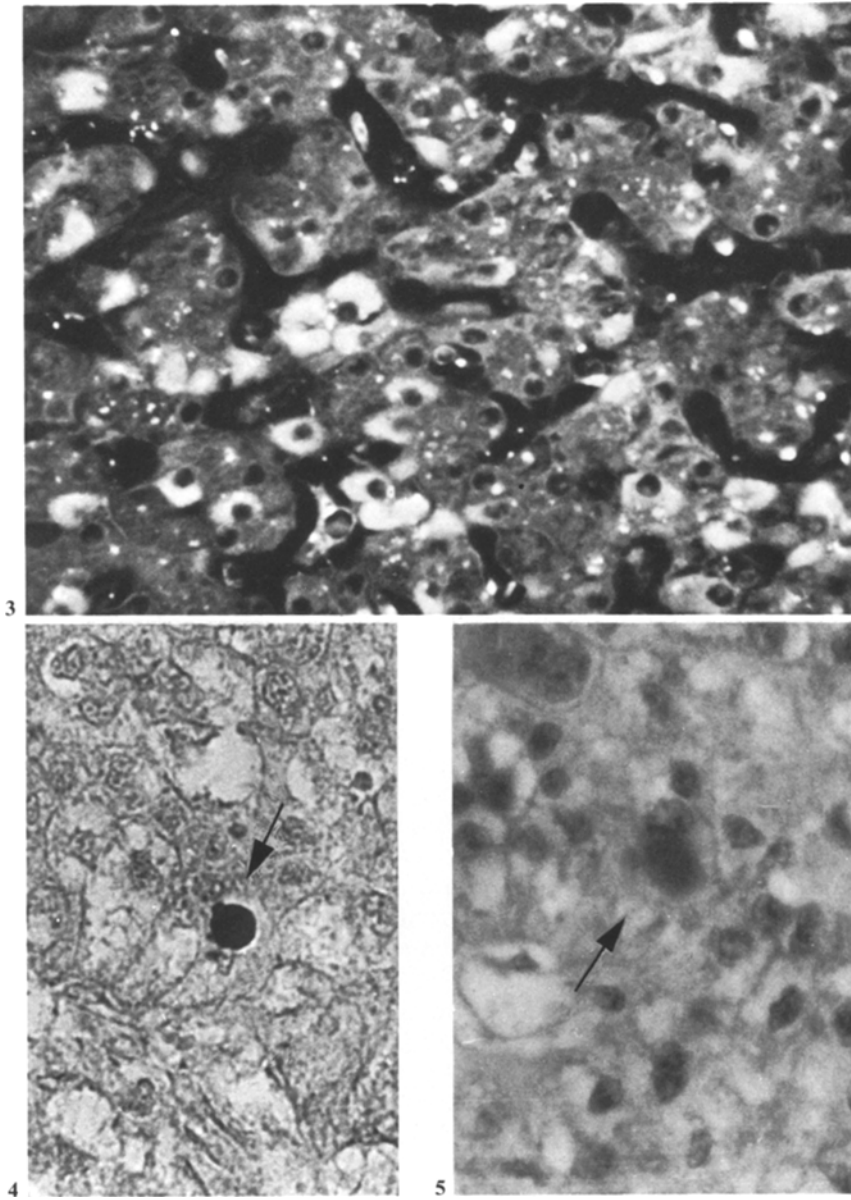


Fig. 3. Positive immunofluorescence in the cytoplasm of cirrhotic liver cells. $\times 450$

Fig. 4. Orcein-positive round inclusion body in a tumor cell

Fig. 5. One immunoperoxidase-positive tumor cell

Discussion

Several immunological techniques have been used during recent years in order to localize HBsAg in hepatic tissue. Shikata and his co-workers in 1974 introduced orcein staining which is now widely used for the detection of HBsAg in conventionally paraffin-embedded liver tissue. Immunostaining methods, such as peroxidase and fluorescence have also been employed on paraffin blocks. Localization of HBsAg has been achieved successfully using these techniques (Nayak and Sachdeva, 1975).

In this study, the three methods have been used and the exclusively cytoplasmic localization of HBsAg confirmed. Of the three techniques, immunoperoxidase was found to be the most sensitive method for detecting HBsAg in cases of cirrhosis. Orcein-staining is a very simple and easy to perform and can be used as a routine screening technique, but it seems not to be specific as orcein-positive material was observed in the parenchymal and the neoplastic tissue in a few cases of seronegative hepatoma; this material was not stained positively by specific immunostaining. A false-positive orcein reaction has also been reported by Kostich and Ingham (1977) in a case of liver-cell injury with methyl dopa. So it becomes reasonable to suggest that we should verify all orcein-positive cases by at least one immunological method in order to exclude rare false-positive results and to confirm positivity.

In specimens from cirrhotic cases all three methods showed a very good correlation between the detection of HBsAg in the liver tissue and the serology. All positive cases came from the group of seropositive cirrhotics, a finding noted by Gerber et al. (1974) using orcein staining, immunofluorescence and immuno-electron microscopy, in cases of chronic hepatitis with and without cirrhosis. Similar results were obtained on the non-neoplastic part of the liver, cirrhotic or not, in cases of hepatocellular cancer. Although positive serology was confirmed by radioimmunoassay, the most sensitive method available nowadays, immunoperoxidase technique detected HBsAg in 84.2% of cases of seropositive cirrhosis and in 94.4% in the non-neoplastic liver tissue of seropositive hepatomas.

The controversial reports from different countries regarding the detection of HBsAg in the neoplastic part of the liver in cases of malignant hepatoma, make the results of this study more interesting. Kostich and Ingham (1977) in preliminary studies reported that 60% of 31 hepatomas stained positively with orcein, and that inclusion bodies, when found, have always been strongly orcein-positive. Kubo and his colleagues (1977), discussing the results of their study stated that in many of the livers bearing hepatocellular carcinoma large amounts of HBsAg stainable by Shikata's orcein stain were demonstrable in the hepatocytes in Japan and South Africa. The same author and Okuda (1978) in a letter to the editor, discuss the findings of Bianchi (1978) in Switzerland and Kew (1978) in South Africa who have demonstrated frequent presence of HBsAg in hepatoma cells. Turbitt et al. (1977) in a retrospective examination in South-west Scotland found by an immunoperoxidase method positive cells in 3 (3.7%) in the tumor part and in 8 (10%) in the surrounding part of the liver out of 81 cases of primary hepatocellular carcinoma, although the staining was less intense in the tumor. Nayak and Sachdewa (1975) in India

found occasional positive tumor cells in 33% and in 91% in the non-tumorous part of the liver of their 13 autopsy cases using immunoperoxidase. Cohen et al. (1978) showed HBsAg positivity in the tumor-cells in 6% and in 36% in the non-tumor liver cell cytoplasm out of the 50 autopsy cases of hepatoma in male black mineworkers from South Africa using orcein staining. Sumithran (1977) from Malaysia studied 10 autopsy cases of hepatocellular carcinoma with cirrhosis and found all positive in the cirrhotic part using orcein, immunoperoxidase and immunofluorescent stainings on examination of multiple blocks, but only small areas of tumor were positive in 2. No positive tumor-cells were seen by Peters et al. (1977) in 168 autopsy cases of hepatocellular carcinoma using orcein and peroxidase techniques in Los Angeles (United States), while HBsAg was demonstrated in liver cells of 21% of the patients in whom carcinomas arose in pre-existing chronic hepatic disease (cirrhosis or precirrhotic fibrosis) and of 5% of the patients who had carcinomas arising in essentially normal livers.

In this study, only occasional tumor-cells were found to be HBsAg – positive by immunoperoxidase in 9 (25%), while HBsAg was detected in the non-tumorous liver in 34 (94%) out of the 36 cases of seropositive primary liver cancer. From the whole number of 99 cases of seronegative and seropositive hepatomas, HBsAg was detected in 9 (9%) in the tumor-cells and in 34 (34%) in the non-tumorous part of the liver (Table 1). These findings are in agreement with those of Cohen et al. (1978) in South Africa. The prevalence of HBsAg in the Greek patients was higher than those in South-west Scotland (Turbitt et al., 1977) and in Los Angeles (Peters et al., 1977) and lower than those in India (Nayak and Sachdewa 1975) and in Malaysia (Sumithran, 1977).

These geographical differences could be attributed to the different prevalence of HBsAg in the general population of the different population groups and to the type of co-existing cirrhosis encountered more often in each country. The prevalence of HBsAg in the general population was considered to be less than 1% by RIA in Great Britain and in the United States (Reed et al., 1973), 7% by counterimmunoelectrophoresis and complement fixation in South Africa (Cohen et al., 1978) and 4.7% by RIA in Greece (Theodoropoulos et al., 1975). The type of cirrhosis that is encountered more often in Greece is the post-necrotic and the prevalence of HBsAg in the serum of Greek cirrhotics was found to be 60.7% (Theodoropoulos et al., 1978).

The frequency of HBsAg in the liver tissue and in the tumor was higher in autopsy cases and surgical specimens than in punching biopsies in all the groups of patients studied (Table 1). This finding confirm those of Deodhar et al. (1975).

The round orcein-positive inclusion bodies seen in some cases of seronegative hepatoma were negative by the specific immunostainings and were probably non-HBsAg cytoplasmic material possessing disulfide bonds.

The results of this study do not support the hypothesis that there is a direct oncogenic effect of hepatitis B antigen on the liver cells, since this antigen was detected mainly in the non-neoplastic part of the liver tissue and only occasionally in the tumor-cells. One cannot exclude the possibility that virus material may be present in the liver in an antigenically incomplete form (Peters et al., 1977).

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Received January 19, 1979