Detection of Hepatitis B Virus Antigens in Paraffin-embedded Liver Specimens from the Amazon Region, Brazil

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Hepatic viscerotomy of paraffin-preserved old specimens, collected in the period from 1934 to 1967, were analyzed by immunohistochemical assays to detect hepatitis B, hepatitis D, dengue and yellow fever virus antigens. The material belongs to the Yellow Fever Collection, Department of Pathology, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil and the cases were diagnosed at that time according to clinical aspects and histopathological findings reporting viral hepatitis, yellow fever, focal necrosis and hepatic atrophy. From the 79 specimens, 69 were collected at the Labrea Region and the other 10 in different other localities in the Amazon Region. The five micra thick histological slices were analyzed for the presence of hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) by immunoperoxidase technique. An immunofluorescence assay was applied to the detection of hepatitis D, yellow fever and dengue virus antigens. Nine (11.4%) histological samples were HBsAg reactive and 5 (6.3%) were HBcAg reactive. The oldest reactive sample was from 1934. Viral antigens related to the other pathologies were not detected in this study. Our results confirm that the methodology described may be used to elucidate the aetiology of hepatitis diseases even after a long time of conservation of the specimens.

Key words: hepatitis B virus - human liver - Amazon - Brazil

According to the World Health Organization, hepatitis B virus (HBV) carriers account for 5% of the world population. These potential infection transmitter individuals can evolve from the asymptomatic condition to a severe hepatic damage and even hepatocellular carcinoma. A similar HBV epidemiological distribution is observed for hepatitis D virus (HDV) that shares the obligatory association to HBV for its replication (Smedile et al. 1981). Frequently related to HBV chronic carriers and less frequently to the acute disease forms (Colombo et al. 1983, Rizzetto et al. 1983), HDV antigen was identified by immunohistochemical assays in liver cell nuclei of patients with chronic persistent or chronic active hepatitis, hepatitis B surface antigens (HBsAg-positive) (Rizzetto et al. 1977). In South America HDV infection was first observed in Venezuela as a severe and fulminant attack among Yucpa indians (Purcell & Gerin 1983) and in Colombia where 60% of the individuals studied were simultaneously HBV and HDV reactive (Ljunggren et al. 1984). In Brazil, the Amazon Region endemicity for both viruses is well known (Figueiredo Mendes et al. 1984, Fonseca et al. 1986, Simonetti et al. 1986) showing high prevalence pattern compared to the other parts of the world (Purcell & Gerin 1983, Nordenfelt et al. 1983, Fonseca et al. 1988). This region is also endemic for yellow fever and it is thought whether another human hepatitis viruses had been introduced in this area by human serum present in vaccines against yellow fever, since there are observations on the occurrence of icterus in England and in Brazil, following vaccination against yellow fever in the 1930s and 1940s decades (Findlay & Mac Callum 1937, 1938, Soper & Smith 1938, Fox et al. 1942). We included the dengue virus antigen research in this study, once the same pathological liver findings are observed in yellow fever and haemorrhagic dengue, such as hepatomegaly, focal visceral haemorrhages, focal necrosis, sinusoidal acidophilic bodies, Kupffer cell hypertrophy and portal tract mononuclear cell infiltration.

To observe the simultaneous HBV and HDV antigen frequency and to detect yellow fever and dengue viral antigens in histological sections we studied 79 hepatic samples by immunohistochemical assays. Hepatic viscerotomy samples belong to the Yellow Fever Collection (Department of Pathology, Instituto Oswaldo Cruz, Brazil) created in the 1930s decade from an agreement between Brazilian Government and the Rockefeller Foundation International Division to study yellow fever in Brazil.

MATERIALS AND METHODS

Material - Seventy-nine paraffin-embedded hepatic samples collected from 1934 to 1967 were analyzed. Sixty-nine samples were from the Labrea Region, State of Amazonas and ten random samples were from other different localities in the same state. The cases were diagnosed at that time as viral hepatitis, yellow fever, focal necrosis or hepatic atrophy according to clinical aspects and histopathological findings.

Methods - Four or five micra thick paraffin-embedded histological sections were prepared for hepatitis B, hepatitis D, yellow fever and dengue virus antigen detection by immunoperoxidase or immunofluorescence techniques. HBsAg and hepatitis B core antigens (HBcAg) were detected by the immunoperoxi-dase assay (Immuno Tag S - Immunon-Lipshaw Corporation). Paraffin sections were deparaffinated and treated with 90% and 85% ethyl alcohol solutions followed by 10% ammonium hydroxide solution. To block endogenous peroxidase, sections were incubated with 3 to 10% hydrogen peroxidase solution for 5 min. Sections were then incubated with peroxidase/anti-peroxidase conjugate, stained with hematoxylin solution (1 min) and treated with 0.2% ammonium hydroxide solution. The reaction colour was developed after 3-amino-9-ethylcarbazole substrate addition. Positive HBsAg and

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HBcAg controls were included for every batch. HDAg was detected by the immunofluorescence assay as follows: 5 µm thick paraffin-embedded sections were deparaffinated, rehydrated and digested with 0.1% protease solution (type XXVII Sigma) in Tris-HCl 0.5M pH 7.4 for 2 h at 37ºC or 0.05% trypsin (Difco) in phosphate buffered saline pH 7.4 for 10 min at 37ºC. Digested sections were incubated at room temperature for 30 min with FITC-anti HD conjugate diluted 1:100 in PBS pH 7.4. Yellow fever and dengue virus antigens were tested by immunofluorescence (Huang et al. 1976, Walker & Cain 1978, Schatzmayr et al. 1984, Barth et al. 1988): 4 µm thick paraffin sections were deparaffinated, rehydrated and digested with 0.1% trypsin with 0.1% $CaCl_2$ solution in PBS pH 7.8 for 2 h at 37ºC. Digested sections were incubated at 37ºC for 30 min with FITC-conjugated yellow fever positive simian serum diluted at 1:20 in PBS pH 7.2. Flavivirus hyperimmune ascitic fluid obtained from mouse was used to detect dengue virus antigen. After washing sections were mounted with buffered glycerol and examined at the UV microscope. Four controls were used for yellow fever virus detection: not infected Vero cells, yellow fever virus (17D strain)-infected Vero cells, not infected liver cells and HBV-infected liver cells. *Aedes albopictus* C6/36 infected cell line was included as positive control for dengue virus detection.

RESULTS

Nine (11.4%) of 79 hepatic samples were HBsAg-reactive and 5 (6.3%) were HBcAg-reactive by immunoperoxidase assay (2 of them showed reactivity for both viral markers). HDAg was detected in none of these reactive samples. HBsAg and HBcAg were more frequently seen in samples from people at lower ages since in both groups examined (male and female) we had a higher number of samples from persons under 21 years old. Two samples showing simultaneous HBsAg and HBcAg reactivity were from boys agged 2 and 6 years. Chronological distribution of viral markers detected in this study showed that old preserved-paraffin liver samples are still suitable for viral markers detection by immunoperoxidase assay. The results are shown in the Table.

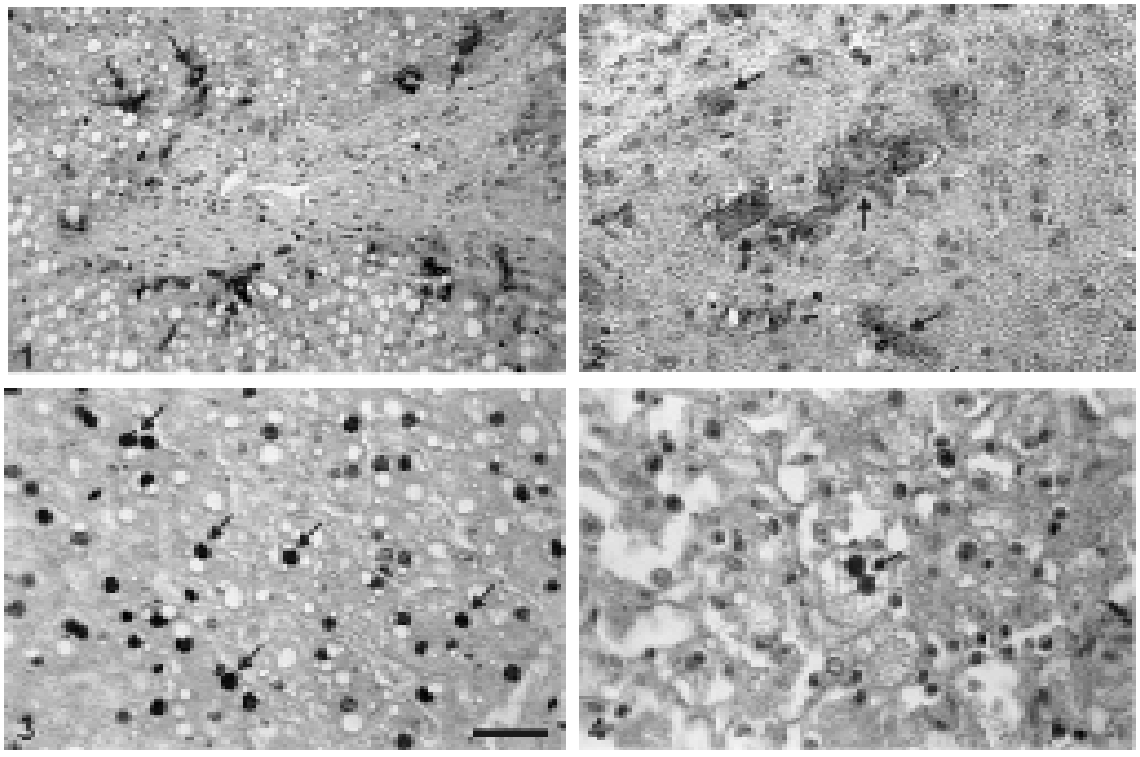
An exclusive cytoplasmic HBsAg localization and an exclusive nuclear HBcAg localization were observed by immunoperoxidase assay. The results are shown in Figs 1-4.

Yellow fever and dengue virus antigens were seen in none of the 79 hepatic samples by immunofluorescence assay.

DISCUSSION

The Labrea Region, State of Amazonas, shows a high HBV endemical level (Figueiredo Mendes et al. 1984, Fonseca et al. 1988); this was an important point in sample selection and could have influence on the uneven age range distribution observed. Hepatitis B virus antigen detection in the liver tissue samples by immunohistochemical assays showed a cytoplasmic HBsAg and a nuclear HBcAg localization.

Only the HBsAg-reactive (9 of 79) and HBcAg-reactive (5 of 79) samples were analyzed for the presence of HDAg. None of them showed reactivity. The possible explanations for this are (1) HDV is mainly found in young adults and 71.3% of people analyzed in our study were children under 14 years old (Fonseca et al. 1988); (2) the immunofluorescence method had low sensitivity that could not detect HDV antigen in low level concentration samples; and (3) the more recent HDV introduction in this region, which would be



Figs 1, 2: hepatitis B surface antigens detected by positive immunoperoxidase assay inside hepatocytes cytoplasm (arrows). Figs 3, 4: hepatitis B core antigens detected by positive immunoperoxidase assay inside hepatocytes nuclei (arrows). Bar: Figs 1, 3 = 140 mm; Figs 2, 4 = 35 mm

TABLE

Data on the specimens positive for hepatitis B virus antigens

Age	Sex	Date of collection	Origin	Original diagnosis	Results
8 mo	F	1948	Urucutuba	Viral hepatitis	HBsAg(+)
12 y	M	1950	Benjamin Constant	Focal necrosis	HBsAg(+)
12 y	M	1950	Benjamin Constant	Hepatic atrophy	HBsAg(+)
6 y	M	1967	-	Viral hepatitis	HBsAg/HBcAg(+)
45 y	M	1950	Lábrea	Viral hepatitis	HBsAg(+)
1 y	F	1952	Lábrea	Viral hepatitis	HBcAg(+)
15 y	M	1963	Lábrea	Hepatic atrophy	HBsAg(+)
9 y	M	1934	Lábrea	Hepatic atrophy	HBsAg(+)
2 y	M	1942	Lábrea	Hepatic atrophy	HBsAg/HBcAg(+)
54 y	F	1945	Lábrea	Hepatic atrophy	HBsAg(+)
5 y	F	1946	Lábrea	Hepatic atrophy	HBcAg(+)
3 y	M	1946	Lábrea	Hepatic atrophy	HBcAg(+)

subsequent to the analysis period of the samples (Fonseca et al. 1986, 1988, Simonetti et al. 1986).

The retrospective analysis evidenced HBsAg preservation in old histological samples. The oldest one, from 1934, was from a 9 year old boy from Labrea Region, Amazonas, with a negative yellow fever diagnosis.

All the 79 samples were analyzed for dengue and yellow fever virus antigen detection. The results were negative. This could be due to the low sensitivity of the method, as discussed for HDV (Fonseca et al. 1988). Alternatively, other viruses causing acute liver inflamation and necrosis, determining similar hepatic lesions to those described, or non-viral fatal infections such as malaria and leptospirosis, could be considered aetiological agents.

Our results confirm that the methodology described may be used to elucidate the aetiology of hepatitis diseases even after long time of conservation of the specimens. Retrospective studies of viral infection of the liver (Schatzmayr et al. 1984) were used in order to acquire a better knowledge of the natural history of these diseases and to may compare the data obtained with the actual distribution of hepatitis in the Amazon region.

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