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Immunohistochemical Patterns of Hepatitis B Surface Antigen (HBsAg) in Patients with Hepatitis, Renal Homografts Recipients and Normal Carriers

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Summary. A series of 180, Bouin-fixed and paraffin embedded liver biopsies obtained from 147 patients was investigated for the presence of hepatitis B surface antigen (HBs) by histochemical and indirect immunofluorescence techniques. A comparison between orcein staining and Masson's trichrome preparations for ground glass hepatocytes, showed that immunofluorescence was both the more reliable and the more specific method for detection of HBsAg in liver tissue. The ability to perform this technique on paraffin sections facilitates systematic studies and allows retrospective work-up.

IF-HBs positive hepatocytes were found in approximately two thirds of all HBs-positive patients in their serum, but never seen in HBs-negative patients.

HBs-positive cells were observed in healthy chronic carriers and in all forms of chronic hepatitis, but never in acute HBs-positive hepatitis.

In patients treated with chronic hemodialysis and in renal homograft recipients, the incidence of positive cells was higher than in the chronic hepatitis groups; this could be correlated with the duration of antigenemia at the time of biopsy.

Key words: HBsAg – Hepatocytes – Immunofluorescence – Orcein stain – HB hepatitis.

Introduction

The cellular localization of the two major antigenic components of Hepatitis B virus (HBV) has been established (Brzosko et al., 1973). According to immunohistochemical, immunoperoxydase and electron microscopic examinations, the

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DNA-associated core antigen (HBcAg) may be present in liver cell nuclei while the lipoprotein surface antigen (HBsAg) can be detected in the cytoplasm (Nowoslawski et al., 1970; Huang, 1971; Akeyama et al., 1972; Krawczinski et al., 1972; Hadziyannis et al., 1972, 1973; Shikata, 1973; Kater et al., 1973; Huang et al., 1974; Gerber et al., 1974, 1975; Sun et al., 1974; Gudat et al., 1975; Huang, 1975; Burns, 1975; Bianchi and Gudat, 1976; Ray et al., 1976). Hadziyannis et al. (1973) and others (Gerber et al., 1974; Korb and Huppertz, 1974; Deodhar et al., 1975) described a characteristic finely granular "ground glass" cytoplasmic appearance as a light microscopic marker for the presence of HBsAg. Subsequently, Shikata et al. (1974) discovered that such cytoplasmic structures are clearly demonstrated by a modified orcein staining method and by Gomori's aldehyde fuchsin stain. Subsequently, these results have been confirmed by Deodhar et al. (1975), Bartok et al. (1976). Filamentous and tubular structures are seen in the cisternae of endoplasmic reticulum by electron microscopy and are generally believed to represent excess virus coat material (Stein et al., 1972; Gudat et al., 1975; Gerber et al., 1974; Winckler et al., 1976). Conflicting reports have been published concerning the association of HBAg components in liver tissue with various types of hepatitis (Gudat et al., 1975; Ray et al., 1976). The variations in the reported incidence may be due to differences in the techniques used. However it has been postulated that the immune response of the host may play a role in the genesis of the diverse pathological patterns. Investigations in this field are of current interest.

Material and Methods

Tissue Specimens

One hundred and eighty biopsies obtained from 147 patients were included in the study. Specimens were taken with a Menghini needle. The material was divided into four groups (Table 1):

Acute Hepatitis Group. This group consisted of 29 biopsies subdivided into "classic lobular hepatitis" (5 cases), "residual acute hepatitis" (20 cases), and "acute hepatitis with signs of possible transition to chronicity" (4 cases).

Chronic Hepatitis Group (CH). This group comprised 31 specimens without cirrhosis, classified as chronic persistent hepatitis (CPH) or chronic aggressive hepatitis (CAH), and 19 with cirrhosis subdivided into cirrhosis with little activity (13 cases including 6 with hepatocellular carcinoma) and active cirrhosis (6 cases). All the biopsies were taken at least 6 months after the onset of the disease.

Healthy Chronic Carriers Group (16 biopsies).

Chronic Hemodialysis and Transplant Group. Eighty-five biopsies were taken from 62 patients, of whom 24 were studied both during hemodialysis and after renal transplantation. In total, the material consisted of 43 specimens obtained during hemodialysis and 42 after transplantation.

Biopsy specimens were immediately fixed in Bouin's fluid for 12–24 h, dehydrated in ethanol, cleared in xylene, embedded in paraffin and sectionned at a thickness of 3 μm using routine procedures. Serial sections were stained by hematin-eosin-safran, Masson's trichrome (Ganter and Jolles, 1970), Gordon-Sweet for reticulin fibers, and periodic acid Schiff (PAS). Neighbouring sections were used for the orcein stain (Shikata et al., 1974).

Table 1. Histological diagnosis

Histological diagnosis	Number	
Acute hepatitis (AH)		29
Classic lobular hepatitis	5	
Residual acute hepatitis	20	
Acute hepatitis with signs of possible transition to chronicity	4	
Chronic hepatitis without cirrhosis		31
Chronic persistent hepatitis (CPH)	17	
chronic aggressive/active hepatitis (CAH)	14	
Chronic hepatitis with cirrhosis		19
Cirrhosis with little activity (6 with hepatocellular carcinoma)	13	
Active cirrhosis	6	
Chronic carriers		16
Hemodialysis and transplant patients		85
Total		180

Immunohistochemical Methods

In the present study, HBsAg was detected in fixed paraffin sections by the indirect fluorescence method. After removal of paraffin by three changes of xylene within 15 min, sections were passed through successive alcohols and washed in phosphate-buffered saline (PBS). Excess saline was removed and sections were covered with diluted rabbit monospecific anti HBs (Behring Werke). Then they were incubated in a moist chamber at room temperature for one hour, washed several times with PBS, and then reacted with fluoresceinated (FITC) goat anti rabbit immunoglobulin for 30 min (Behring Werke). The slides were then mounted in buffered glycerine and examined under UV light (Leitz Ortoplan-Ortomat equipped with epiillumination arrangement according to Ploem, a HBO 200 W lamp (Osram) as light source). Fluorescent cells were directly counted in at least six high-power fields ($\times 250$). A photographic record was kept of the results (Ektachrome HS 125 ASA).

Appropriate controls performed: specific abolition of fluorescence by absorption of the rabbit anti-HBs with known HBsAg (HBs positive serum Behring Werke) and abolition of fluorescence when specific antiserum was replaced by normal serum and when inappropriate conjugated antiglobulin was used.

Serology

Serum samples obtained within one week of biopsy were tested for HBsAg. The seropositive group comprised 103 cases in whom the serum was positive by electroimmunodiffusion or radioimmunoassay. The seronegative group consisted of 77 cases in which the serum was negative by all techniques, including radioimmunoassay.

Results

Immunohistochemical Appearance of HBsAg

HBsAg was generally distributed randomly throughout the liver, being present in individual cells separated from each other, or in focal areas of neighbouring hepatocytes (Fig. 1). Positive cells rarely occurred in large clumps with interven-

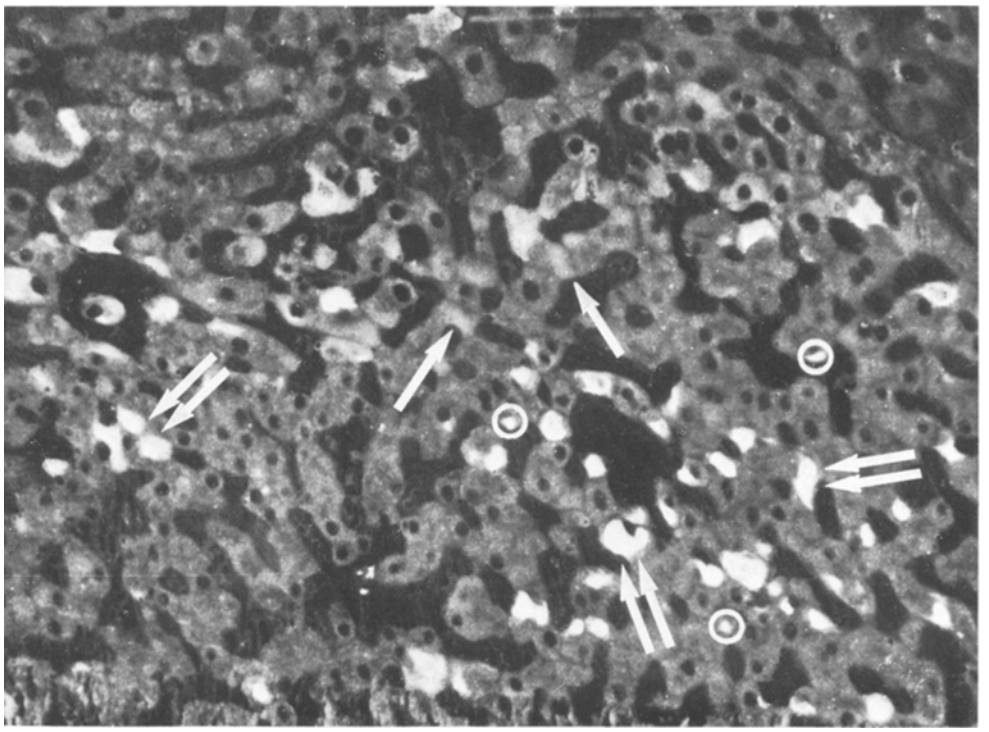


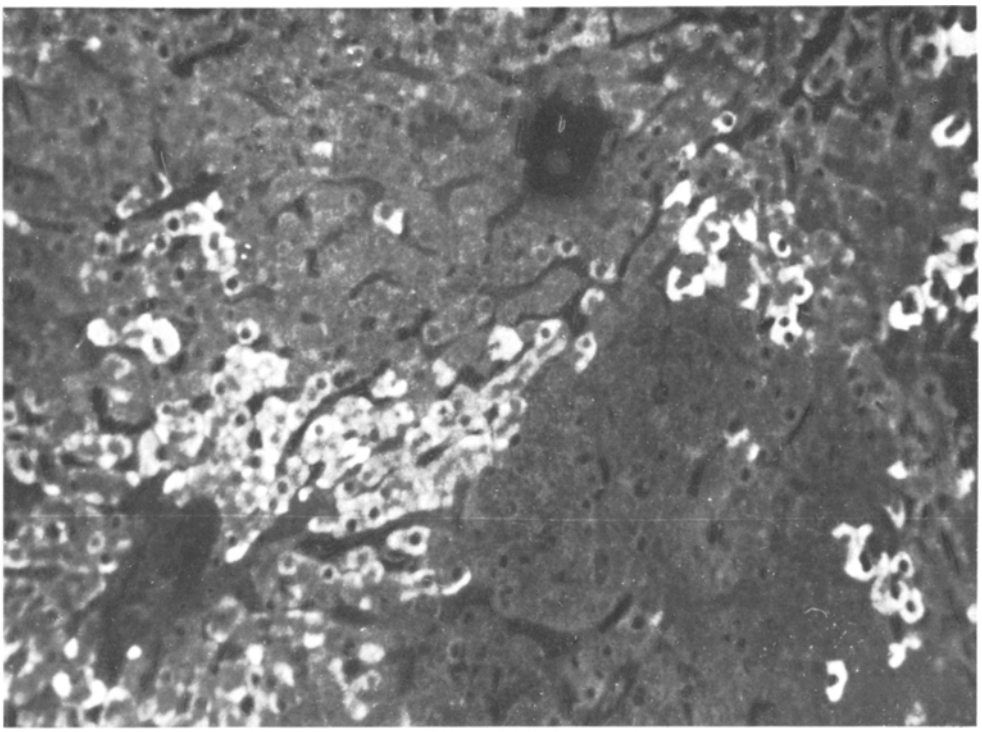
Fig. 1. Chronic persistent hepatitis: IF-positive hepatocytes scattered throughout the liver. The antigen can be seen in different patterns: diffuse homogeneous fluorescence (↗ ↗), clearly outlined discrete inclusions (circled), faint focal fluorescence restricted to the sinusoïdal border or the perinuclear area (↗). Immunofluorescence, ×400

ing areas of negative hepatocytes (Figs. 2 and 3). No predilection of HBsAg for any specific vascular territory was observed.

The antigen appeared at the hepatocellular level in three distinct morphologic patterns. In most cells, it was diffusely distributed throughout the cytoplasm and the nuclei appeared to be displaced to the periphery of the cells. In other antigen-carrying cells, the antigen was localized in clearly outlined discrete inclusions. Occasionally, only part of the cytoplasm showed specific fluorescence which was restricted to the sinusoidal border or the perinuclear area. On close examination, the fluorescent areas often appeared to be in a reticular or a vacuolar pattern; on occasion, the fluorescence had a dense homogeneous appearance and was of very strong intensity. When the hepatocytes showed cytoplasmic vacuolation or steatosis, the antigen was found around the vacuoles in the form of thin rings.

Fig. 2. Chronic persistent hepatitis: Numerous IF-positive cells in large clumps with areas of negative hepatocytes. Immunofluorescence, ×300

Fig. 3. Liver biopsy from a healthy carrier of HBsAg. Immunofluorescence, ×500



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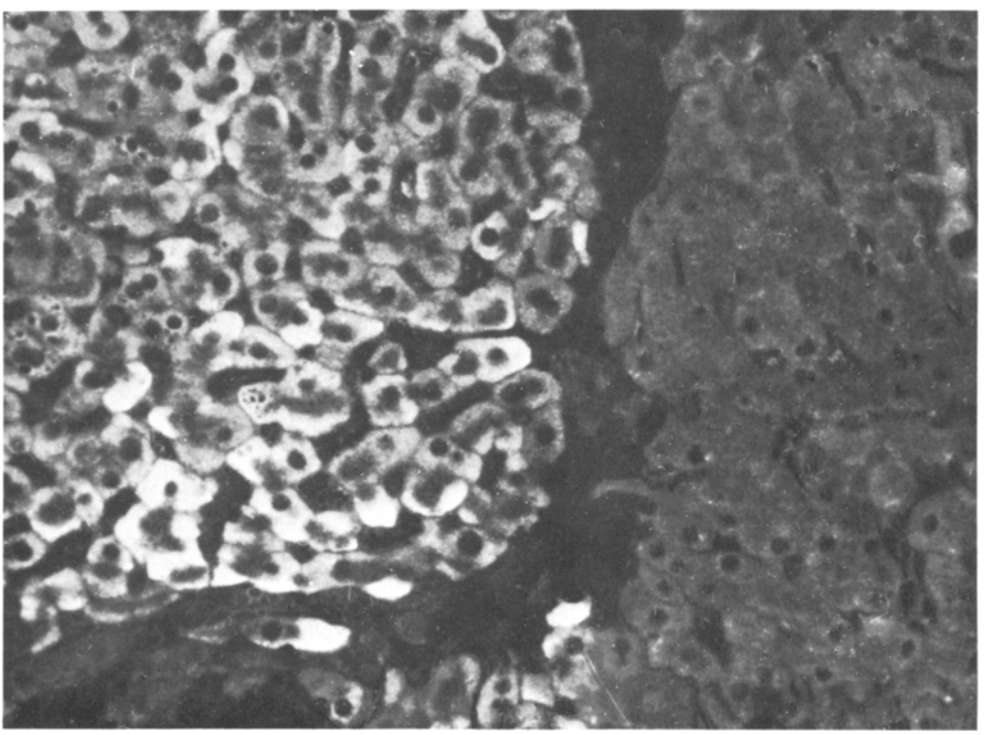

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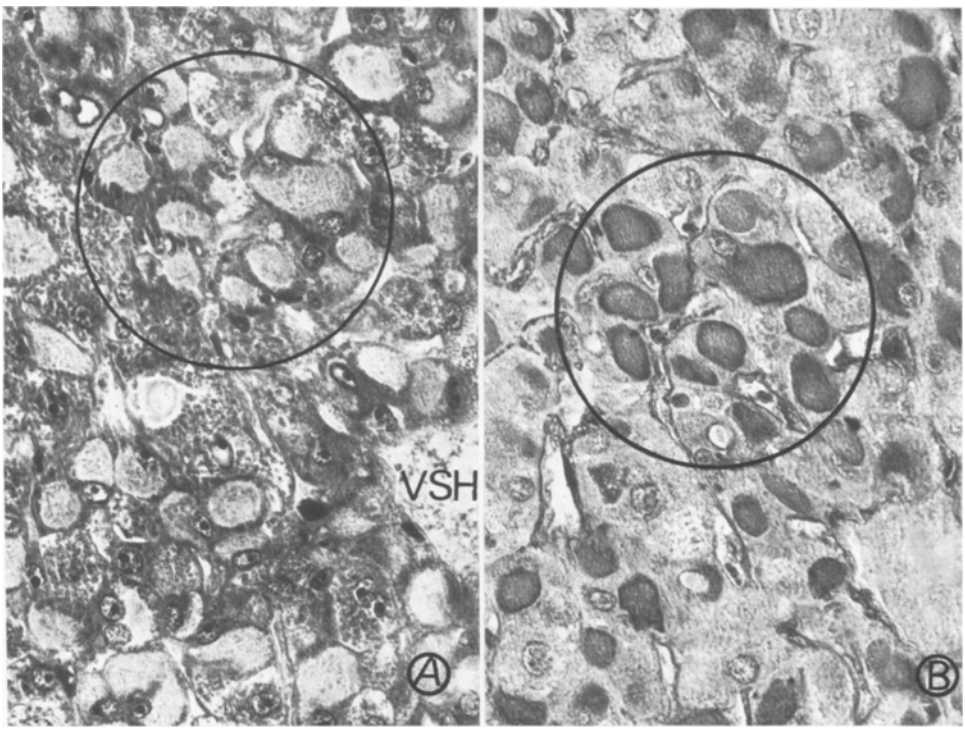

Fig. 4. Liver biopsy from a healthy carrier, processed by Masson's trichrom (a) and orcein (b). ×550

In some cases, HBsAg was occasionally localized in Kupffer cells. In these instances, nuclear fluorescence was consistently absent.

Under the light microscope, diffuse homogeneous fluorescence corresponded to the so-called "ground glass" hepatocytes (GGH) in subsequent hematin-eosin-safran stained sections. The peculiar glassy, finely granulated, appearance of the cytoplasm was easily distinguished from acidophilic or Mallory bodies. However, the differentiation of true GGH from similar hepatocellular changes observed in toxic and drug lesions was sometimes difficult. On orcein staining, only HBs positive cytoplasm with GGH change was dark-yellowish brown (Fig. 4). Such a reaction was never found in seronegative patients. Moreover, in our material, staining with Masson's trichrome showed an excellent correlation with the orcein staining technique, the sensitivity being almost as high, and the method more stable. On the other hand, the counter-part of discrete spotty inclusions and the faint focal fluorescence patterns were difficult to identify in conventional lightmicroscopy. Rates of detection were noticeably less by hematin-eosin, trichrom and orcein stains, as compared to immunohistochemical techniques. Of the 180 biopsies examined, 103 (57%) were from patients who were positive for HBsAg in the serum, 66 (36%) showed HBsAg in liver tissue by immunohistochemical techniques and only 46 (25%) showed GGH with Masson's trichrom and/or orcein positive material in the cytoplasm of

Table 2. Frequency of HBsAg obtained by serology and immunofluorescence

	Number	Liver IF+	GGH+
Serum+	103 (57%)	66 (36%)	46 (25%)
Serum−	77 (43%)	0	0
	180	66	46

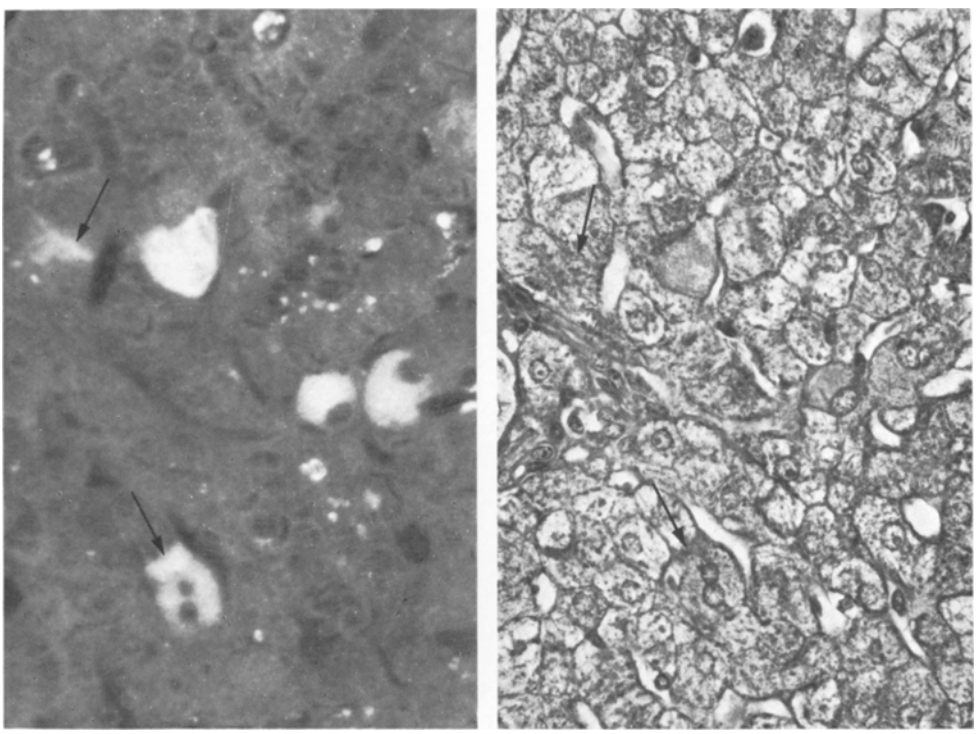

Fig. 5. Chronic aggressive hepatitis: matched photographs of the same liver section processed by immunofluorescence and subsequently stained with Masson's trichrom. There is locational identity between the diffuse homogeneous pattern and the ground glass appearance. On the opposite hand, the counterpart of the focal fluorescence patterns is difficult to identify in Masson's trichrom stain (arrows). ×600

hepatocytes (Table 2). No HBs antigen could be detected by histochemical and immunofluorescence methods in seronegative patients. The correlation between the immunofluorescent and conventional stains is shown in Figure 5.

Distribution of HBsAg in Liver Tissue

In acute hepatitis (AH) HBsAg was present in the serum in 18 out of 29 patients (62%). No antigen could be found in any of these biopsies (Table 3).

Table 3. Acute hepatitis

Histological diagnosis	Nb	Serum+	Liver+	GGH+
Classic lobular hepatitis	5	1	0	0
Residual acute hepatitis	20	15	0	0
Acute hepatitis with signs of possible transition to chronicity	4	2	0	0
Total	29	18	0	0

Table 4. Chronic hepatitis

Histological diagnosis	Number	Serum+	Liver*	GGH+
Chronic hepatitis without cirrhosis	31	18 (58%)	13 (42%)	7 (22%)
CPH	17	11	8	5
CAH	14	7	5	2
Chronic hepatitis with cirrhosis	19	12 (63%)	9 (47%)	4 (21%)
Cirrhosis with little activity	13	7	6	3
Active cirrhosis	6	5	3	1

In chronic hepatitis without cirrhosis, 18 out of 31 biopsies (58%) were obtained from seropositive patients (Table 4). HBsAg was detected in 13 (42%) with immunofluorescence. Generally, positive hepatocytes were few and randomly scattered throughout the parenchyme. Ground glass hepatocytes (GGH) were present in 7 (22%). The biopsies were classified as chronic persistent hepatitis (CPH) and chronic aggressive or active hepatitis (CAH) on the basis of their hepatic morphology as previously described by De Groote et al. (1968). The incidence of HBsAg in liver tissue was comparable in both subgroups. However, a high frequency of GGH was found in CPH patients. Furthermore, an attempt was made to correlate the HBsAg status to the natural history of the disease. The mean interval between the onset of the disease and the biopsy was 12 months in serum positive but liver negative patients, and 28 months in serum and liver positive patients. After two years of antigenemia, all the biopsies were found to contain positive hepatocytes.

Chronic hepatitis with cirrhosis was subdivided into active cirrhosis (6 cases) when the morphology showed signs of histological activity (piecemeal necrosis) and cirrhosis with little activity histologically comparable to CPH (13 cases). Of the latter, 6 had developped a hepatocellular carcinoma. HBsAg was present in the serum in 12 (63%) and in liver tissue in 9 (47%). GGH could be found in 4 (21%). No clear-cut difference was observed on comparing the appearance seen in active forms and less active forms. In cirrhosis with carcinoma, only

Table 5. Chronic HBs carriers

Number	Serum+	Liver+	GGH+
16	16 (100%)	15 (98%)	12 (75%)

Table 6. Chronic hemodialysis and renal transplant patients

Diagnosis	Number	Serum+	Liver+	GGH+
Hemodialysis	43	15	9	6
Renal transplantation	42	24	20	17
Total	85	39 (45%)	29 (34%)	23 (27%)

surrounding non-neoplastic cells were HBs IF-positive. Fluorescence was consistently absent in tumour cells.

In chronic carriers, HBs positive cells were distributed in numerous neighbouring hepatocytes. Large sheets of positive cells were adjacent to negative areas (Fig. 3). At the cellular level, the antigen was generally distributed diffusely in the cytoplasm. Occasionally, the antigen was present in smaller amount and localized in the form of a few scattered cytoplasmic inclusions. Among the 16 specimens obtained from chronic carriers, 15 contain positive hepatocytes. In most of them (12), large clumps of GGH were found in the same location as IF-positive cells. Identity could be demonstrated by subsequent trichrome staining of slides containing IF-positive hepatocytes.

In chronic hemodialysis and renal transplant group (85), 39 (45%) were from seropositive patients. HBsAg was found in 29 (34%) and GGH were present in 23 (27%) (Table 6). The incidence of the antigen was higher in patients followed after transplantation than in those during hemodialysis. Similarly, HBsAg positive hepatocytes were more often observed and GGH more numerous in the transplant group. Twenty-four patients were studied both during hemodialysis and after transplantation. At the time of transplantation, 11 were HBs positive in the serum and only 6 in liver tissue. After the first year following transplantation, the antigen was detected in 13, and the biopsy from five patients previously negative for HBsAg had become positive. Of the two positive in the serum but persistantly negative in the liver, one became positive within two years. In the remaining case, antigenemia had been intermittent. Moreover, the number and distribution of positive hepatocytes in serum positive patients of these two categories were not related to histological data (four nearly normal livers, eight with minimal changes and/or focal parenchymal necrosis, 24 chronic persistent hepatitis, three chronic active hepatitis).

Discussion

Immunofluorescence appears to be a reliable method, with regard to its sensitivity, for the detection of HBsAg or surface coat antigen in liver tissue. It has

been shown that certain viral antigens are not inactivated by fixation and processing for paraffin embedding (Nairn, 1969) and this is true for the specific antigenic determinants of HBsAg (Nayak and Sachdeva, 1975). The ability to perform immunofluorescence on fixed paraffin-embedded sections, which are always available in routine histopathologic liver biopsies, facilitates systematic studies and allows retrospective work-up (Ray and Desmet, 1975; Huang, 1975; Portman et al., 1976). Paraffin sections can be easily stored and the size of specimens available both for immunofluorescence and conventional staining procedure is greater than with other methods.

In contrast to initial reports (Coyne et al., 1970), it has been shown recently that the surface antigen can only be observed in the cytoplasm (Hadziyannis et al., 1972; Shikata, 1974; Gudat et al., 1975; Ray et al., 1976). The characteristic ground glass appearance, described by Hadziyannis et al. (1973), is generally considered to be evidence of the presence of this antigenic determinant in liver cells. There is complete locational and morphological identity between the ground-glass hepatocytes (GGH) and the orcein stained intracellular material (Shikata et al., 1974; Deodhar et al., 1975; Bartok et al., 1976). In our experience, Masson's trichrome stain was a useful tool for the differentiation of true GGH from cytoplasmic induction changes sometimes observed in liver lesions following toxins and drugs, e.g. chlorpromazine, barbiturates, and alcohol. However, it must be stated that the Masson's trichrome and orcein stains appear to be less sensitive than the immunofluorescence technique.

There is a good correlation between the presence of HBsAg in the serum and the presence of the antigen in liver cells. Our results showed no antigen in specimens obtained from seronegative patients. They are comparable with those of others (Hadziyannis et al., 1972; Gudat et al., 1975; Gerber et al., 1975; Portman et al., 1976). However, Ray et al. (1976) reported cases in which HBsAg could be detected in liver tissue by immunofluorescence but not in the serum by radioimmunoassay. Such discrepancies may be ascribed to the different procedures used (Ray et al., 1974).

The frequency of IF-positive cells in biopsies from seropositive patients has also been the subject of conflicting reports. It now seems likely that the quantity of antigen in liver tissue varies, and this variation depends on the host-virus interactions and the diverse histological pictures (Dudley et al., 1972; Gudat et al., 1975; Ray et al., 1976).

Thus, in acute hepatitis type B, the absence or rarity of the antigen in hepatocytes is consistent with the hypothesis that the HBs-containing cells can be promptly cleared by the immune reactions resulting in hepatocellular necrosis and a selflimited disease (Gudat et al., 1975). In contrast, almost all the biopsies obtained from chronic HBs carriers are found to contain numerous positive hepatocytes with a diffuse pattern. The large results of fluorescent cells observed in these cases suggest a direct cell-to-cell spread of HB virus (Portman et al., 1976). Supporting evidences indicate that this carrier state may be due to an unresponsiveness to HBs (Hoofnagle et al., 1973). It is accompanied by an absence of, or mild, inflammatory changes in the liver.

Similarly, in patients treated with chronic hemodialysis and renal homografts recipients, the weakening of the host immune system allows the frequent develop-

ment of HBV hepatitis. Though the persistance of antigenemia is generally accompanied by clinically silent liver impairment, serious cases of hepatitis appear to be more frequent than in chronic carriers. In the present work, a chronic active liver disease has been observed in three cases. It seems likely to depend on the grade of the immunologic deficiency state. In these patients, a particular immunomorphologic pattern has been observed and is characterized by a low incidence of HBsAg and a more pronounced core expression (Gudat et al., 1975). In the present investigations, two particular points need to be stressed. Firstly, both the frequency and the number of HBs positive hepatocytes were higher in the transplant group than in the hemodialysis one. Secondly, the quantity of the antigen was not related to the serum titre of HBsAg, but was correlated with the duration of antigenemia at the time of biopsy. Thus, all except one of the specimens obtained from the renal homografts recipients have become IF-positive. The remaining one was from a patient with intermittent antigenemia. This high incidence of HBsAg detected by immunofluorescence in transplant patients is comparable to that reported by Orfila et al. (1976).

In chronic hepatitis, it is generally assumed that HBs containing hepatocytes are found in discrete pattern and randomly distributed throughout the liver lobule. The number and distribution of HBsAg in liver cells in various groups of chronic hepatitis remain a matter of debate. Our results showed HBs-positive cells in two thirds of the sero positive patients and are in general agreement with those of other authors (Gudat et al., 1975; Portman et al., 1976). However, Ray et al. (1976) found HBsAg in IF-positive cells in 100% of the seropositive patients and according to these authors, no patient had the antigen solely in the serum. Aside from the serious problem of sampling error in liver biopsy, further investigations are needed to clarify this point. Moreover, in our experience, the presence of HBsAg in liver tissue seems likely to depend also upon the duration of antigenemia. Again, different patterns have been described. Several authors have stressed the inverse relationship between the presence of HBs as detected by immunofluorescence and areas of piecemeal necrosis (Hadziyannis et al., 1972; Shikata, 1973; Gudat et al., 1975). More recently, Ray et al. (1976) have reported that in active forms of chronic hepatitis there is a prominent membrane expression of HBsAg and that the localization of HBs antigen in the membrane of liver cells could be correlated with the presence of HBc in the nuclei. In the present study, we were unable to find any relationship between the immunohistochemical pattern and the histological activity of the disease. Sometimes, IF-positive cells were detected close to areas of piecemeal necrosis and no membrane localization could be observed with the procedure used.

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