HBsAg and HBcAg in the livers of asymptomatic hepatitis B antigen carriers

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SUMMARY Asymptomatic blood donors with persistent HBs antigenaemia have a variety of histological lesions in the liver, and serial biopsies indicate that, in some, these lesions may progress. The immunoperoxidase technique was found to be a sensitive method for the histological demonstration of HBsAg and HBcAg. Livers showing minor histological changes contained more HBsAg than those with active lesions and there appears to be an inverse relationship between the amount of HBsAg in the liver and the severity of the histological damage. In the carriers who had more than one biopsy, the presence of stainable HBsAg, irrespective of the initial histological diagnosis, was associated with a greater likelihood of progression of the histological lesion. HBcAg was found only in the hepatocyte nuclei of carriers with chronic aggressive and chronic persistent hepatitis.

Hepatitis B antigen (HBAg) was first identified in the liver by immunofluorescence (Millman et al., 1969; Nowoslawski et al., 1970). More recently, similar techniques have been used to demonstrate the surface antigen (HBsAg) in the cytoplasm of hepatocytes and core antigen (HBcAg) in the nuclei of hepatocytes (Gudat et al., 1975; Ray et al., 1976; Trepo et al., 1976). A disadvantage of immunofluorescence is that the level of sensitivity is such that the most intense fluorescence is produced in sections of fresh frozen tissue (Portmann et al., 1976). Moreover, the fluorescent stains are themselves only transitory, and these sections must be examined soon after staining. Consequently, the use of orcein-stained paraffin sections of liver (Shikata et al., 1974; Deodhar et al., 1975) has some potential advantages over immunofluorescence. A further advance in the identification and localisation of the hepatitis virus antigens was made when it was shown by Burns (1975) and by Nayak and Sachdeva (1975) that they could be stained in paraffin sections using the immunoperoxidase technique. This technique combines the specificity of immunological methods with all the advantages inherent in the use of paraffin sections for the study of the antigen.

In this study of the liver biopsies from asymptomatic blood donors with persistent HBsAg antigenaemia, the immunoperoxidase, orcein, and electron optical methods have been compared and used to identify HBsAg. HBcAg was demonstrated in sections by both immunoperoxidase and electron microscopy.

Material and methods

Liver biopsies from a group of 33 asymptomatic blood donors, 22 of whom had had at least two biopsies over periods of two to four years, were reviewed. In most of these biopsies (48/55) there was sufficient material for immunoperoxidase and orcein staining. The investigation of the blood donors has been described previously (Woolf et al., 1974; Tapp et al., 1976).

IMMUNOPEROXIDASE

Paraffin sections were stained using the type of sandwich technique described by Mason et al. (1975). The following sera were applied to the sections in turn:

(a) for HBsAg—normal porcine serum—specific anti HBsAg raised in rabbits (Hoechst)—antibody to rabbit serum raised in pigs (Mercia Diagnostics)—the peroxidase-anti-peroxidase complexes (Mercia Diagnostics);

(b) for HBcAg—normal porcine serum—human serum containing high titre anti HBcAg and devoid of anti HBs and antibody to e antigen—anti human IgG serum raised in rabbits—antibody to rabbit
Fig. 1 The cytoplasm of the majority of the cells in some lobules is stained. Immunoperoxidase × 145.

Fig. 2 Small groups of cells show positive cytoplasmic staining. Immunoperoxidase × 400.
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serum raised in pigs—the peroxidase-anti-peroxidase complexes.

The optimal dilutions for the anti HBs and anti HBC that produced least non-specific staining were found by titration. The specificity of the HBsAg staining was confirmed in control sections where staining was abolished when anti-HBs previously absorbed with HBsAg was used. Specificity of the HBCAg staining was demonstrated by treating control sections with normal human serum instead of anti HBC. The anti HBC serum was tested for anti HBs by haemagglutination and for anti e antigen by immunodiffusion.

In both (a) and (b) the peroxidase and hence the antigen was visualised by the development of the antibody-bound peroxidase using diamino benzidine.

ORCEIN
Paraffin sections were stained by the method described by Deodhar et al. (1975).

ELECTRON MICROSCOPY
A part of each biopsy was processed separately for electron microscopy, and ultrathin sections were stained with urinylacetate and lead citrate.

Results

IMMUNOPEROXIDASE STAINING FOR HBsAg
HBsAg staining was seen in the cytoplasm of hepatocytes in 34 of the 48 biopsies which were examined. The biopsies from 12 of the 33 carriers did not have demonstrable HBsAg in the first or subsequent biopsies. The amount of cytoplasm staining in the individual cells varied from almost the whole to only

<table>
<thead>
<tr>
<th>Liver lesion</th>
<th>No. of biopsies</th>
<th>HBsAg staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diffuse</td>
<td>Focal</td>
</tr>
<tr>
<td>Chronic aggressive hepatitis</td>
<td>7</td>
<td>1 (14%)</td>
</tr>
<tr>
<td>Chronic persistent hepatitis</td>
<td>17</td>
<td>6 (35%)</td>
</tr>
<tr>
<td>Focal parenchymal necrosis</td>
<td>17</td>
<td>9 (53%)</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
<td>2 (50%)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>20 (42%)</td>
</tr>
</tbody>
</table>

Table HBsAg staining and liver lesions

Fig. 3 The two nuclei in the centre of the field are stained heavily. Other nuclei show lighter staining. Immunoperoxidase × 400.
small parts of the cell. The intensity of the staining varied from a dark brown to a pale yellow, and it is assumed that this is a reflection of the amount of HBsAg in the cytoplasm. Where there were lipid droplets in the cytoplasm these were unstained, giving rise to a foamy appearance of the cytoplasm. There was no evidence of HBsAg limited to the margins of the cells in these biopsies.

The number of cells which contained HBsAg varied in different biopsies, and two main groups could be identified. In the first, there was diffuse staining of sheets of cells affecting up to two-thirds of the liver lobule, while in the second considerably fewer cells were affected (Figs. 1 and 2). There was no particular distribution of HBsAg staining within the lobule in either the diffuse or the focally stained sections.

**HBsAg staining and liver lesions**

The type of HBsAg staining in biopsies showing

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**Fig. 4** There is marked dilatation of the endoplasmic reticulum and part of the cytoplasm contains very few mitochondria (upper part of photograph). EM × 5400.
different histological lesions is given in the Table. Diffuse staining was more common in livers with focal parenchymal necrosis and was seen also in two of the three biopsies showing cirrhosis. Focal staining was seen more commonly in livers showing chronic aggressive hepatitis. Biopsies with the histological lesion of chronic persistent hepatitis showed both patterns of HBsAg staining with equal frequency.

**HBsAg staining in sequential biopsies**

There was little variation in the pattern of HBsAg staining in the first and subsequent biopsies from each individual.

In these carriers, 2/4 of those having a focal distribution and 5/8 of those having a diffuse distribution of HBsAg showed some deterioration of the histological appearances in the second biopsy; this occurred in only 1/10 carriers in whom HBsAg was not demonstrated in the liver.

**IMMUNOPEROXIDASE STAINING FOR HBcAg**

Staining was seen only in the nuclei of the hepatocytes. Of 48 biopsies that were stained for HBcAg, in only eight, from four donors, could positively stained nuclei be identified with certainty. In these biopsies only relatively few nuclei stained, and the

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**Fig. 5** Round particles (single arrow) and filaments (double arrow) are seen within the dilated endoplasmic reticulum. EM × 53 250.
degree of HBcAg staining in individual nuclei in each biopsy varied from brown to black (Fig. 3).

The biopsies from one donor showed chronic persistent hepatitis and, in three, chronic aggressive hepatitis; in two donors, the lesions had increased in severity during the period of study.

There were four other biopsies with occasional nuclei which stained faintly and were regarded as doubtful positives.

ORCEIN STAINING FOR HBsAg
Biopsies stained with orcein showed the same variability in the extent and degree of cytoplasmic staining in hepatocytes as was seen in those stained for HBsAg using the immunoperoxidase method and could be divided into the same two main groups. Of 20 biopsies showing diffuse staining by the immunoperoxidase method, 10 were negative when stained by orcein. Nine of 14 biopsies with focal immunoperoxidase staining failed to stain by the orcein method and 14 biopsies were negative by both methods. Orcein staining was clearly the less sensitive method.

ELECTRON MICROSCOPY
Filaments and tubules could be identified in the cytoplasm of hepatocytes in some biopsies. The areas containing these could be identified at low magnification by the presence of 'clear areas', the latter representing parts of the cytoplasm where the endoplasmic reticulum was dilated and there was a reduction of other cytoplasmic components especially mitochondria (Fig. 4). At higher magnification the filaments and tubules could be seen within the dilated endoplasmic reticulum (Fig. 5). These areas could be found more easily in biopsies which had stained diffusely with the immunoperoxidase technique for HBsAg.

Particles measuring about 20 nm in diameter were seen in the nuclei in six of the 37 biopsies which were examined (three carriers each with two biopsies). The biopsies containing nuclear particles all stained for HBcAg with the immunoperoxidase technique, and there was a good correlation between the number of nuclei staining with the latter and the ease with which 20 nm particles could be found. In two biopsies which stained for HBcAg, intranuclear particles could not be found but these contained fewer stained nuclei than the other positive biopsies.

Discussion
With the immunoperoxidase technique specific staining of HBsAg and HBcAg was obtained. The morphological appearance of HBsAg in sections stained by the immunoperoxidase technique is similar to that described by other workers using this method (Burns, 1975; Nayak and Sachdeva, 1975) or immunofluorescent techniques (Akeyama et al., 1974; Ray et al., 1976). However, staining of the peripheral part of the cytoplasm or of the cell membrane described by the latter two groups of workers was not seen in the present formalin-fixed material.

The immunoperoxidase technique was more time-consuming than orcein staining but was more sensitive and gave clearer staining. Orcein staining was variable in quality because some commercial orcein preparations stain better than others. Afroudakis and his co-workers (1976) also found that immunoperoxidase staining for HBsAg was more sensitive than orcein, although this was not the experience of Nayak and Sachdeva (1975).

In ultrathin sections, tubular and circular structures were seen in the cytoplasm of hepatocytes in biopsies which were positive for HBsAg with the immunoperoxidase technique. Gerber and his co-workers (1974), using direct immuno-electron microscopy, have shown that these structures contain HBsAg determinants, and there is little doubt that these bodies represent cytoplasmic HBsAg. Examination of the sections by electron microscopy is laborious and only small areas of the liver can be examined by this method. In view of the focal nature of HBsAg in some sections it is clearly a less satisfactory method for the quantitation of HBsAg in the liver.

Using the immunoperoxidase technique, HBsAg was found in the cytoplasm of 71% of biopsies. However, the type of distribution of HBsAg in the biopsies varied, a focal distribution predominating in those showing the histological lesions of chronic aggressive hepatitis while a diffuse distribution of HBsAg was seen more commonly in those showing none or only minor histological abnormalities. A similar distribution of HBsAg in patients with chronic aggressive and persistent hepatitis has been described by Ray et al. (1976). Portmann and his co-workers (1976) also found large numbers of positive hepatocytes in chronic carriers showing only minor histological lesions, and it would appear in general that there is an inverse relationship between the amount of HBsAg in the cytoplasm and the activity of the disease in the liver. The relative amounts and distribution of HBsAg and HBcAg in the liver have been related to the different immunological responses to hepatitis B virus infection (Gudat et al., 1975).

In those carriers having two or more biopsies the presence of demonstrable HBsAg in the initial biopsy was more likely to be associated with some deterioration in the histological appearances in the second biopsy. This appeared to be so irrespective of the amount of HBsAg in the liver or the initial
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histological diagnosis.

The immunoperoxidase technique was also used to demonstrate HBcAg in the nuclei of hepatocytes. There was a good correlation between the incidence of positive staining and the presence of the 20 nm core particles. It is probable that the increased sensitivity of the immunoperoxidase technique is responsible for the finding of two biopsies positive for HBcAg by the immunoperoxidase method in which core particles could not be found in the nuclei.

All the biopsies that were positive for HBcAg showed either chronic aggressive or chronic persistent hepatitis. These HBcAg positive biopsies came from four blood donors in whom significant numbers of Dane particles had been found repeatedly by electron microscopy of the serum and who are also persistently e antigen positive (Tapp et al., 1974).

References


