Methods for detection of hepatitis B surface antigen in paraffin sections of liver: a guideline for their use

E. SUMITHRAN

From the Department of Pathology, Medical Faculty, University of Malaya, Kuala Lumpur, Malaysia

SUMMARY Methods for the localisation of hepatitis B surface antigen (HB_sAg) in paraffin sections of the liver include the detection of ground-glass hepatocytes and the use of Shikata's orcein stain, and of immunoperoxidase and immunofluorescent techniques. A comparative study of the different methods on 20 livers shows the orcein stain to be the method of choice for routine use. The Shikata stain is not only specific but is relatively inexpensive, easily performed, and stains out distinct cytoplasmic inclusions even in stored formalin-fixed livers, old paraffin blocks, and autolysed livers. Since HB_sAg is irregularly distributed in the liver, adequate sampling is necessary to prevent false negatives; when sufficient tissue is available at least five blocks should be examined before a case is labelled as HB_sAg-negative.

In recent years there has been a growing interest in the detection of hepatitis B surface antigen (HB_sAg) in paraffin-embedded liver tissue. Methods for the localisation of HB_sAg in paraffin sections of the liver include the detection of ground-glass hepatocytes in haematoxylin and eosin preparations (Hadziyannis *et al.*, 1973) and the use of Shikata's orcein stain (Shikata *et al.*, 1974) and of immunoperoxidase (Burns, 1975) and immunofluorescent (Akeyama *et al.*, 1972) techniques. These methods have been invaluable as research tools in the retrospective detection of HB_sAg in stored formalin-fixed livers and in old paraffin blocks containing liver tissue. They are also of value in clinical diagnosis. The specificity and reliability of the methods have previously been compared (Nayak and Sachdeva, 1975; Portmann *et al.*, 1976) but few guidelines for their use are available.

We have used the above methods in retrospective studies on liver tissue from Malaysian Aborigines (Orang Asli) who have a very high incidence of primary hepatocellular carcinoma (Sumithran and Prathap, 1976) and have found attributes and deficiencies in all the methods. A major problem is that HB_sAg may be sparsely distributed in the liver and fail to be detected unless an adequate number of blocks are examined. How many blocks constitute an 'adequate number' has not been established. In view of the great use being made of these methods for the detection of HB_sAg, there exists a need for

proper guidelines to their use. This paper, the result of a comparative study of the different methods, outlines suggestions for their use.

Material and methods

Twenty formalin-fixed cirrhotic livers from necropsied Orang Asli subjects were used in the study. Ten of the livers had associated primary hepatocellular carcinomas. Two livers, one of which contained a liver cancer, were badly autolysed: cell outlines were indistinct and nuclei were lost. The livers had been preserved in formalin for from one to 10 years. Paraffin blocks from all the livers made at the time of necropsy were available. The number of such blocks available from each liver ranged from two to five (average three).

Serial sections, 3 μ m, were cut, and four consecutive sections from a ribbon were picked up on glass slides numbered 1 to 4. Slide 1 was stained by haematoxylin and eosin, slide 2 by orcein, slide 3 by the immunoperoxidase method, and slide 4 by the immunofluorescent method.

From each formalin-fixed liver 10 fresh square pieces of tissue were randomly taken from different areas. Each piece, measuring 1.5 $\times$ 1.5 $\times$ 0.3 cm in size, was blocked in paraffin. The 10 blocks from each liver were randomly tagged A to J. Serial sections, 3 μ m, were cut from each block, and four consecutive sections from a ribbon were picked up on glass slides numbered 1 to 4. As before, the slides were stained by the four methods.

Orcein staining was carried out using the method of Shikata *et al.* (1974). The deparaffinised sections were oxidised in a 0.15% solution of potassium permanganate and sulphuric acid (0.15 g KMnO_4 + 100 ml distilled water + 0.015 ml conc. H_2SO_4) for five minutes. (Oxidation gives good contrast in the final product.) The sections were then decolourised in 2% oxalic acid (10 minutes), washed in water, and placed in a 1% solution of orcein (BDH) in 70% alcohol at pH 1–2 (1 g orcein + 100 ml 70% alcohol + 2 ml conc. HCl) for four hours. The sections were finally differentiated in absolute alcohol, dehydrated in xylene, and mounted. Orcein-positive cells stain dark brown against a background of very light brown negative cells.

Immunoperoxidase staining was carried out by the indirect sandwich technique after endogenous peroxidase had been blocked by immersing the sections in methanol containing 0.3% hydrogen peroxide. Specific rabbit antiserum against HB_sAg (anti-Au/SH serum from Behringwerke AG, Germany) and a swine antirabbit immunoglobulin conjugated with horseradish peroxidase (obtained from Dakopatts AS, Denmark) were used. The tissue-bound peroxidase-conjugated antibody was stained with diaminobenzidine (DAB from BDH Chemicals, England).

Immunofluorescent staining was also carried out by an indirect technique using the same rabbit antiserum against HB_sAg as above and sheep anti-rabbit immunoglobulin tagged with fluorescein (Wellcome Research Laboratories, England).

Results

All the livers were found to contain HB_sAg on examination of multiple blocks (Table 1). There was little to choose from between the orcein stain and the immunoperoxidase method; each method distinctly stained out the positive areas. The HB_sAg -positive cells, when abundantly present, were readily appreciated by the immunofluorescent method, but when HB_sAg was sparsely present the background

fluorescence made the few positive cells difficult to detect.

As previously described (Nayak and Sachdeva, 1975), the material which stained positively with the empirical orcein stain showed complete identity with that stained by the immunospecific methods. Localisation both within focal areas and in individual cells matched extremely well with those seen in the immunoperoxidase (Figs 1 and 2) and immuno-

Fig. 1 Orcein-positive inclusions in hepatocytes ($\times 450$)

Fig. 2 Immunoperoxidase-positive inclusions in section contiguous to that illustrated in Fig. 1 ($\times 450$)

In less than one-half of the livers was HB_sAg so abundant as to be detected on examination of a single block. In some livers HB_sAg was confined to parts of small nodules. The number of positive cases increased as more blocks were examined (Table 2). On examination of one block per liver, only 45% of the livers were found to be positive. On examination of five blocks 95% of the livers were found to

Table 1 Number of livers showing ground glass, orcein, peroxidase, and immunofluorescent positive hepatocytes in relation to type of block examined

Type of block examined	Number of livers (total 20)			
	Ground-glass	Orcein	Peroxidase	Immunofluorescent
Stored paraffin (av. 3 per liver)	9	15	15	15
Fresh (10 per liver)	12	20	20	20

Table 2 Number of livers with HB_sAg -positive hepatocytes in relation to number of blocks examined

No. of blocks examined per liver	No. of livers positive (total 20)	% of livers positive
1	9	45
2	11	55
3	16	80
4	17	85
5	19	95
6	19	95
7	20	100

contain HB_sAg. The difference in the number of positive cases between the fresh blocks and stored blocks (Table 1) was probably due entirely to the greater number of the former.

The two extremely autolysed livers surprisingly contained material which stained strongly positive with orcein and the immunospecific methods (Figs 3 and 4).

Fig. 3 Autolysed hepatocytes (Haematoxylin and eosin $\times 500$)

Fig. 4 Orcein-positive inclusions in autolysed hepatocytes ($\times 500$)

Although the orcein and immunologically stained material was present mainly in the noncancerous parenchyma of the livers with hepatocellular carcinoma, in two such livers small focal areas of tumour contained orcein, immunoperoxidase, and immunofluorescent-positive cytoplasmic inclusions, as has previously been described (Nayak and Sachdeva, 1975).

Typical ground-glass hepatocytes, as described by Hadziyannis *et al.* (1973), were present in only small numbers in 60% of the livers. The majority of

hepatocytes with HB_sAg-positive inclusions did not have a typical ground-glass appearance.

Discussion

HB_sAg may be detected in hepatocytes using immunospecific methods or by the empirical orcein stain. There is complete identity between the material which stains positive with orcein and that stained by immunofluorescent and immunoperoxidase methods. Interference by background fluorescence decreases the value of the immunofluorescent technique, especially when HB_sAg is sparsely present, but both the orcein and immunoperoxidase methods stain out the inclusions distinctly. Since the products of the latter two procedures are equally satisfactory, the very much lower cost of the orcein method and the relative simplicity of its technique make it the method of choice for the routine detection of HB_sAg in fixed liver tissue.

The immunoperoxidase and immunofluorescent methods may, however, be useful for further studies on livers containing HB_sAg. Nuclear staining has never been observed with orcein, and should the demonstration of hepatitis B core antigen become an important research or diagnostic tool, then immunospecific methods may have to be used. Ray *et al.* (1976) have found a number of different staining patterns by immunofluorescence in chronic hepatitis and in acute hepatitis, which may prove to be helpful in the histological diagnosis of different types of HB_sAg-positive hepatitis.

HB_sAg appears to be rather stable; neither formalin-fixation for several years nor paraffin-embedding seems to inactivate its antigenic determinants. Material used in this study has been in formalin or has been paraffin-embedded for up to 10 years. Shikata (1974) has stained specimens from patients with cirrhosis that have been in the files for as long as 50 years. Moreover, HB_sAg is readily detected even in badly autolysed livers.

The ground-glass hepatocyte is little more than a useful diagnostic hint for the presence of HB_sAg. It may occur in other conditions, such as drug-induced liver injury, cholestasis, and alcoholic hepatitis (Popper, 1975). These cells were present in small numbers in only 60% of livers carrying the hepatitis B surface antigen. The majority of hepatocytes with HB_sAg-positive inclusions did not have a typical ground-glass appearance. Nayak and Sachdeva (1975), who also based their work on necropsy material, were unable to identify ground-glass hepatocytes in liver tissue containing orcein, immunoperoxidase, and immunofluorescent-positive hepatocytes. This observation differs from the findings of Portmann *et al.* (1976), who found

ground-glass hepatocytes in all the needle liver biopsies which were positive for HB_sAg by immunofluorescence or orcein methods; the ground-glass cells corresponded well with those containing HB_sAg. Their study, however, did not include postmortem material. The ground-glass appearance is thought to be due to an increase in the smooth endoplasmic reticulum of the affected hepatocytes. Since cell organelles autolyse fairly rapidly after death, it is possible that HB_sAg-positive hepatocytes may lose their ground-glass appearance within a few hours of death. This may be the explanation for the paucity of ground-glass hepatocytes in necropsy material.

Owing to the irregular distribution of HB_sAg in the liver, adequate sampling of tissue is required before a case is labelled as antigen-negative. When limited material is available, as in needle liver biopsies, the number of false negatives due to sampling error is likely to be very great. Examination of only one block from each liver resulted in more than 50% of false negatives. When a large amount of tissue is available, it is imperative that multiple blocks be examined before a case is labelled as antigen-negative. Examination of five 1.5 cm square blocks from each liver resulted in failure in detection of HB_sAg in only one of the 20 livers.

In conclusion, for the routine detection of HB_sAg in paraffin sections of the liver, Shikata's orcein stain is the method of choice. It is not only specific but is relatively inexpensive and easily performed, and stains out distinct cytoplasmic inclusions even in stored formalin-fixed livers and old paraffin blocks. Autolysis does not invalidate orcein staining. Adequate sampling is necessary to prevent false negatives. When large amounts of liver tissue are

available, at least five blocks should be examined.

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