Localization of Hepatitis B Surface Antigen in Conventional Paraffin Sections of the Liver

Comparison of Immunofluorescence, Immunoperoxidase, and Orcein Staining Methods With Regard to Their Specificity and Reliability as Antigen Marker

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Hepatitis B antigen (HBAg) has been demonstrated in conventional formalin-fixed paraffin-embedded liver tissue by peroxidase and fluorescent immunostaining as well as by orcein. Complete locational and morphologic identity is seen between material stained by specific immunologic methods and by orcein. The antigen is restricted to the cytoplasm and is generally observed in the hepatocyte; it is present in three morphologic forms. Certain morphologic forms can even be identified in hematoxylin and eosin-stained tissue. Results of immunostaining procedures indicate that the antigen demonstrated in this study consists entirely of surface coat of hepatitis B virus (HB_sAg). This seems to be the only component revealed by orcein staining. The latter is considered to be a good marker of the surface antigen and to have certain advantages over immunostaining. It is suggested that suitability of conventional paraffin sections for the detection of HBAg has wide and important implications. (Am J Pathol 81:479–492, 1975)

DEMONSTRATION of hepatitis B antigen (HBAg) in the serum or liver is generally considered to be evidence of persistence of the virus in the body. Though detection of the antigen in the serum by various methods is simple and yields consistent results, its localization in the liver has, until recently, proved relatively difficult. Immunofluorescent staining has been reported to have shown antigen within the hepatocyte in either its cytoplasm or nucleus or in both.[1-6] Electron microscopic studies reveal small spherical particles in the nucleus, and larger tubular, filamentous, and spherical structures in the endoplasmic reticulum in the cytoplasm of the hepatocytes.[7-10] These seem to correspond with particles and structures observed in the sera of patients with antigenemia,[11,12] as well as with those seen in liver tissue homogenates.[13] Immunoagglutination electron microscopy and immune electron microscopy using either ferritin- or peroxidase-conjugated antibody show distinct antigenic differences between the intranuclear core particles and the cytoplasmic coat material.[4,8,12] This duality of the hepatitis B virus has been confirmed by

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Accepted for publication July 21, 1975.
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separation of the two components from Dane particles on treatment with detergents.[14,15] Hoofnagle and his colleagues [16] and Brzosko and co-workers [17] have recently demonstrated different antibody systems specific against these two components of the virus in animal and human sera. It has been suggested that the commonly recognized HBAg (Australia antigen or hepatitis-associated antigen), in fact, represents the surface coat material of the virus present in the cytoplasm of the liver cells.[14,17-19]

The techniques utilized in the above-mentioned studies on tissue localization, while providing important information, are time consuming and unsuitable for application in large-scale studies. Shikata and his co-workers [20] very recently demonstrated a simple and reproducible technique for the detection of HBAg in conventional paraffin sections by staining with orcein. They partly confirmed the specificity of this staining by *in vitro* tests on the antigen and by fluorescent stains on frozen sections of fresh tissue. Considerable evidence is now available to indicate that several forms of fatal chronic liver diseases, including primary cancer of the liver, show strong association with infection by hepatitis B virus.[21-26] Such association has up to now been established mainly by high rate of detection of HBAg in the serum. Availability of a simpler technique for the demonstration of HBAg or the virus in the liver of such patients would undoubtedly provide a useful tool for the examination of large numbers of material in retrospective and prospective studies. While pursuing such an investigation, we found that conventional paraffin sections are also quite suitable for specific immunologic staining of HBAg. This finding provided means to test with greater accuracy the specificity and reliability of orcein staining and compare them with those of the immunospecific stains.

Materials and Methods

The material consisted of paraffin blocks of formalin-fixed liver tissue collected at autopsy from 10 cases of cirrhosis of the liver, 5 cases of acute fulminant hepatitis, 13 cases of primary hepatocellular carcinoma, and 15 miscellaneous hepatic lesions such as chronic venous congestion, liver abscess, extrahepatic portal vein obstruction and extra hepatic biliary obstruction. In some cases of cirrhosis and primary carcinoma of the liver, only paraffin sections of autopsy material were available. The paraffin blocks were anywhere between a few months to several years old.

Wherever paraffin blocks were available, 3-μ serial sections were cut, and six consecutive sections from a ribbon were picked up on glass slides numbered 1 to 6. In those cases where only limited number of paraffin sections were available, a few selected staining procedures were carried out.

In the serially sectioned material, Slide 1 was stained by hematoxylin and eosin and the remaining slides were stained for HBAg by various procedures. Slide 2 was stained for the antigen by orcein. Slides 3 and 4 were used for immunofluorescence staining as test and control, respectively. Slides 5 and 6 were stained by the immunoperoxidase method, again as test and control, respectively.

Orcein staining was carried out according to the method of Shikata *et al.*[20] with a light coloring of the nuclei by hematoxylin. Paraffin sections of formalin-fixed brain tissue from autopsies of subjects with rabies and herpes encephalitis, and of surgical skin biopsy material from patients with molluscum contagiosum and verruca vulgaris were also stained by orcein.

Immunofluorescence was carried out by the indirect technique using rabbit antiserum against HBAg (anti-HB_s/ar which was kindly supplied by Dr. Holland of the National Institutes of Health, USA) diluted 1:16 and goat anti-rabbit globulin tagged with fluorescein isothiocyanate (FITC) (Cappel Laboratories Inc.). The slides were examined under a Zeiss fluorescent microscope using a dark field condenser, BG 12 excitation filter and 44/50 interference filters. Control sections were treated with normal rabbit serum instead of the specific antiserum.

Immunoperoxidase staining was performed using the same dilution of antiserum either by the double sandwich method described for the localization of α-fetoprotein and albumin in the liver by Nayak and colleagues,[27,28] or by a single sandwich method where the later steps of the previous technique were eliminated by using peroxidase-conjugated goat anti-rabbit globulin (Kindly prepared for us by Ms. Jill Curtis of the Department of Biochemistry according to the method of Avrameas [29]). As for immunofluorescence, the control section was treated with normal rabbit serum instead of the specific anti-HBAg antiserum.

Comparison of Antigen Localization

With each staining procedure, the attempt was made to demonstrate material positively stained for the antigen, observe its morphology and distribution, and map out regions of localization in relation to the cell and also to the area of the tissue section. The section stained with orcein was compared with its immediately preceding serial section stained with hematoxylin and eosin and its immediately following one stained for immunofluorescence. Comparison was also made between sections stained by the orcein and immunoperoxidase techniques. The aim of this comparative study was to determine the identity of antigen by different staining techniques. However, due to large size of the tissue block and prolonged storage of some of these blocks, serial sections failed to show exactly identical cells in some instances, though identical areas were always represented.

To ensure more precise localization, the following procedures were adopted. Test sections used for immunofluorescence, after examination and photography, were washed and stained by the orcein technique. Similarly sections stained with hematoxylin and eosin after examination and photography were decolorized and then stained by the orcein technique. In selected cases, thin serial sections were obtained fresh from the paraffin blocks; one of these was stained by the immunoperoxidase method and the two contiguous ones, preceding and following, were stained with orcein.

Immunoprecipitation and Staining

Sera known to be positive for HBAg, the same sera treated with equal volumes of 10% formalin solution for 2 to 4 hours and HBAg-negative sera were run in counter-immunoelectrophoresis (CIEP) against the anti-HBAg rabbit antiserum used for immunostaining. After the precipitation lines developed, sandwich CIEP was done by refilling the anode wells with FITC-conjugated goat anti-rabbit globulin and running the apparatus for 1 hour. The slides were then left at room temperature from 4 to 18 hours, after which they were examined under a fluorescent microscope. The same slide was then washed in phosphate-buffered saline for 4 hours, dried, and stained by the orcein technique.

Results

Antigen was observed in livers from several patients with cirrhosis and primary liver cancer but in none of the other livers including ones from patients with acute fulminant hepatitis. Wherever a liver was positive for HBAg, localization could be generally uniformly seen by the immunofluorescence, immunoperoxidase, and the orcein technique, though positivity was higher and detection simpler by the latter two methods (Table 1). Certain forms of antigen could also be easily appreciated in the conventional hematoxylin and eosin preparation. In immunofluorescent staining, background fluorescence appeared to be somewhat in excess of what is encountered in sections of directly frozen tissues. However, when compared with the control sections, specific immunofluorescence of medium brightness could be easily appreciated, being particularly clear when it presented as condensed inclusions (Figure 1). Whether in this form or in the more diffuse form, positive fluorescence was limited exclusively to the cytoplasm, predominantly of hepatocytes.

Immunoperoxidase staining done either by the double or the single sandwich technique presented much more convincing localization of the antigen (Table 1). The latter stained brownish black to very dark brown and showed morphologic variations to be described later. Assessment being much easier than in the immunofluorescent preparation, comparison with the orcein-stained material was considerably simpler. As in the immunofluorescent staining, antigen localization was always cytoplasmic, being mostly observed in the hepatocytes and very occasionally in the Kupffer cells.

Table 1—Demonstration of Hepatitis B Surface Antigen in Paraffin Sections of Cirrhosis and and Primary Hepatocellular Carcinoma by the Three Different Methods

Hepatic lesion	No. examined	Tissue localization of antigen* (No. positive/No. examined) Immuno-fluorescence	Immuno-perioxidase	Orcein
Cirrhosis	10	3/7 − to − − −	6/10 − to − − − −	7/10 − to − − − −
Primary hepatocellular carcinoma				
Nontumorous part of liver	13	6/8 − to − − −	10/11 − to − − − −	13/13 − to − − − −
Tumor	13	1/4 −	4/12 − to − −	6/13 − to − −

* Approximate quantity of demonstrable antigen has been indicated as follows: − = very small, − − = small, − − − = moderate, − − − − = large.

Orcein-stained preparations showed localization of antigen as described by Shikata and his colleagues.[20] Localization both within individual cells and in geographic areas of the tissue section in this preparation matched extremely well with those seen in the immunofluorescence (Figures 1 and 2) and immunoperoxidase (Figures 3 and 4) preparations. There was complete identity of material stained positively by orcein and by the two immunostaining techniques. With orcein staining, the antigen presented an "amber" color and was again exclusively cytoplasmic in location, being predominantly in the hepatocyte and only occasionally in the Kupffer cell.

Morphology and Structure of HBAg

This could be best appreciated in the orcein-stained preparations and to a large extent in the immunoperoxidase preparations (Figures 5 and 6). In the former, the amber staining of the antigen stood out prominently against the beige cytoplasmic background and the hematoxylin staining of the nucleus (Figure 7). On the other hand, in immunoperoxidase-stained tissue the antigen often took up a dense staining (Figures 5 and 6), and the finer structure could not be appreciated adequately against the lighter brown staining of the cytoplasm. The antigen was distributed very randomly in the liver, being present in a number of neighboring hepatocytes over focal areas or in individual liver cells widely separated from each other. There was no predilection of these antigen-carrying cells to be distributed around any of the vascular territories. The antigen presented essentially three distinct morphologic patterns: a) large, clearly outlined, discrete inclusions (Figures 2, 4, 7, and 8) of different shapes and sizes occupying a large portion of, or occasionally, the entire cytoplasm; b) one or more round to oval vesicles and short tubules (Figures 7 and 9, inset) measuring approximately 3 to 7 μ in width or diameter; and c) granules or short, fine fibrils distributed diffusely along one or both sinusoidal borders or throughout the cytoplasm of the hepatocyte (Figures 7 and 8). When examined closely, the inclusion form was seen to be comprised of a densely packed meshwork of fine fibrils (Figures 6 and 8). The pattern of diffusely distributed granules and fine fibrils was almost exclusively observed in areas where a group of neighboring hepatocytes contained the antigen.

Generally, hepatocytes containing the antigen appeared healthy, though degenerating and atrophic cells did in some instances show the antigen. Whenever a hepatocyte showed cytoplasmic vacuolation, in addition to the antigen, the latter was localized around the vacuoles in the form of small or large thin rings, or chains.

Comparison of the section stained first by hematoxylin and eosin and subsequently by orcein revealed that the inclusion variety of antigen

could often be demonstrated in the conventional hematoxylin and eosin stain (Figures 9 and 10). It appeared as very light, purplish red, relatively ill-defined, translucent inclusions having a finely fibrillar internal structure, different from other hyaline inclusions observed in the hepatocytes (Figure 10). The vesicular and tubular forms of antigen could also be seen in the hematoxylin and eosin preparations (Figure 10, inset). On the other hand the counterpart, in hematoxylin and eosin preparations, of the granular and finely fibrillar form could not be appreciated. On the whole, rates of detection and appreciation of the amount of antigen was less by immunofluorescence as compared to immunoperoxidase and orcein staining (Table 1). The highest success was with orcein stain.

The only other orcein-positive material that was occasionally encountered consisted of coarse, "caramel" colored granules in the cytoplasm of degenerating hepatocytes. These were homogenous and had tinctorial and structural characteristics easily distinguishable from those of HBAg described previously. We are presently investigating the nature of this material.

In some cases where unequivocal demonstration of antigen was made, the latter could not be localized in sections from all the blocks. Sometimes, only one of several blocks showed the antigen. Also, different sections from a particular block did not all show the antigen.

Intracellular inclusions in samples from subjects with rabies, herpes encephalitis, or viral epidermal lesions of the skin did not stain with orcein.

Immunoprecipitates and Their Staining

Precipitation lines formed with both antigen-positive serum as well as with formolized serum. These lines gave positive staining by both immunofluorescence and orcein techniques, though the staining appeared slightly fainter in case of the formolized antigen.

Discussion

The results of this study convincingly demonstrate that the HBAg can be clearly localized in the liver cells in formalin-fixed, paraffin-embedded material. Even paraffin blocks preserved for several years can conveniently be examined for the presence of this antigen. Localization can be successfully achieved not only by the empirical orcein staining but also by the specific immunostaining methods using fluorescence and peroxidase. Positive immunostaining indicates that the specific antigenic determinants of the HBAg are not inactivated by formalin fixation and processing for paraffin embedding. That formalin treatment of the antigen does not

eliminate its ability to react with specific antiserum is also evident from the results of *in vitro* immunoprecipitation reactions. Precipitation lines with both untreated and formalin-treated antigen are stained well with both immunofluorescence and orcein. Of the two immunostaining techniques (i.e., fluorescence and peroxidase), the latter has been shown to offer certain distinct advantages over the former.[27] When working with conventionally fixed paraffin sections, interpretation of immunofluorescence can pose greater difficulties than frozen sections of fresh tissue, principally due to an increased background autofluorescence. Our study, however, shows that the antigen, particularly the large inclusion form, can be easily observed by immunofluorescence. It is also clear that not only immunoperoxidase staining can be reliably used for localization of this antigen but also formalin-fixed material can conveniently be utilized in this procedure. We have earlier shown that, in case of other antigens such as α-fetoprotein and albumin, identical localization in the liver cell can be achieved by these two immunostaining techniques.[27,28] These antigens are well preserved, even after processing through alcohol, xylol, and paraffin. One of them, α-fetoprotein, is in fact not properly localized in frozen sections of fresh tissue.[30] It appears that HBAg remains well preserved, not only after fixation in formalin but also after subsequent processing through alcohol and xylol.

Several reports are available on localization of HBAg in liver cells by immunofluorescence in frozen sections.[1-6] Though the antigen has been located in the cytoplasm alone or in both cytoplasm and nucleus, the large inclusion variety as seen in our study has not been shown earlier with immunofluorescence. It is possible that these might have been missed because of the use of small needle biopsies or because formalin treatment somehow alters the morphology of the antigen and condenses it to yield the inclusion form. The latter, however, seems somewhat improbable in view of the demonstration of fluorescent inclusions in the recent report by Shikata and his colleagues.[20]

The material which stained positively with orcein showed complete identity with that stained by the immunospecific methods. Shikata and his colleagues [20] have shown that serum HBAg can be stained by orcein, and that positive immunofluorescence for antigen in frozen tissue also reveals positive orcein staining after formalin treatment of the same section. Our study, in addition to confirming these findings, has extended the specificity of orcein staining further by demonstrating its identity with both immunostaining procedures performed on the same sections. This observation seems to us to be of great significance because of the simplicity and several other advantages of the orcein staining procedure over the immuno-

staining procedures. It is true that the latter, particularly the immunoperoxidase method, can be conveniently used for studies on paraffin-fixed material. However, for large scale retrospective and prospective studies, orcein staining offers distinct advantages.

Some recent studies using electron microscopy and immunoelectron-microscopy (IEM) to study HBAg have shown that the antigen in the cytoplasm is generally located inside the cisternae of the endoplasmic reticulum [6-10] in the form of tubular and circular structures. These are either packed or diffusely scattered in the entire or part of the cytoplasm of the hepatocyte.[8,9] The location and morphology of the antigen observed by various techniques used in the present study possibly represent the light microscopic counterpart of these electron microscopic observations. The predominantly fibrillar structure of the antigen observed by us suggests that it may be contained in the endoplasmic reticulum.

We did not find antigen in the nucleus of the hepatocyte either by orcein staining or by the two immunostaining procedures. It is being increasingly realized that the hepatitis B virus consists of two structural elements: the core particle resembling enteroviruses, which is possibly the true infective form, and the coat material surrounding the core.[14,16-19,31,32] The virus material can thus be observed in the hepatocyte in three distinct morphologic forms; the spherical core, replicating and generally seen in the nucleus; the vesicular and tubular coat protein; and the Dane particle, representing the virus core complete with its coat. The latter two are exclusively seen in the cytoplasm.[13] It has been suggested that the core particle gains a coat in the liver cell cytoplasm and is released into circulation as a Dane particle along with excess coat material.[13,32] The core and the coat appear to be antigenically distinct [16-18] and the HBAg commonly detected in the serum (Australia antigen or hepatitis-associated antigen) is considered to represent the surface or coat material (HB_sAg) and not the core virus proper (HB_cAg).[17-19] Antiserum collected from human patients is likely to contain antibodies against either or both of these antigenic components,[14-16] while antiserum raised in animals against HBAg reacts almost exclusively with coat material produced in the cytoplasm of the hepatocyte.[17] This duality of hepatitis B virus antigen–antibody system may explain the variations in nuclear versus cytoplasmic localization of hepatitis B antigen reported in the various studies employing immunofluorescent techniques.[1-6] In our immunostaining procedures, failure to localize any antigen in the nucleus is almost certainly due to the fact that the rabbit antiserum used by us contains antibody only against the coat material (HB_sAg).

Orcein-stained sections also showed entirely cytoplasmic localization of

antigen similar to what has been reported by Shikata and his colleagues.[20] Orcein positivity appears to be fairly specific for HBAg, since in our material it matched extremely well with the specific immunologic localization; other nonhepatic viral inclusions did not stain with it and false positivity was not encountered. Though the exact basis of the preferential selectivity for HBAg by orcein is not yet understood, it is clear that this stain under the circumstances is specific for the coat material (HB_sAg), thus accounting for the exclusive cytoplasmic localization of antigen. If the core particle is equally resistant to formalin fixation and subsequent processing as the coat material, with regard to its antigenic property, it would be possible to demonstrate nuclear localization of this antigenic component of the virus by immunoperoxidase or immunofluorescence studies in formalin-fixed tissue sections, when specific antiserum against the core (anti-HB_cAg) is used. This is presently being investigated by us.

An interesting finding in the present study was the identification of the inclusion and vesiculotubular forms of the antigen in preparations routinely stained with hematoxylin and eosin. Identity of location and morphology of these structures with those subsequently seen to be positively stained by orcein in the same section proves that they are indeed antigen material. The finer, fibrillar form was not detected with the hematoxylin and eosin stain. Shikata and his colleagues [20] stressed that the antigen could not be detected in the hematoxylin and eosin–stained tissue, while Hadziyannis *et al.*[33] described antigen-containing cells in such preparations as "ground glass" hepatocytes. We have not been able to confirm the findings of the latter group in our autopsy material (though in an occasional needle biopsy of the liver we have seen such cells that contain antigen), and the appearance of such hepatocytes illustrated in their report is different from that of those seen in our material. Their study, however, does not necessarily indicate that the cells showing positive immunofluorescence were the very same ones that exhibit the "ground glass" appearance in hematoxylin and eosin–stained preparations, since the former was performed on fresh frozen tissue and the latter apparently on paraffin sections. In a simultaneous light and electron microscopic study, Sun and co-workers [10] did not also observe significant number of "ground glass" hepatocytes, even when the proliferated cisterns of the endoplasmic reticulum were loaded with coat material. They in fact postulated that such cisterns are likely to be represented as dense eosinophilic material on light microscopy. Notwithstanding the fact that HBAg is relatively difficult to appreciate with the hematoxylin and eosin stain as compared to the orcein stain, we have been able to demonstrate some of its morphologic forms in all our cases showing antigen positivity on orcein

staining. We therefore believe that proper screening of the conventionally stained paraffin sections may help to suggest the presence of antigen in them. The comparison between immunochemical and orcein staining of the HBAg shows that the latter can be used as a specific and reliable antigen marker in conventional paraffin sections (Table 1). Most of the cytoplasmic antigen is likely to be easily revealed by this stain with an additional advantage of quick screening of sections made possible by the good contrast staining. As indicated earlier, however, the sampling of liver tissue has to be adequate before labeling a case as antigen negative. This fact has to be borne in mind while evaluating needle biopsy and autopsy material with regard to their antigen positivity. Shikata and co-workers [20] observed that livers from all cases with antigenemia at the time of autopsy were antigen positive. Presence of coat material in the cytoplasm of hepatocytes as revealed by its orcein positivity may indirectly indicate continued presence of the hepatitis B virus. Tapp and co-workers [34] found good correlation between presence of intranuclear core particles and the titer of HBAg in serum.

Mass screening of stored paraffin-embedded liver tissue, particularly samples obtained at autopsy, would therefore yield important information on the association of HBAg with various forms of acute and chronic liver diseases, including primary liver cancer. Preliminary studies carried out by us indicate that the antigen is observed not only in the nonneoplastic part of the liver in a very large proportion of primary liver cancers seen in this part of India, but also in the tumor cells themselves, though much less frequently [35] (also see Table 1).

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Acknowledgments

The authors are grateful to Asha Mittal and Lok Nath for technical asistance.

Legends for Figures

Figure 1—Positive immunofluorescence of intracytoplasmic HBAg in hepatocytes (*arrows*) shown in paraffin section of nontumorous cirrhotic liver in a case of primary hepatocellular carcinoma (× 600).

Figure 2—Section illustrated in Figure 1 stained with orcein after fluorescence microscopy. Note antigen inclusions (*arrows*) identical in site and morphology to those in Figure 1. (× 600)

Figure 3—Immunoperoxidase-positive HBAg in the cytoplasm of hepatocytes (*arrows*). Paraffin sections of cirrhotic liver counterstained with methyl green. (× 450)

Figure 4—Orcein-positive antigen (*arrows*) in section contiguous to that illustrated in Figure 3. Its identify of location and very close similarity of morphology in the two preparations is apparent. (× 450)

Figure 5—Intracytoplasmic peroxidase-positive HBAg in a group of hepatocytes from the nontumorous cirrhotic liver in a case of primary hepatocellular carcinoma. (Paraffin section, × 100)

Figure 6—Positive immunoperoxidase staining of HBAg seen predominantly in two morphologic forms—the dense, well-defined inclusions and lighter staining granular and fibrillar form. The latter is mostly distributed along the margins of the hepatocytes. Paraffin section of a cirrhotic liver counter stained with methyl green. (×450)

Figure 7—HBAg stained with orcein. All morphologic forms and inclusions—granular and fibrillar and vesciculotubular (*arrows*)—can be clearly made out in nonneoplastic hepatocytes from a liver with a primary hepatocellular carcinoma. (Paraffin section, × 450)

Figure 8—Inclusion form (*straight arrows*) and granular, short, fibrillary form (*curved arrows*) of HBAg. Note the fibrillary meshwork comprising the inclusion forms. Paraffin section of liver illustrated in Figure 7. (Orcein stain, × 1000)

Figure 9—Three inclusion forms of HBAg (*straight arrows*) in the cytoplasm of nonneoplastic hepatocytes in a tumor-bearing liver. **Inset** shows the vesiculotubular form of antigen (*curved arrows*). (Paraffin section, orcein stain, × 450)

Figure 10—Hematoxylin and eosin staining of the same sections illustrated in Figure 9, done prior to orcein staining. Inclusion forms of antigen are clearly seen (*straight arrows*) presenting identical location and morphology as in Figure 9. **Inset** shows the vesiculotubular form (*curved arrows*) identical to those seen in the inset of Figure 9. (× 450)

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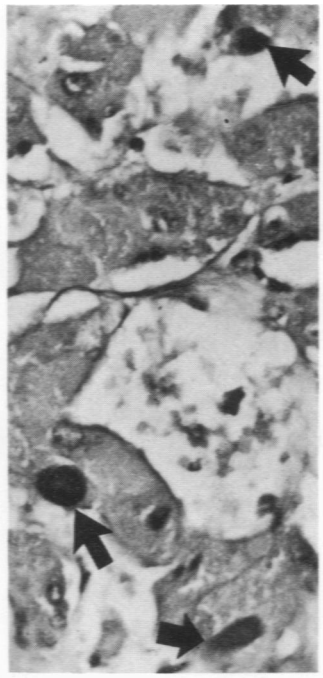

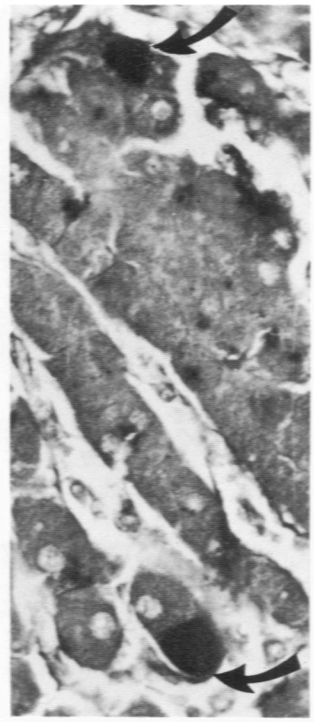

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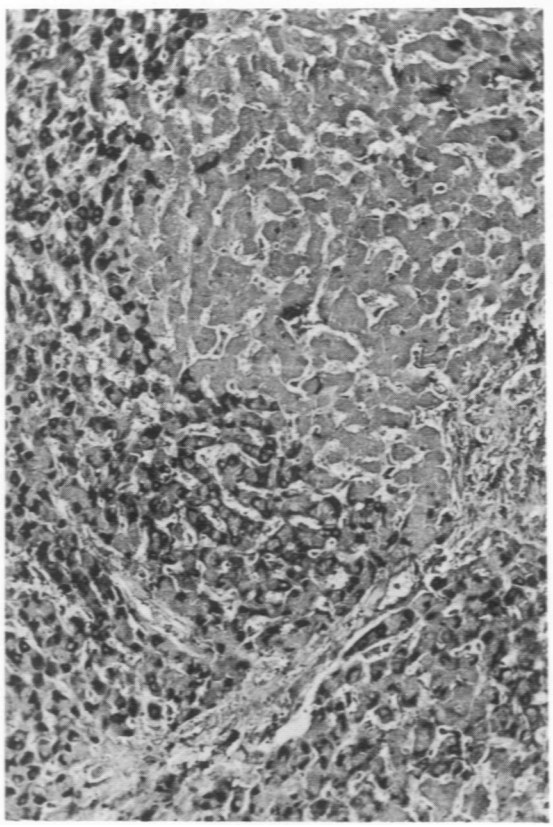

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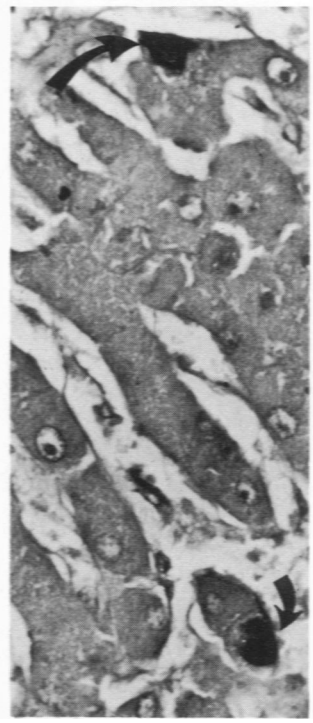

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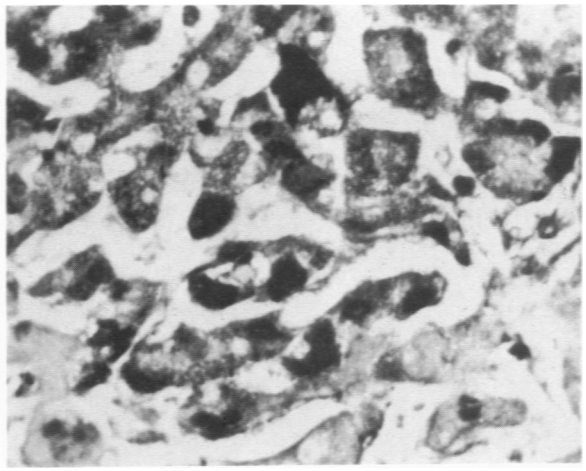

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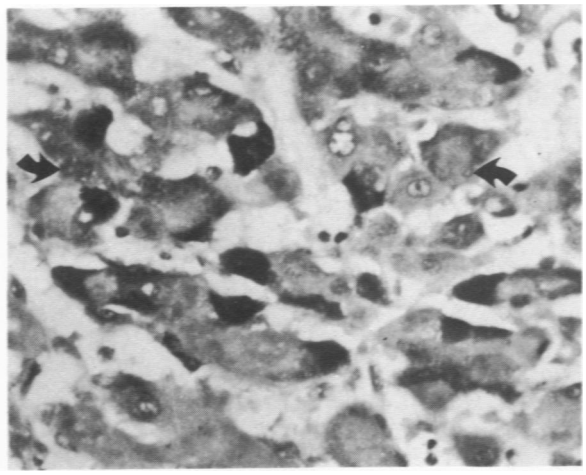

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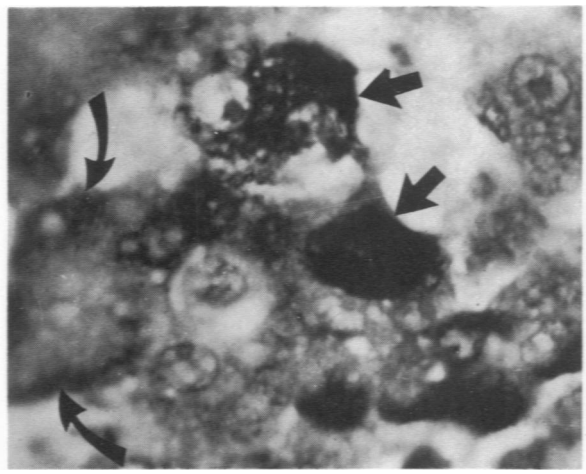

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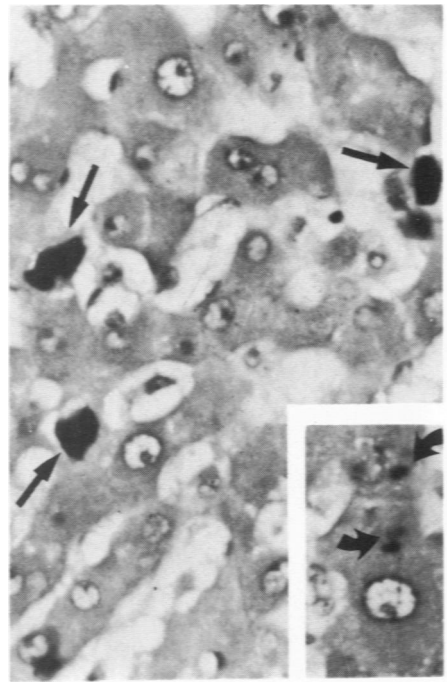

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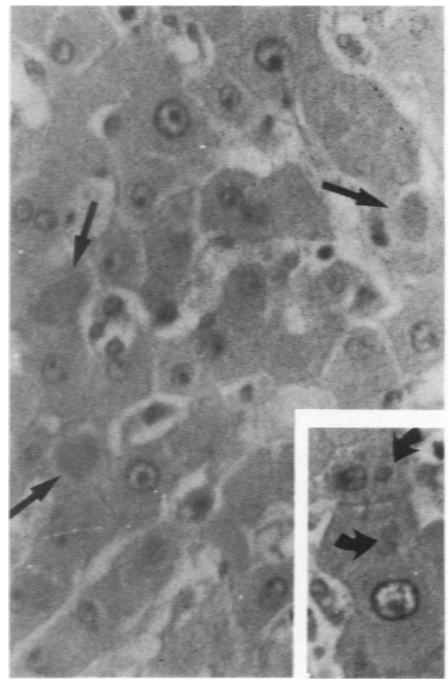

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