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INVESTIGATIONS OF LIVER BIOPSIES FOR AUSTRALIA ANTIGEN BY IMMUNOFLUORESCENT TECHNIQUE

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SUMMARY

134 liver biopsies obtained from 124 patients with various diseases of the liver were examined by the direct immunofluorescent technique for the Australia antigen. FITC-labelled IgG fractions prepared from three different sera containing antibody to the Australia antigen were used. Controls included FITC-labelled IgG fractions of sera containing antibodies against human immunoglobulins, complement factors and herpes simplex virus.

The three antibodies to the Australia antigen employed showed identical fluorescent patterns and reacted with the same biopsies. In 68 biopsies prepared and examined within 12 hr, speckled or granular fluorescence was noted in the nuclei of the hepatocytes in all cases irrespective of the clinical or histological diagnosis or the serological reaction for the Australia antigen. The antigen in the nuclei of the hepatocytes proved to be very labile.

It is concluded that IgG prepared from sera containing antibodies to the Australia antigen may have two antigenic specificities, one precipitating with the Australia antigen in serum and another reacting by immunofluorescence with a labile antigen which appears to be a constituent of normal liver cell nuclei.

INTRODUCTION

The association of the Australia antigen (Blumberg, Alter & Visnich, 1965) with viral hepatitis is now well established. The role of this antigen in the aetiology and pathogenesis of acute viral hepatitis, chronic hepatitis and cirrhosis of the liver, however, is still far from clear, although the accumulated evidence suggests that the Australia antigen is closely related to a hepatitis virus. This hypothesis is based primarily upon epidemiological investigations (Giles *et al.*, 1969; London, Sutnick & Blumberg, 1969) and it is supported by electronmicroscopic studies (Bayer, Blumberg & Werner, 1968; Almeida *et al.*, 1969) and,

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to some extent, by immunofluorescent studies (Millman *et al.*, 1969; Nowoslawski *et al.*, 1969; Coyne *et al.*, 1970). Using FITC-labelled immunoglobulins containing antibodies to the Australia antigen, Millman *et al.* (1969) demonstrated the presence of fluorescent granules in the nuclei of hepatocytes of four patients with acute hepatitis in whom the Australia antigen was detected in the serum, while such fluorescence was not observed in five patients without hepatitis and without the antigen in the serum. Similar results were obtained by Nowoslawski *et al.* (1969) in a study of livers examined at autopsy of six antigen positive and six antigen negative patients. Using an identical technique, Coyne *et al.* (1970) recently examined sixty-one liver biopsies from patients with various diseases. Fluorescence in the nuclei of the hepatocytes was observed in twenty-six patients with Australia antigen in the serum and in a further fifteen patients without detectable antigen in the serum. Evidence of previous hepatitis was not found in nine of these cases nor including patients with normal livers.

In the present report an attempt was made to detect the Australia antigen in 134 liver biopsies by the fluorescent antibody technique and using antibody to the antigen from different sources.

MATERIALS AND METHODS

Tissue examined, 134 liver biopsy specimens were obtained by Menghini's method from 124 patients. At the time of liver biopsy, a serum sample from each patient was tested for the Australia antigen by the micro-Ouchterlony technique as described by Prince (1968).

The following tissues were examined by the immunofluorescent technique: buccal smear cells (one case), skin and muscles (two cases), kidney (one case), isolated leucocytes (three cases) originating from three patients with positive serological reaction for the Australia antigen, rat livers, spleens, thymus, kidneys (two cases), and the same tissues from mice (two cases). All biopsies were prepared and fixed with acetone within a few hours as described by Millman *et al.* (1968). The slides were examined immediately or after storage at 4°C. In addition some of the biopsies were frozen at −70°C with dry ice and examined unfixed after cutting in a cryostat. Some biopsies, prepared as cell suspensions by Millman's method were also examined unfixed.

The antisera to the Australia antigen employed were (1) antibody from a patient with aplastic anaemia, who had received multiple transfusions, (2) antibody obtained 21 weeks after an attack of acute viral hepatitis from a patient who had not received transfusions, (3) antibody produced in a rabbit by immunization with Australia antigen purified by density gradient centrifugation in sucrose. These three antibodies showed identical precipitation lines with reference antisera kindly supplied by Dr D. J. Gocke, Dr A. Prince and Dr R. W. McCollum. The IgG fractions of these antisera were precipitated with 50% ammonium sulphate followed by chromatography in DEAE Sephadex. The purity of the IgG fractions was confirmed by immunoelectrophoresis. The IgG fractions which formed precipitation lines with the Australia antigen, were labelled with FITC (16 mg FITC /g protein, 25°C, 30 min). All biopses were examined against this panel of conjugates. The slides were incubated at room temperature for 30 min and then washed.

Treatment with DNA-ase and RNA-ase. Four biopsies with positive fluorescence reaction were treated with DNA-ase and with RNA-ase for 60 min at 37°C (0·5 mg enzyme/ml Wilbur's buffer) and subsequently washed with Coon's buffer.

Fluoresence microscopy. The slides were read within a few hours using a Leitz Zernicke microscope equipped with a Tiyoda carcoid condenser. An iodine quartz lamp was employed together with a primary interference filter (495 nm) with a high transmission of light (Rygaard & Olsen, 1969) and a secondary filter cutting off at 530 nm.

The histological examination was performed in the Institute of Pathological Anatomy, The Municipal Hospital, Copenhagen, by Professor Hemming Poulsen and Dr Per Christoffersen.

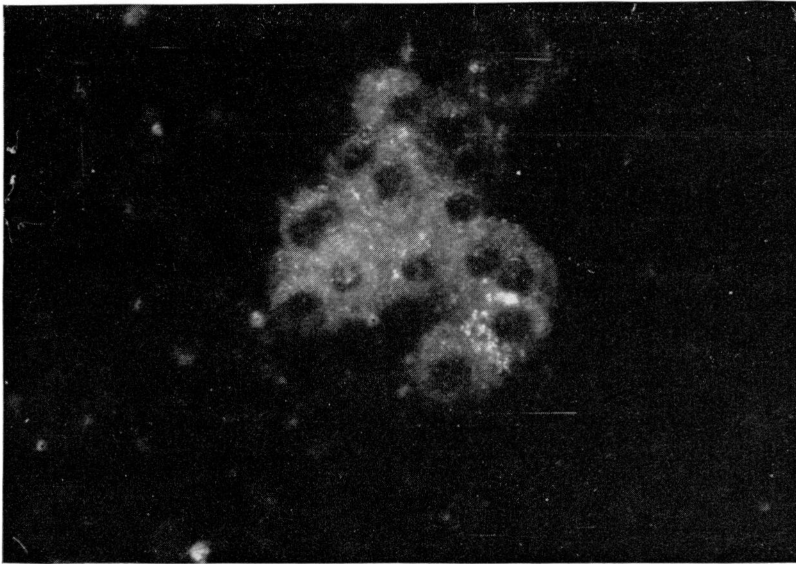

FIG. 1. Isolated hepatocytes after storage for 2 weeks and pretreatment with FITC-labelled anti-Australia-antigen immunoglobulin: slight cytoplasmic and none nuclear fluorescence.

RESULTS

The biopsies collected early in this investigation were frequently stored as described from 3 to 12 weeks before staining with the conjugates. Examination of such stored biopsies indicates that the antigen in the nuclei was very labile. If stored for a few weeks, most biopsies which exhibited positive fluorescence when tested immediately, frequently showed no fluorescence when retested 1–2 weeks later. Fluorescent granules were not found in any biopsies examined 8 weeks after storage at 4°C.

Fig. 1. Reproducible results were only obtained when biopsies were examined on the day they were taken. The results are shown in Table 1. These correspond to the results previously obtained by us in a small series of biopsies (Nielsen & Elling, 1970). All 68 biopsies examined immediately showed fluorescent particles localized in the nuclei of the hepatocytes irrespective of the clinical diagnosis, the histological findings or the serological reaction for the Australia antigen. Fig. 2a, b. Fluorescent particles were also found in four liver biopsies with normal histology. The three FITC conjugated antibodies to the Australia antigen reacted with the same biopsies and showed identical fluorescent patterns. In all specimens, nearly all the nuclei of the hepatocytes showed distinct granular or speckled fluorescence. The cytoplasm often showed a discrete granular fluorescence.

The nuclear particles resisted treatment with DNA-ase and RNA-ase. On one occasion similar fluorescent particles were found in the gastric epithelium of man, but not in the following human tissues examined: buccal smear cells, skin and muscles, kidney and leucocytes. Fluorescent particles were also found in rat livers and spleens, but not in rat thymus, kidney or in any tissues from mice examined immediately after death.

TABLE 1. The histologic diagnosis and the Au-seroreactions in relation to immunfluorescence in all 134 liver biopsies

Histological diagnosis	Total No. of biopsies	No. of biopsies† investigated after storage		No. of biopsies investigated immediately	
		Fluorescence positive	Fluorescence negative	Fluorescence positive	Fluorescence negative
Acute					
viral	Au(+)23	10	5	8	—
hepatitis	Au(0)51	15	10	26	—
Hepatitis	Au(+)—	—	—	—	—
sequelae	Au(0) 3	1	1	1	—
Chronic					
persistent	Au(+) 1	—	1	—	—
hepatitis	Au(0) 4	1	1	2	—
Chronic					
aggressive	Au(+) 3	1	—	2	—
hepatitis	Au(0) 4	2	2	—	—
Liver	Au(+)—	—	—	—	—
cirrhosis	Au(0)12	—	7	5	—
Mild					
inflammatory	Au(+)—	—	—	—	—
changes	Au(0) 6	—	2	4	—
Steatosis	Au(+)—	—	—	—	—
	Au(0) 9	—	2	7	—
Metastatic liver	Au(+)—	—	—	—	—
	Au(0) 5	1	1	3	—
Mononucleosis	Au(+)—	—	—	—	—
	Au(0) 2	—	1	1	—
Liver stasis	Au(+)—	—	—	—	—
	Au(0) 1	—	—	1	—
Sarcoidosis	Au(+)—	—	—	—	—
	Au(0) 1	—	—	1	—
Extrahepatic					
bileduct	Au(+)—	—	—	—	—
obstruction	Au(0) 3	—	—	3	—
Normal livers	Au(+)—	—	—	—	—
	Au(0) 6	—	2	4	—

† Stored 1–7 weeks, after 8 weeks storage no fluorescence were observed in any of the biopsies.

Thirteen liver biopsies which showed positive fluorescent reaction if examined immediately after the biopsies were obtained did not show any fluorescence when FITC-labelled antisera against human immunoglobulins were used. Similarly, two liver specimens from patients with hepatitis did not show any fluorescence with FITC-labelled antisera against human beta I-C-globulin or herpes simplex virus. In two cases (not recorded in the table), fluores-

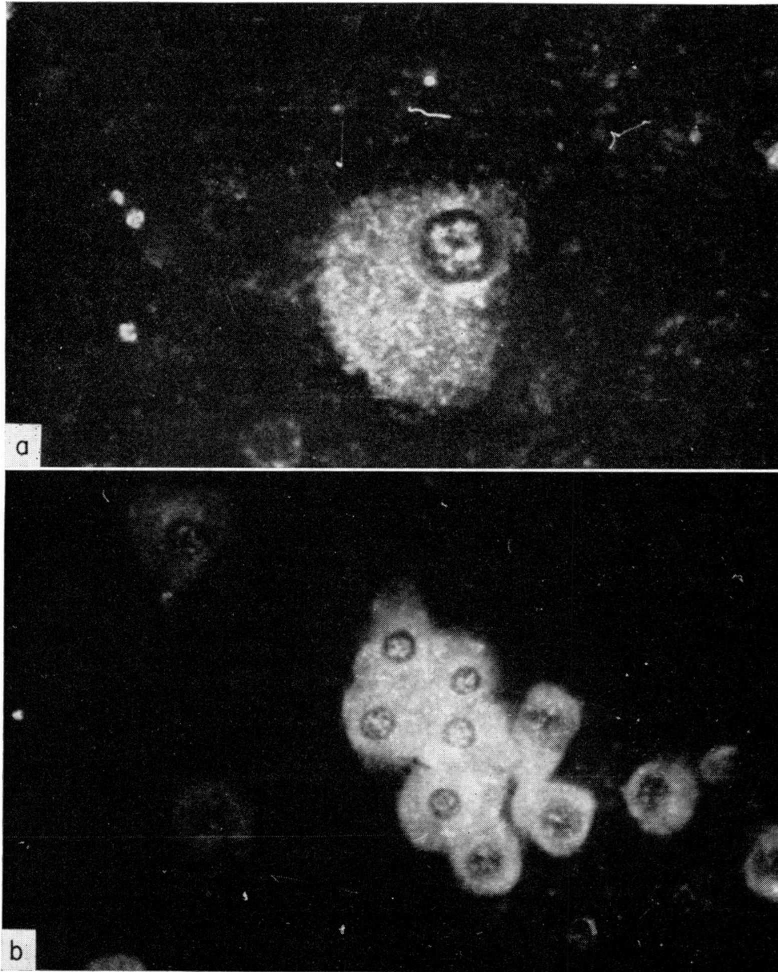

FIG. 2a, b. Isolated hepatocytes treated with FITC-labelled anti-Australia antigen-immunoglobulin: fluorescence particles in nuclei and cytoplasm.

cent particles were found in liver specimens when FITC-labelled antisera against human IgM were used. These liver biopsies were derived from patients with Felty's syndrome.

DISCUSSION

The results of these studies indicate that IgG fractionated from sera containing antibody to

the Australia antigen may have two antigenic specificities. One precipitating with the Australia antigen in serum and another reacting by immunofluorescence with a labile antigen which appears to be constituent of normal liver cell nuclei. The question whether the precipitating and the fluorescent antigen are identical has not yet been resolved. Identity is suggested, however, since the same patterns of fluorescence were obtained with three different antibodies.

The fluorescent pattern obtained may result from a liver specific antinuclear antibody not related to the antibody to the Australia antigen, though occurring simultaneously in the serum. There is some evidence to support this view. Thus, Elling & Faber (1968) have reported a high incidence of speckled antinuclear antibodies in patients with acute hepatitis and with infectious mononucleosis. This antinuclear antibody reacted almost exclusively with liver cell nuclei. Speckled antinuclear antibodies have also previously been found in sera from animals subjected to experimental immunological liver cell-damage (Popper *et al.*, 1963). Similarly, the lability of the fluorescent nuclear particles to freezing and thawing is in accordance with the biochemical characteristics described for the speckled liver specific nuclear antigen, and differ from the stability of the Australia antigen.

However, attempts so far to absorb this antinuclear factor with isolated liver nuclei have failed. Furthermore, is it difficult to explain why our rabbit anti-Australia-immunoglobulin showed identical fluorescent patterns with the human anti-Australia-immunoglobulins, since this presupposes the existence of a speckled nuclear antigen in purified Australia antigen used for immunization.

Coyne *et al.* (1970) have demonstrated a significant association between the presence of fluorescent particles in liver cells and the presence of Australia antigen in serum and the diagnosis of viral hepatitis. The finding of fluorescent particles in livers from patients without hepatitis and from patients with normal livers was explained on the basis that it might be difficult to exclude previous subclinical hepatitis. However, our results show clearly that fluorescent particles are present in all human livers and that these are not related to the serological or histological diagnosis. Our findings may be attributed to lability of the nuclear antigen which is present in the hepatocytes.

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