Localization of Liver Antigens Produced by Poisoning with Alpha-Naphthyl-Isothiocyanate.* (32262)

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It has been shown that alpha-naphthyl-isothiocyanate (ANIT) causes a progressive hyperplasia of the bile ductures in the rat's liver(1,2). More recently we demonstrated that the liver of ANIT fed rats contains additional antigens which are not present in the normal rat's liver(3). The purpose of the present work was to try to localize these antigens in the liver of ANIT fed rats by immunohistochemical methods.

Material and methods. Twenty albino rats of a random stock bred at the Hebrew University were used in this experiment. A group of 10 rats was fed on a low riboflavin diet to which 0.1% ANIT was added, and another group of 10 rats received a low riboflavin diet only, and served as a control (3).

The animals were sacrificed by decapitation 28 days after the beginning of the experiment. Residual blood was removed from the livers by perfusion with cold buffered saline. The livers of each group were pooled. Part of each liver was taken for regular histology and immunohistochemical studies, and the rest was processed according to Baldwin's method(4) to obtain a cell sap, which was stored at −20°C until used.

Preparation of antisera. Eight rabbits were divided into 2 equal groups. Four animals were immunized with the liver sap of the ANIT fed rats and 4 with liver sap of the control group. Each rabbit received, at weekly intervals, 4 intradermal injections of 20 mg liver cell sap protein in complete Freund's adjuvant. Ten days after the last injection, the animals were bled and their sera preserved at −20°C.

The individual sera were tested separately by a modification of the double diffusion technique of Ouchterlony, as described previously(3). Sera displaying a good antibody response, were absorbed as described according to Asherson *et al*(5) by normal rat liver sap to obtain a specific antiserum to the ANIT induced liver antigen.

The following antisera were used for fluorescent studies: 1. Rabbit-anti ANIT rat liver sap, absorbed with normal liver sap (R.A.A.R.L.); 2. Rabbit-anti-normal rat liver sap (R.A.N.R.L.); 3. Fluorescein isothiocyanate conjugated goat anti-rabbit globulin (Fl.G.A.R.G.), obtained from Microbiological Associates, Bethesda, Md.

Immunohistochemical studies. Liver blocks from both groups of rats were fixed in a mixture of ethanol and dry ice and were kept at −20°C, until used. Frozen sections, 4-5 μ thick, were prepared in an International Harris cryostat. The sections were fixed for 10 minutes in 50% ethanol at room temperature, then for 20 minutes in 96% ethanol at 37°C. They were stained by the sandwich technique, first with either R.A.A.R.L. or R.A.N.R.L., and after thorough washing in buffered saline, with Fl.G.A.R.G. Other sections were stained directly with serum No. 3 only.

Results. Fig. 1 illustrates immunodiffusion studies carried out with an antiserum against liver of ANIT fed animals in the center well (to the left), which reacted with the ANIT liver sap in the top well and the normal liver sap in the lower well (to the right). One can clearly see one, and in some animals, 2 additional bands with the liver sap of the ANIT fed animals as compared with the controls. After absorbing the ANIT liver antiserum with normal liver cell sap, the precipitin bands obtained with the normal liver sap disappeared completely, and with the ANIT liver sap one, and sometimes 2 precipitation lines were present. (Fig. 2).

After 28 days of ANIT feeding the histo-

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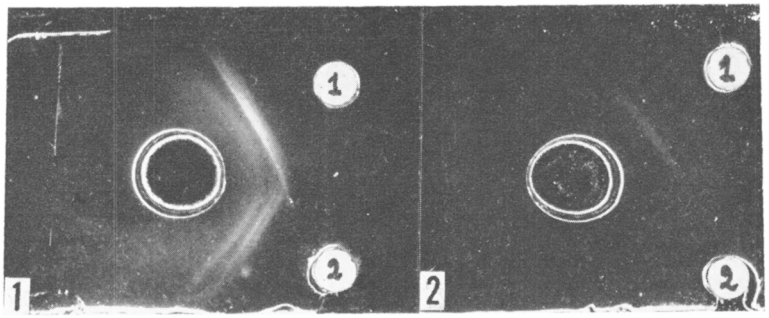


FIG. 1. Double diffusion in agar. Large well contains anti-ANIT serum. Well 1 contains ANIT liver and Well 2 control liver. Note additional lines at Well 1.

FIG. 2. Same arrangement as in Fig. 1. except that the anti-ANIT serum was absorbed by normal liver sap.

logical picture of the liver was as follows (Fig. 3): The lobular structure was preserved. In the portal spaces there was an abundance of bile ducts of different sizes, which showed a tendency to infiltrate in between the liver cells and here the usual cuboidal epithelium of the cells became flattened. The connective tissue was not increased in quantity as compared to the normal liver.

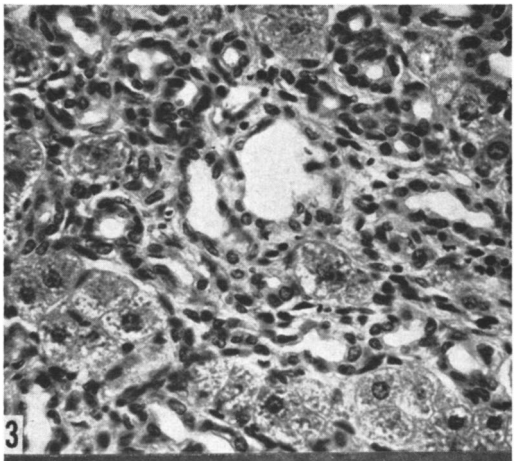
Sections of livers of rats fed with ANIT stained by the sandwich technique with the absorbed rabbit anti ANIT rat liver (R.A.A.R.L.) and the fluorescent goat anti rabbit globulin (Fl.G.A.R.G.) sera, showed a bright green-yellowish fluorescence of the basal membrane of the bile ducts (Fig. 4). Staining of the sections with Fl.G.A.R.G. alone did not show any fluorescence. Sections of normal livers stained with R.A.A.R.L. and Fl.G.A.R.G. gave a very faint fluorescence of the basal membrane of a few large bile ducts. No fluorescence was seen when these sections were stained with Fl.G.A.R.G. only. Sections of ANIT livers and normal control livers stained by the sandwich technique with R.A.N.R.L. and Fl.G.A.R.G. gave identical results, in both instances a bright fluorescence of the nuclei of the liver cells was observed (Fig. 5).

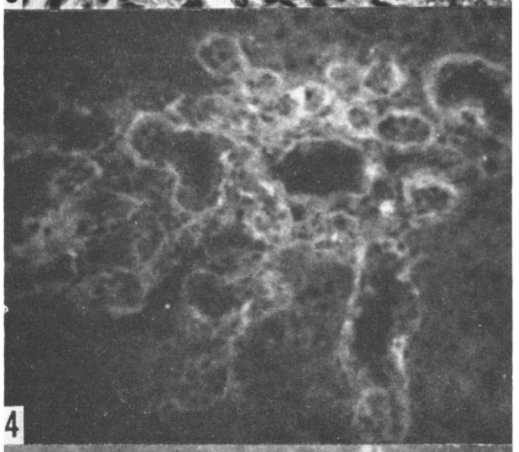
Discussion. From these experiments it is evident that rats which develop bile duct hyperplasia as a result of continuous ANIT feeding, develop one or two additional antigens which cannot be detected by the same immunological methods in the normal liver. Using the immunofluorescent technique, it was possible to show that these antigens are located in the basal membrane of the hyperplastic bile ducts of the ANIT liver.

This finding may indicate that ANIT modifies the antigenicity of the rat liver and thus elicits, in the rabbit, an immune response which cannot be obtained with the normal liver. Another possibility to be considered is that the extensive proliferation of the bile ducts caused by ANIT, increases the quantity of a ductular antigen which is also present in the normal liver but in an amount too small to elicit an immune response in the rabbit.

The presence of faint fluorescence in the basal membrane of some bile ducts of the normal rat liver stained with the antiserum against ANIT liver, indicates that the basal membrane of the biliary ducts of both the ANIT treated rat liver, and the normal rat liver may have some common antigenicity. From the above experiments it is impossible to determine whether these antigens are identical or cross react with the basal membrane antibody found in the sera of rabbits immunized with liver of ANIT treated rats.

It is of interest to note that the antiserum to normal liver contains antinuclear antibodies, as shown by the fluorescence of the nuclei of both the normal and ANIT treated rat livers, when this serum was applied on them. A similar observation was made by Green and Ghose(6) who obtained perinuclear staining of the parenchymal rat-liver





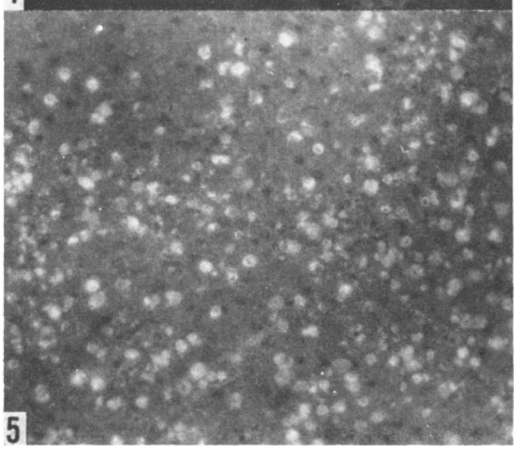


FIG. 3. Histology of liver after 28 days of ANIT feeding. Note abundance of bile ducts.

FIG. 4. Frozen section of rat liver after 28 days of ANIT feeding, stained by the sandwich technique with rabbit anti-ANIT rat liver and fluorescent goat anti rabbit globulin. The basal membrane of the bile ducts is clearly stained.

FIG. 5. Frozen section of normal liver stained by the sandwich technique with rabbit anti normal rat liver and fluorescent goat anti-rabbit globulins. Note nuclear staining.

cells with a rabbit antinormal rat-liver serum.

Summary. Rats that received alphanaphthyl-isothiocyanate (ANIT) in their food developed intrahepatic bile duct hyperplasia. Immunochemical studies revealed additional antigens in the ANIT treated liver compared to that of the normal liver. These antigens were shown to be localized in the basal membrane of the intrahepatic bile ducts.

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