THE EFFECT OF INTERMITTENT ADMINISTRATION OF ALPHA-NAPHTHYL ISOTHIOCYANATE TO RATS

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It was reported in previous experiments that the administration of a single dose of alpha-naphthyl isothiocyanate (ANIT) to rats caused acute necrotizing cholangitis and obstruction of the interlobular bile ducts accompanied by biochemical evidence of severe obstructive jaundice. The biliary obstruction subsided within 1 to 2 weeks as a result of recanalization of the obstructed bile ducts and early proliferation of bile ductules.[1-3] If ANIT was given continuously, fibrous organization of the exudate in the bile ducts could be observed at 1 to 3 weeks after the beginning of the experiment. After this period, despite the continuous feeding of ANIT, the jaundice gradually disappeared[1,4] and bile drainage was restored by recanalization of the fibrous obstruction; also manifest was abundant bile ductule hyperplasia in the portal and intralobular areas.[1,3,5,6]

Among several obscure points in the understanding of the action of ANIT in these experiments were the occasional presence of jaundice without complete obstruction of the intrahepatic bile ducts and the absence of acute inflammation during the long-term experiment. In addition it was considered desirable to determine whether the marked ductular proliferation was due to metabolic changes related to the obstructive jaundice or to the direct cholangioplastic action of ANIT. The present experiments were performed in an attempt to elucidate some of these problems.

MATERIAL AND METHODS

One hundred and fifty-five male albino rats of a random stock bred at the Hebrew University were used. The rats weighed between 150 to 200 gm. at the beginning of the experiment and were maintained on Purina Laboratory Chow (Ralston Purina Company) and tap water *ad libitum*. Alpha-naphthyl isothiocyanate (ANIT) was administered intermittently over a period of 16 weeks, one week of treatment being alternated with weekly intervals free of treatment. A 16 per cent solution of ANIT in neutralized olive oil was given by mouth once daily, in a dose of 100 mg. per kg. of body weight. Groups of animals were sacrificed by exsanguination at the end of each week of ANIT administration and at the end of each treatment-free interval. Sixteen

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groups of animals (5 to 24 in each group) were thus available for histologic and biochemical study.

In a further group of 5 animals the above schedule of treatment was continued to the end of 21 weeks (i.e., 11 alternate weekly periods of ANIT administration). One other group was sacrificed after a treatment-free interval of 1 month following the eighth course of ANIT.

Sixty additional untreated rats served as controls for the biochemical examinations.

The following blood examinations were performed: total and direct bilirubin,[7] total cholesterol [8] and alkaline phosphatase.[9]

For histologic examination liver tissue was fixed in Zenker-acetic acid solution, and paraffin sections were stained with hematoxylin and eosin, the McManus periodic acid-Schiff (PAS) procedure controlled by saliva digestion for 1 hour at 37° C., and by the Feulgen technique followed by Mallory's aniline blue-orange G stain.

Results

Body Weight

At the end of the first acute course of ANIT poisoning the animals lost an average of 25 to 50 gm. in weight (about 10 to 20 per cent). Following the first treatment-free interval the animals returned to their initial weight. A lesser loss of weight (15 to 20 gm.) was observed at the end of the second course of ANIT administration. A restoration in body weight was again observed after the second treatment-free interval. Each of the later courses of weekly administration of ANIT produced a minimal loss of weight. During the treatment-free intervals the animals grew and developed normally.

Biochemical Results

The results of the biochemical examinations are shown in Text-figure 1.

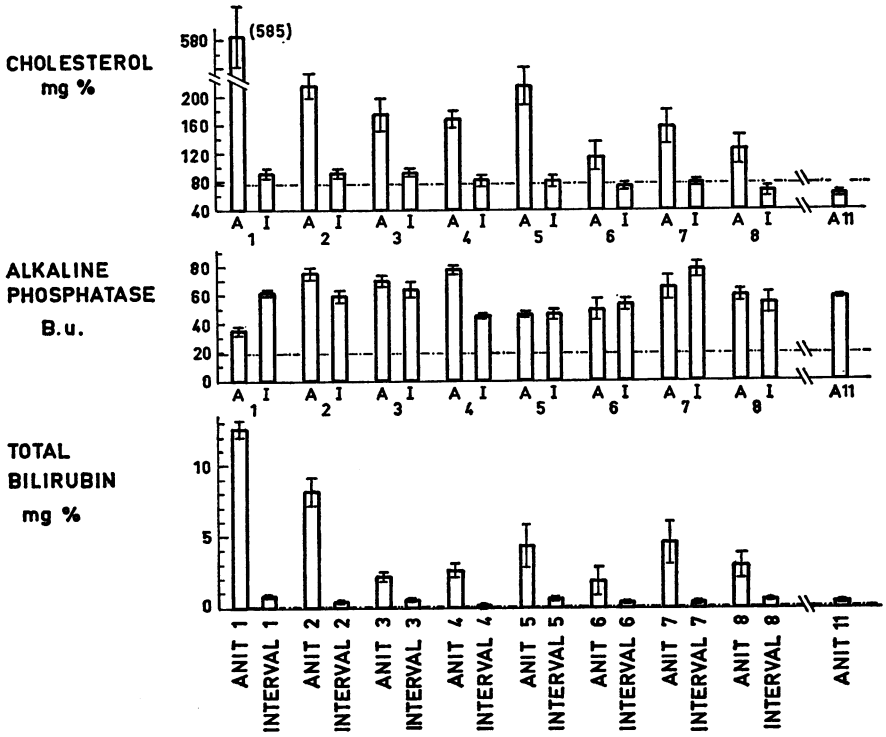
The average value of the total bilirubin was found to be very high at the end of the first period of treatment (12.6 ± 0.6 mg. per 100 ml. of serum). After the first treatment-free interval the average value had dropped to a near normal level (0.8 ± 0.09 mg.). The second course of ANIT resulted in a less severe hyperbilirubinemia (8.2 ± 1.0 mg.). With each consecutive period of administration of ANIT the total bilirubin values rose moderately (1.8 mg. to 4.5 mg.) in comparison to the rise following the first and second courses of treatment. During the treatment-free intervals the total bilirubin values repeatedly returned to within normal limits. The direct fraction of bilirubin constituted about 70 to 80 per cent of the total bilirubin at the end of all periods of treatment.

The average value of total cholesterol was very high (585 ± 43 mg. per 100 ml. of serum) after the first course of ANIT administration. Each of the following courses of ANIT treatment produced comparatively lesser increases to values varying from 215 ± 15 mg. per 100 ml.

of serum. The treatment-free intervals were accompanied by average values within the normal range (63 to 90 mg. per 100 ml. of serum).

Following the eleventh period of treatment there was no rise in total bilirubin or total cholesterol levels.

In the animals sacrificed one month after the eighth period of treatment, normal bilirubin and cholesterol values were observed.



TEXT-FIG. 1. Total serum bilirubin, alkaline phosphatase and cholesterol mean values following weekly, intermittent administration of ANIT to rats. The dotted lines represent the average values in control rats. A = end of the one-week courses of ANIT. I = end of the treatment-free intervals.

Serum alkaline phosphatase was elevated at the end of the first period of treatment, attaining a level of 35 ± 3 Bodansky units (B.u.) as compared with the normal of 19 B.u. In contrast to the bilirubin and cholesterol levels, the values of alkaline phosphatase continued to rise during the first treatment-free interval and reached an average of 62 ± 2 B.u. High levels of alkaline phosphatase (46 to 78 B.u.) were observed during the whole period of the experiment, and there were no constant differences between the levels at the end of the treatment and treatment-free periods (Text-fig. 1). High values were also obtained after the eleventh course of ANIT (58 ± 1 B.u.) and one month after

the eighth course (64 ± 5 B.u.) in the absence of hyperbilirubinemia and hypercholesteremia.

Histologic Findings

At the End of the First Two Acute Courses. Signs of acute cholangitis were found in many portal areas. Large bile ducts contained necrotic epithelium, inflammatory cells and PAS-positive amorphous material (Fig. 1). The portal tracts also showed a considerable number of small bile ducts with narrow lumens (Fig. 2). These lesions were observed with irregular distribution and were of varying intensity in the individual animals. Obstructive cholangitis was, however, constantly severe in the hilum of the liver and the adjacent large portal tracts (Fig. 3). These signs of severe acute inflammation almost obscured the outlines of the biliary ducts. The walls of the arteries in the portal tracts were thickened and exhibited hyaline areas and endothelial swelling.

The intralobular bile ductules were slightly increased in number and showed acute inflammation and edema. Focally their epithelium was swollen or missing (Fig. 4). Throughout the liver parenchyma there were scattered small foci of recent necrosis adjacent to the inflamed portal and intralobular bile ductules. A scanty leukocytic infiltration was seen about these areas (Fig. 4).

At the End of the First Two Treatment-free Intervals. The main hepatic ducts in the hilum were replaced by a considerable number of small bile ducts with patent lumens which formed a labyrinthine pattern (Fig. 5). Their epithelium was flat and irregular. These ducts were surrounded by concentric layers of fibroblasts and adult fibrous tissue. At the end of the treatment-free intervals bile ducts in the portal areas showed a marked regression of the inflammatory process. Their number was slightly increased and their general appearance was similar to that seen in the liver hilum. In some areas their lumens were narrowed. The presence of dilated lymphatics in the portal spaces was a prominent finding at this stage (Fig. 6). Extensions of small bile ductules into the liver lobules were observed. These intralobular ductules were slender, narrow, compressed by edema and accompanied by cells with oval hyperchromatic nuclei which in many instances gave the impression of being connected with the bile ductular epithelium (Fig. 7). The liver cells were of normal appearance.

After the Repeated Courses of ANIT Administration. The hilar and portal bile ducts showed progressive organization of the inflammatory process and periductal fibrosis. Their lumens were narrowed and lined by regenerated hypertrophic epithelium with large hyperchromatic and irregular nuclei (Fig. 8). These ducts were surrounded by small satellite bile ductules. In the intralobular bile ductules an inflammatory re-

action accompanied by progressive proliferation was a constant finding in animals sacrificed after the third to the eighth courses of ANIT (Fig. 9). There were only rare small foci of recent liver cell necrosis in the vicinity of the intralobular bile ductules.

However, in the animals examined after the eleventh course of ANIT administration, inflammation was entirely absent, the main picture being the extensive proliferation of the intralobular ductules, which were patent and lined by flat regular epithelium (Fig. 10). The liver cells were normal.

At the end of the later (third to eighth) treatment-free intervals, moderate fibrotic narrowing of the portal bile ducts and an increasing number of intralobular bile ductules were constantly seen. The inflammatory reaction had regressed. The lumens were patent and the lining epithelium flattened with pyknotic nuclei, often resembling "oval cells" (Fig. 11). With each successive interval between the acute weekly courses of ANIT these changes in the intralobular bile ductules became progressively more impressive. The proliferated ductules formed convolutions enmeshed in fibrillary connective tissue and extended throughout the liver parenchyma. At the end of the eighth treatment-free interval, many hepatic lobules were encircled and penetrated by convolutions of bile ductules with the formation of pseudolobules (Fig. 12).

However, 3 weeks later (i.e., 1 month after the eighth course of ANIT treatment) a marked involution of the biliary hyperplasia was seen. In the areas in which bile ductular hyperplasia had been present, clusters of "oval cells" were found (Fig. 13). At this stage there was no regression of the changes in the large portal bile ducts.

Discussion

In these experiments, biliary obstruction due to acute cholangitis was observed at the end of the first and second periods of ANIT administration, and the changes were identical to those described in earlier articles.[1,3] In addition, it was observed that the lesions were predominantly situated near the hilum of the liver just before the hepatic ducts merged into the common bile duct. This peculiar localization of the obstruction was overlooked in our earlier studies in which intrahepatic obstructive cholangitis was not constantly demonstrated despite the presence of jaundice. The preponderance of obstruction in the hilum appeared to be the main cause of the severe obstructive jaundice seen at the end of the first and second periods of ANIT administration. Obstruction of the larger hepatic ducts by organized exudate did not, however, appear to be the sole cause of the jaundice. In the later stages of the experiment, after obliteration of the hilar and interlobular bile ducts

had been relieved by new formation of satellite ductules, milder jaundice was observed. It was likely that the inflammation observed in the small intralobular bile ductules at this stage was responsible for the jaundice.

The absence of epithelial necrosis and of inflammatory reaction in the interlobular bile ducts when acute intermittent poisoning with ANIT was renewed after the first two weekly intervals of recovery, suggested that the regenerated epithelium in these had at this stage acquired resistance to the ANIT. A similar process of acquired resistance of regenerated epithelium to repeated injury, when separated by periods of recovery, has been observed in liver cells following administration of toxic drugs at intervals long enough to allow regeneration.[10]

It appears that the adaptation of the biliary epithelium to cholangiotoxic effect of ANIT is a gradual process and is delayed in the smaller intralobular ductules in which inflammatory exudate was observed at periods when lesions in the interlobular bile ducts had ceased to recur. Eventually, at the end of the eleventh period of ANIT intoxication, proliferation of the small intralobular ductules was present in the complete absence of inflammation or edema. Accordingly, normal values of total bilirubin and total cholesterol were found.

While the interlobular bile ducts had acquired resistance towards the phlogistic and necrosis-producing effect of ANIT, repeated courses of administration continued to stimulate proliferation of the intralobular bile ductules. The finding of biliary obstruction in the acute periods of the experiment had lent support to the assumption that the cholangioplastic effect of ANIT in the initial stages was the result of metabolic changes related to bile stasis,[11,12] as is believed to occur following obstruction[13] or experimental ligation[14] of the common bile duct. The progressive proliferation of bile ductules encountered in the ultimate stage, in the absence of inflammatory reaction, hyperbilirubinemia or hypercholesteremia, suggested an independent growth-stimulating effect of ANIT or of some as yet unknown metabolite of this agent.

The continuously elevated values of the serum alkaline phosphatase may be regarded as an expression of regurgitation during the periods of jaundice.[1,2] Through most stages of the experiment the elevated values of the enzyme might have been the result of overproduction by the active hyperplasia of biliary epithelium.[1,2,15] At the end of the later treatment-free intervals it was apparent that one week was not sufficient to allow involution of the bile ductular hyperplasia induced during the repeated intermittent periods of ANIT administration. At the end of one month free of treatment, however, following 8 intermittent weekly periods of ANIT administration, there was an almost complete involution of the tremendous bile ductule proliferation observed at the end of the eighth

"acute course." On the other hand, periductal fibrosis about the interlobular bile ducts was still present. The reversibility of ANIT-induced bile ductular hyperplasia was also observed in the long-term experiments reported by Cameron, McLean and Prasad[16] who found regression of the process beginning as early as 2 weeks after withdrawal of ANIT.

The finding, one month after the cessation of long-term intermittent ANIT administration, of sheets of oval cells in the areas in which the bile ductular hyperplasia had been seen, suggested that these cells might represent involutional forms of the earlier bile ductular proliferation. The elevated values of alkaline phosphatase at this period, in the absence of biochemical or histologic evidence of bile retention, probably indicated that the involutional cells were able to produce alkaline phosphatase as in the case of biliary epithelium during periods of proliferation.[15]

Summary

Alpha-naphthyl isothiocyanate (ANIT) was given to rats in weekly courses interrupted by equal treatment-free intervals allowing for recovery of the lesions. The cholangiotoxic effect of ANIT on the interlobular bile ducts was only observed after the first two courses of treatment. The most severe changes were found in the hepatic hilar area.

With repeated intermittent administration, the regenerated epithelium of the interlobular bile ducts was found to be resistant to the toxic agent. This was observed to be correlated with a lessening icterogenic effect of the drug.

Proliferation of intralobular bile ductules was found to be progressive and reversible. A direct cholangioplastic effect of ANIT, independent of bile stasis, was suggested.

A constantly raised level of serum alkaline phosphatase was observed throughout the experiment and was thought to be due mainly to overproduction by proliferating ductular epithelium.

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The photomicrographs were made by Mrs. Hannah Weinmann and Mr. H. Reuveni.

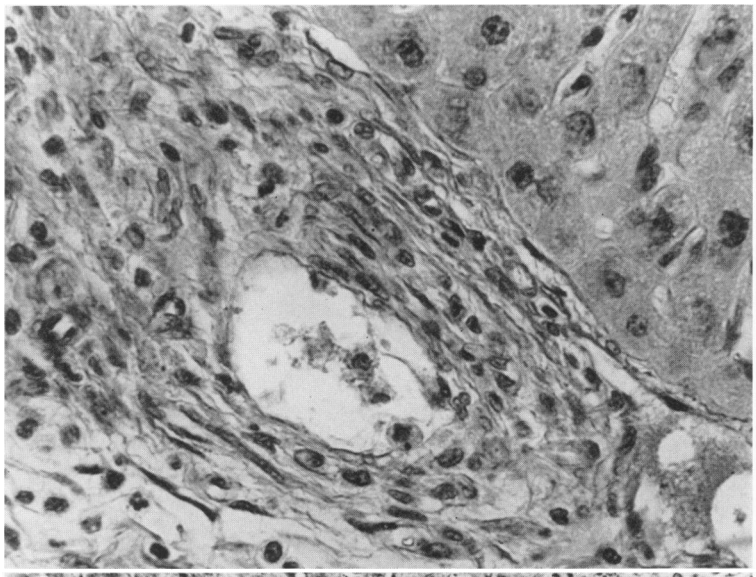
LEGENDS FOR FIGURES

Photomicrographs were prepared from sections stained with hematoxylin and eosin. Figures 1 to 4: End of first course of ANIT.

FIG. 1. Acute cholangitis in a portal area. A large bile duct in the center exhibits desquamation of its lining epithelium. The lumens contain necrotic material. × 530.

FIG. 2. Numerous small satellite bile ducts appear in the portal tracts. × 160.

FIG. 3. An intense acute and chronic cholangitis is encountered in the liver hilum. × 170.



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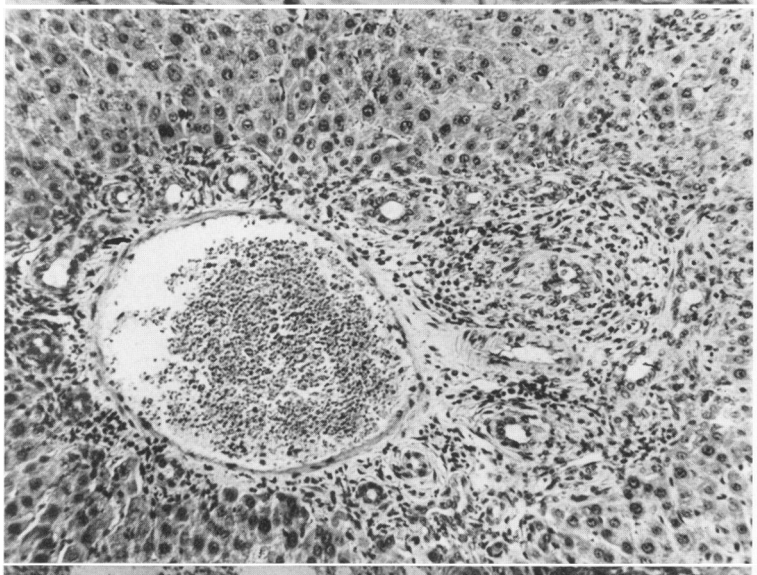

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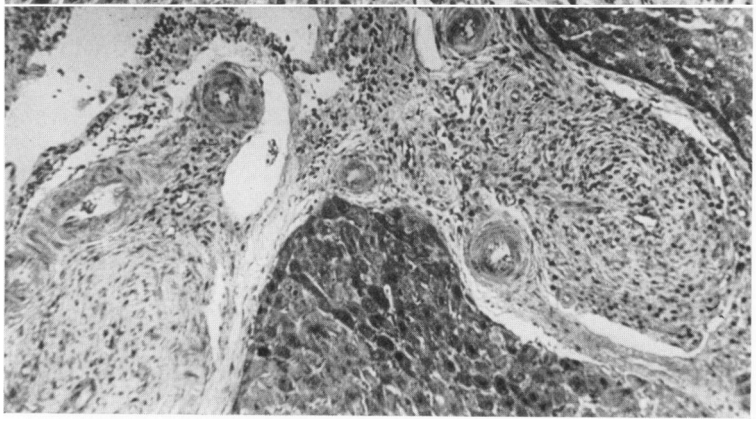

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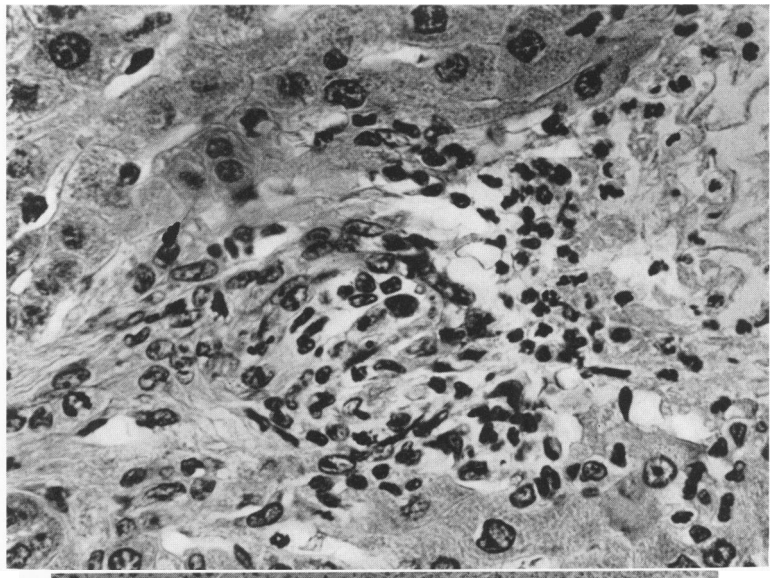

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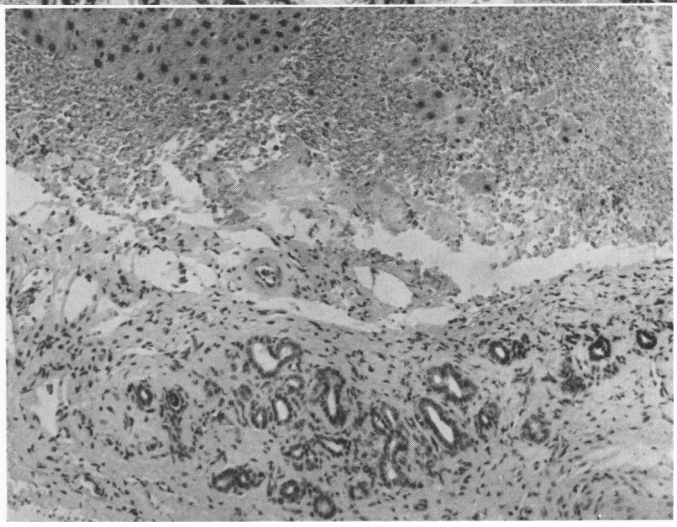
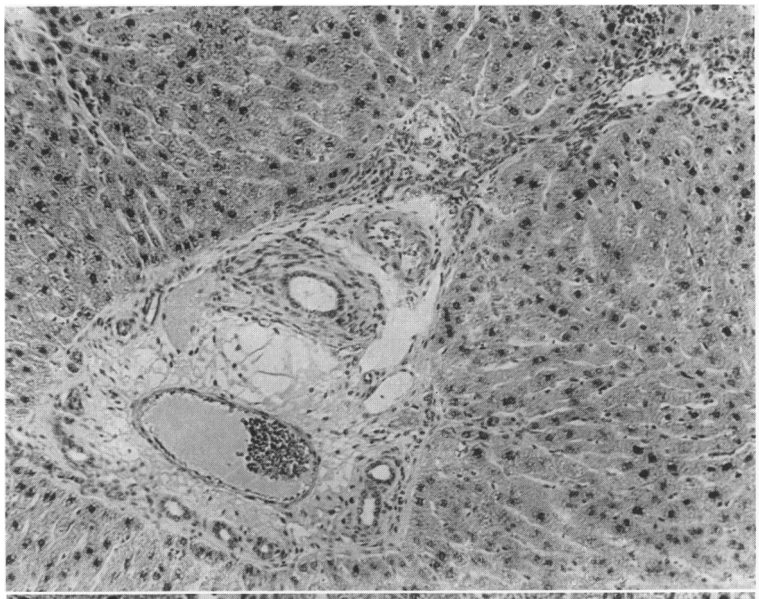


FIG. 4. Intralobular bile ductules are slightly increased in number and are surrounded by edema and acute inflammation. The epithelium is swollen and the lumens are narrow. Nearby there is recent liver cell necrosis. × 530.

Figures 5 to 7: End of the first and second treatment-free intervals.

FIG. 5. The hilum of the liver exhibits regression of the inflammatory reaction. Increased numbers of bile ducts have patent lumens and are surrounded by mature fibrous tissue. × 130.



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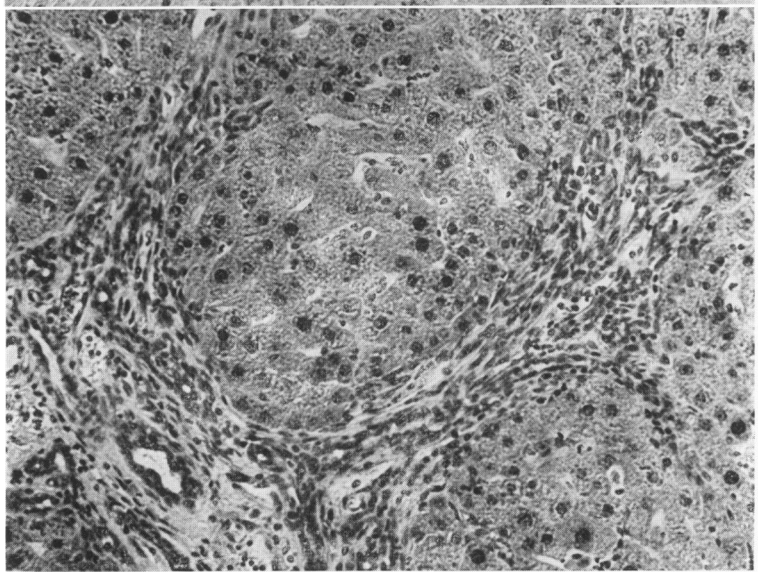

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FIG. 6. The portal tracts show complete re-epithelization of the bile ducts which are surrounded by fibrous "collars." Extending from the portal space, small bile ductules accompanied by oval cells penetrate the liver lobules. × 160.

FIG. 7. Intralobular extensions of bile ductules. Oval cells with hyperchromatic nuclei appear in relation to the ductules. × 240.

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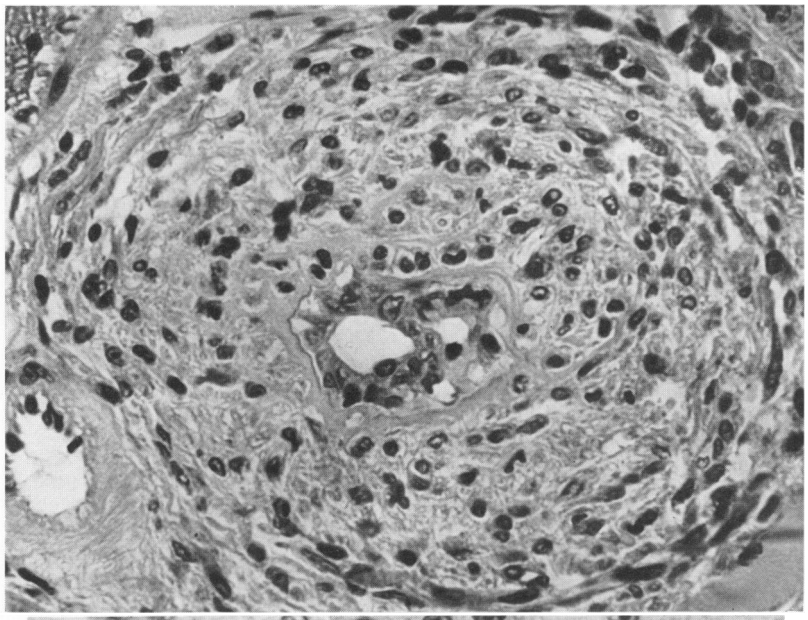

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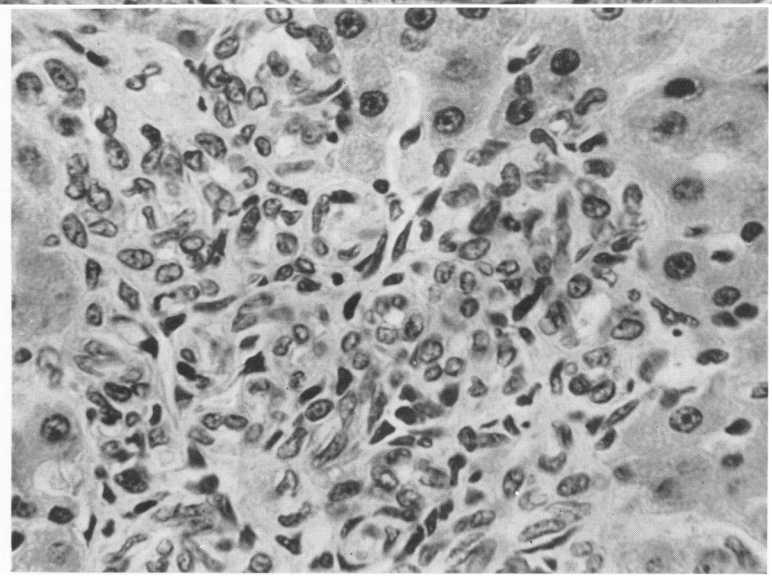

Figures 8 and 9: End of the fourth course of treatment.

FIG. 8. Periductal fibrosis is evident in a portal space. The duct lumen is narrowed and lined by regenerated epithelium. × 560.

FIG. 9. Intralobular bile ductular proliferation is associated with an inflammatory reaction. × 630.

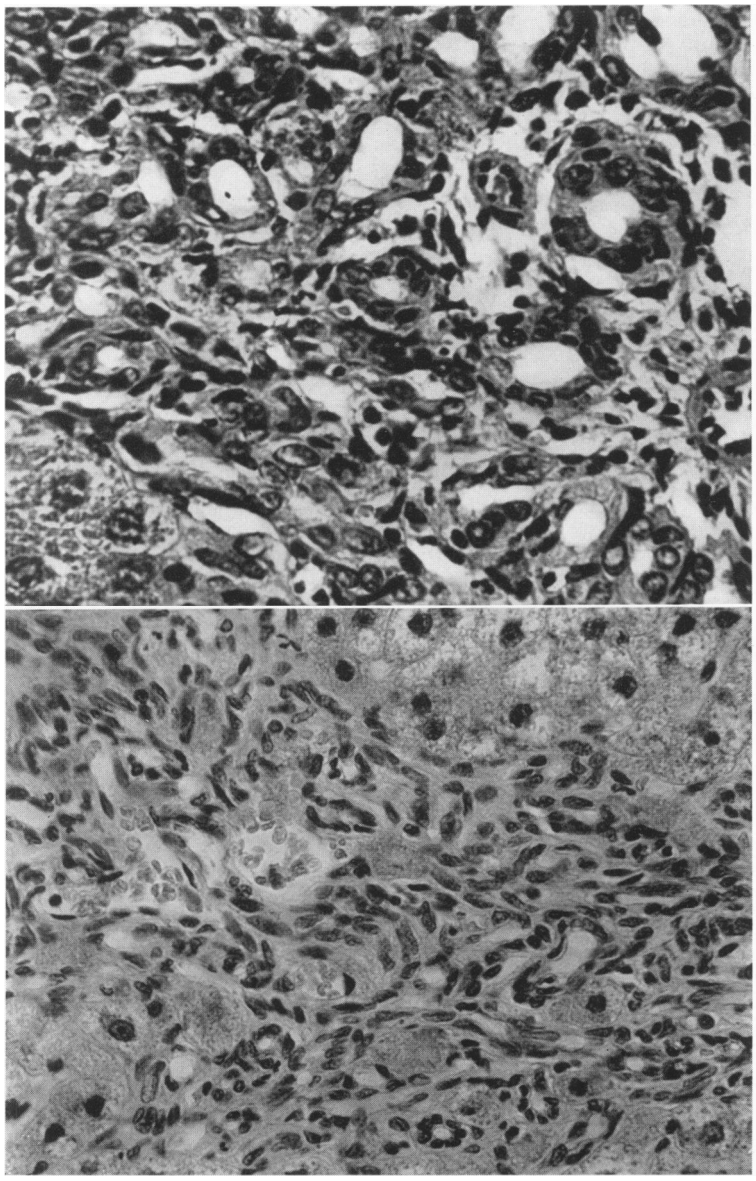

FIG. 10. At the end of the eleventh course, the multiplied intralobular bile ductules have patent lumens. Signs of inflammation are lacking. × 630.

FIG. 11. End of the fifth interval. Intralobular bile ductules are multiplied, and their patent lumens are often lined by flattened epithelium. The inflammatory reaction has regressed. × 420.

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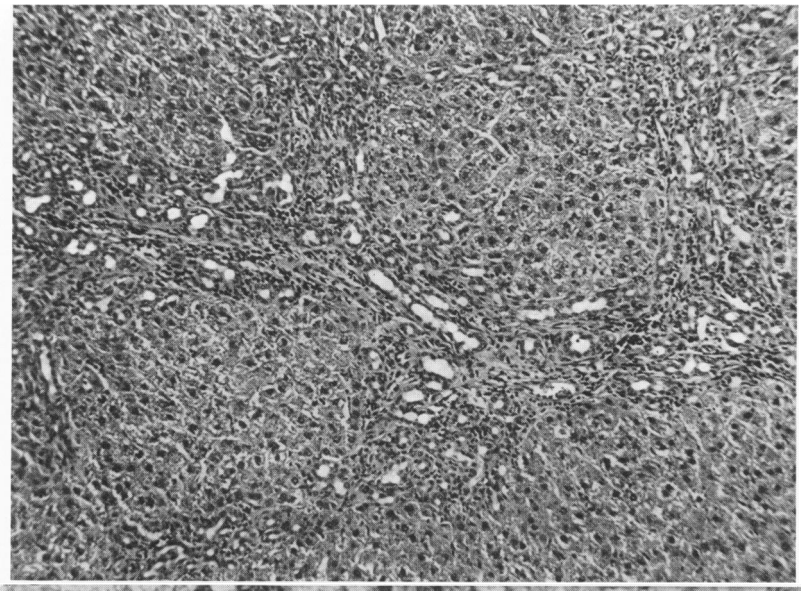

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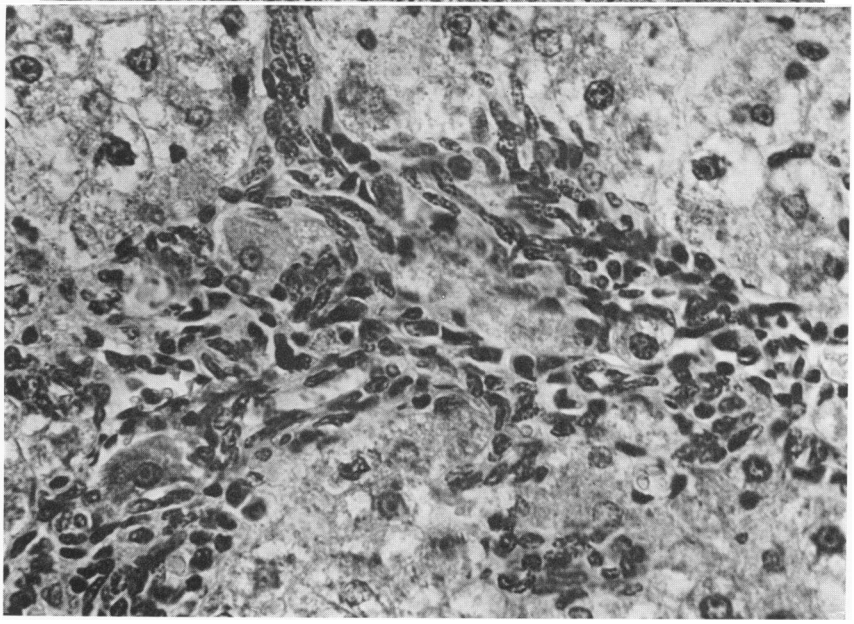

FIG. 12. End of the eighth interval. Marked intralobular bile ductular proliferation is manifest. Enmeshed in a delicate fibrillary connective tissue, convolutions of bile ductules extend and penetrate the liver parenchyma and form pseudolobules. × 100.

FIG. 13. One month after 8 weekly intermittent courses of ANIT treatment. Intralobular bile ductules are replaced by clusters of "oval cells." × 530.