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Experimental induction of biliary cystadenoma in rats: a morphological study

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Summary. This paper presents the experimental results obtained in the hepatic parenchyma of Wistar rats subjected to a combined treatment with a hepatic neoplasia inducing agent (N-Nitrosodienthylamine) and a promotor (Phenobarbital), which gave rise to a neoplastic process classed as biliary cystadenoma.

Key words: Cystadenoma, Liver, Rat, N-Nitrosodiethylamine, Phenobarbital, Histology, Ultrastructure

Introduction

The use of nitrosamines in experimental carcinogenesis, irrespective of whether they are associated to promoters like phenobarbital, usually leads to the development of hepatic neoplasia. The induction of biliary cystadenomas is relatively rare, and few reports of its structural and ultrastructural features are to be found in the literature. The cystadenoma is a benign proliferative epithelial neoplasm, generally of a cystic or polypoid nature (Short et al., 1971). It most commonly occurs in the intrahepatic bile ducts and less frequently in the extrahepatic bile ducts and the gallbladder (Ishak et al., 1977; Beretta et al., 1986).

The origin of this neoplasia has not been precisely determined though various hypotheses have been advanced. It has been suggested that they may originate in certain anomalies in the development of the liver, the cysts occurring as a result of malformations of the bile ducts (Short et al., 1971; Ishak and Rabin, 1975; Ishak et al., 1977). In additon to congenital anomalies, certain acquired causes have been adduced, including the use of oral contraceptives (Suyama et al., 1988) and the administration of B_1 aflatoxin to Sprague-Dawley rats (Cruickshank and Sparshott, 1971).

Bannasch and Reiss (1971) and Bannasch and Massner (1976) suggest that the formation of biliary cystadenomas consists of three separate phases. In the first phase, necrosis of various areas of hepatic parenchyma is accompanied by a proliferation of mesenchymal cells and ducts. The onset of mucous cholangiofibrosis in the second phase is followed in the third by the development of two types of benign neoplasm: cystadenoma (or cystic cholangioma) and cholangiofibroma. Cystadenoma development is accompanied by a series of cellular changes including the flattening of mucosecretory cylindrical cells and the interruption of mucous substance production.

The carcinogenic capacity of N-Nitrosodiethylamine at hepatic level has been reported by various authors (Kelly et al., 1966; Windholz, 1983), and hepatocarcinomas have been experimentally induced in rats by intraperitoneal administration of 200 mg/kg live weight (Ogawa et al., 1979). Thomas (1961), however, detected development of cholangiocellular neoplasia when using this substance.

The role of phenobarbital has been examined by a number of authors, some of whom consider it to be a promoter exclusively in the liver (Peraino et al., 1973; Devita et al., 1988). The combined action of this drug with N-Nitrosodiethylamine has been found to increase the formation of hepatic neoplasia (Weisburger et al., 1975).

The purpose of this study was to analyse the histological, histopathological and ultrastructural changes induced in the hepatic parenchyma of Wistar rats by the combined administration of N-Nitrosodiethylamine and phenobarbital, this last one as promoter.

Materials and methods

72 Wistar rats, of average weight 150 g (females) and 250 g (males) were used for this experiment. Animals were housed in individual cages, in optimum light and temperature conditions. Food and water were administered ad libitum throughout the experiment.

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Animals were divided into three groups, according to the treatment received:

Group I: Control group, consisting of 18 animals receiving no treatment at all.

Group II: 18 animals receiving phenobarbital (500 ppm/Kg liver weight), dissolved in drinking water, throughout the duration of the experiment.

Group III: 36 animals receiving two intraperitoneal injections of N-Nitrosodiethylamine dissolved in sterile physiological serum. The two doses (200 mg/Kg live weight) were separated by a 7-day interval. One week after the second injection, animals received phenobarbital following the schedule used for Group II.

Animals were divided into six homogeneous batches, which were slaughtered after 4, 6, 8, 10, 11 and 13 months of treatment. Livers were extracted for histopathological examination, and samples were taken for histological and ultrastructural analysis.

Liver samples for histological analysis were fixed in 10% buffered formol, processed using routine light microscopy techniques, and finally embedded in paraffin. 5 μm sections cut from the blocks thus obtained were stained with H-E, PAS, Mayer mucicarmine, Van Gieson and Gomori reticulin stains.

Samples for TEM analysis were fast-fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer, and then washed. After postfixing in phosphate-buffered 2% osmium tetroxide, samples were dehydrated through a graded acetone series (Sabatini et al., 1963), immersed in propylene oxide and successive mixtures of propylene oxide and Araldite, and finally embedded in pure Araldite. Sections 60 nm thick were counterstained using 2% uranyl acetate and lead citrate.

Results

Gross examination

On gross examination, the livers of group I (control) and II (phenobarbital treatment) animals were of normal appearance. In group III livers, however, lesions were observed from 6 months onwards. These affected a third of the batch slaughtered at 6 months, and took the form of small cysts with a smooth whitish-yellow outer covering, which tended to protrude onto the liver surface. Cysts contained a fluid of a similar colour. At this stage, cysts were distributed in fairly small numbers throughout the hepatic parenchyma.

As the duration of treatment increased, lesions became progessively more apparent, affecting an increasing number of animals from each batch (Fig. 1); lesions were thus most manifest in the final batch (13 months' treatment), in which 100% of animals were affected. No significant differences were recorded between sexes as regards the onset and evolution of the process.

In order to determine whether cysts were related to the biliary tract, a Chinese ink solution was perfused through the common hepatic duct of three animals; the solution spread through the bile-duct system and the fluid within the cysts assumed a blackish-blue colouring (Fig. 2).

Histological analysis

Light microscopic analysis revealed parenchyma of totally normal appearance in groups I and II. Alterations were observed, however, in group III parenchyma after four months' treatment. Initial changes took the form of widespread and diffuse foci of cell proliferation (Fig. 3). Cells tended to form canalicular structures located at various sites within hepatic lobules. They were composed of simple cuboid

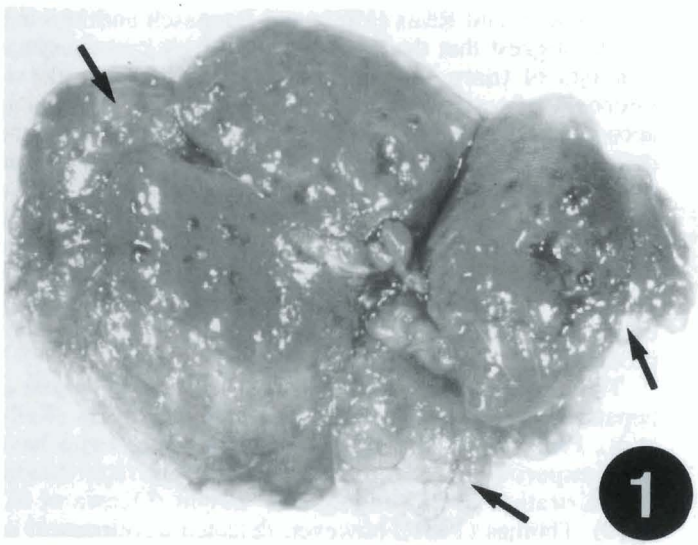


Fig. 1. Group III (8 months). Multiple neoplastic cyst formations affecting a large area of hepatic parenchyma (arrows).

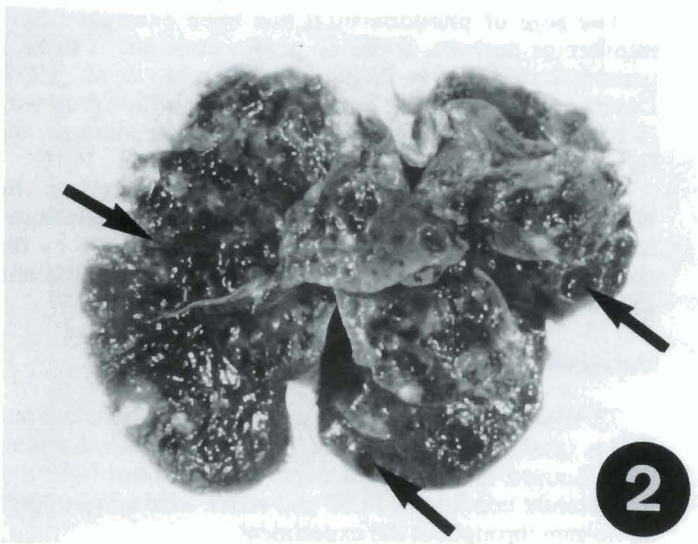


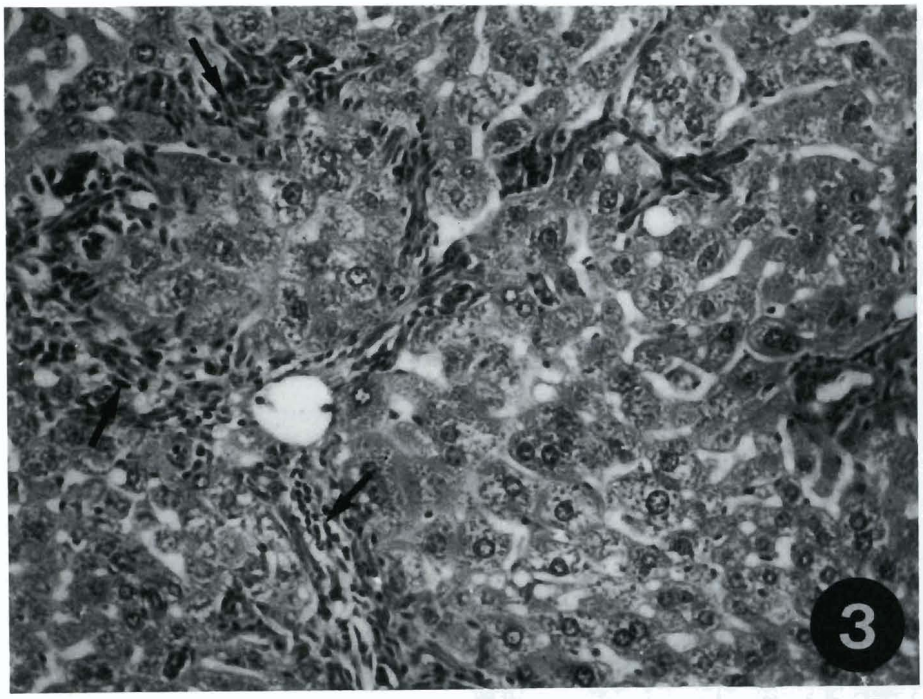
Fig. 2. Group III (11 months). View of the cyst formations after perfusion of Chinese ink (arrows).

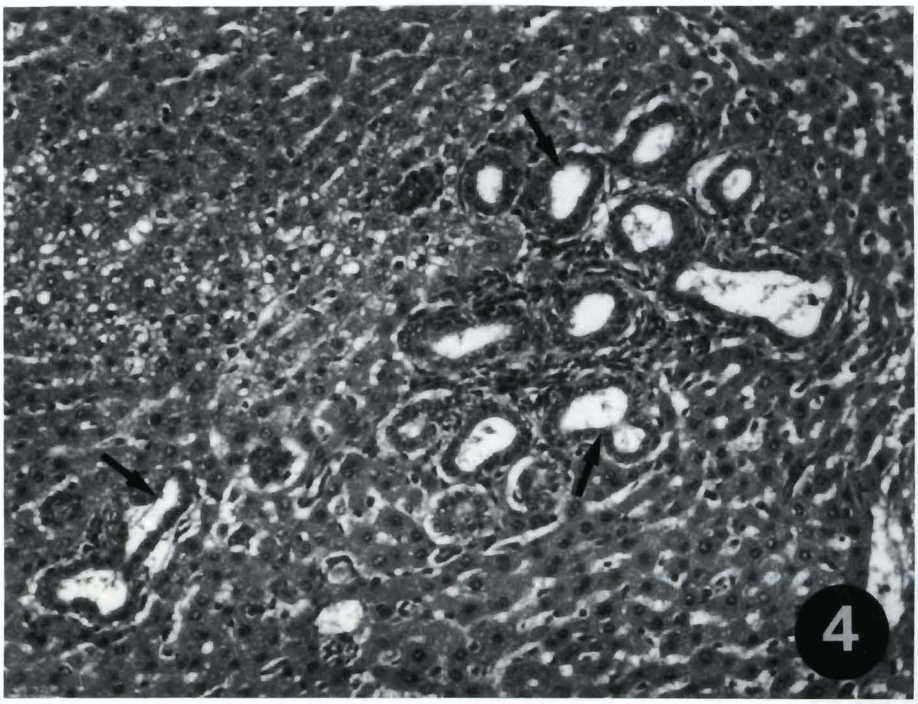
epithelium (Fig. 4).

From 6 months onwards, these structures gradually evolved to form cysts or polycysts, consisting of a sinuous lumen of varying width lined by a simple epithelium varying in appearance between columnar, cuboid and flat. Epithelium was surrounded by connective tissue made up of a fine network of collagen and reticulin fibres (Fig. 5).

The cell population within the connective stroma comprised spindle cells with vesicular nuclei, spheroid cells with pyknotic nuclei, some eosinophils and a certain number of hepatocytes which tended to form small, diffusely-distributed clusters. Both the fluid contained within the cystic cavities and the inner epithelial lining of cyst walls stained negative to PAS and Mayer mucicarmine.

Observation of the hepatic parenchymas from all six group III batches revealed that all animals presented epithelio-proliferative lesions. These lesions were the only finding at 4 months p.i.. After six months, lesions were accompanied by cystic and polycystic lesions characteristic of biliary cystadenomas. 25% of group III animals were affected at 6 months, 45% at 8 months, 66% at 10 months, 75% at 11 months and 100% at 13 months of treatment.





Ultrastructural analysis

No alterations were recorded in the hepatic parenchyma of group I or group II animals. Indeed, there was a striking absence of changes in the biliary tract in the latter group. Alterations observed in animals treated with N-Nitrosodiethylamine were compatible with the histological findings described earlier.

Cells proliferating in parenchyma after fourth months treatment shared similar characteristics in all cases; though slightly irregular in shape, cells tended towards a roughly cuboid appearance and contained a large nucleus which accounted for a large proportion of cell volume, the amount of cytoplasm thus being relatively small. Nuclei were manifestly irregular in shape. Cells were surrounded by a delicate network of collagen fibres (Fig. 6).

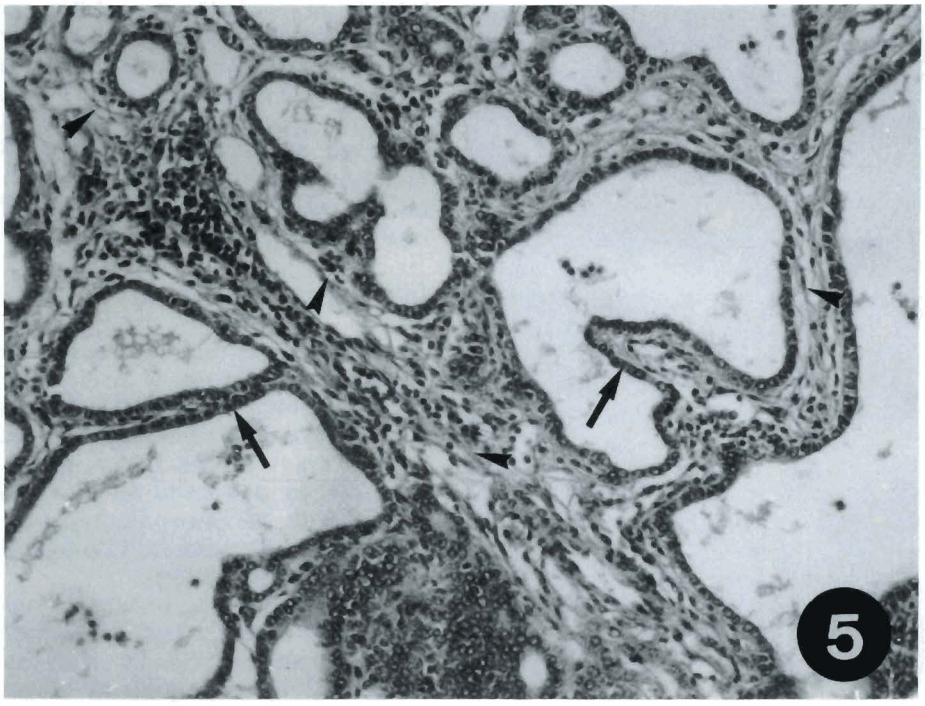
A further change concerned the formation of canalicular structures. These were composed of a simple cuboid epithelium, in which cytoplasm was largely occupied by an irregular-shaped nucleus. Short projections protruded from the apical border into the lumina which it

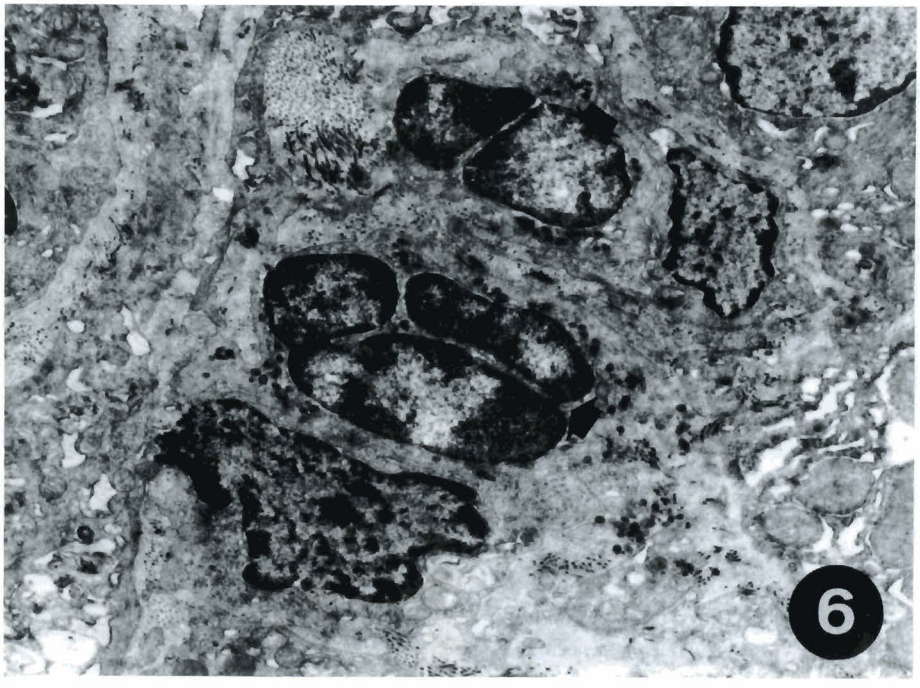
Fig. 3. Group III (6 months). Extensive cell proliferation distributed diffusely through hepatic parenchyma (arrows). H-E x 320

Fig. 4. Group III (8 months). Numerous canalicules with highly dilated lumen, lined with simple cuboid epithelium (arrows). H-E x 270

delimited. Lumina were of varying size. The basal border was supported by a delicate basement membrane with an underlying connective stroma (Fig. 7).

Light microscopic analysis of cysts developing from 6 months onwards showed the cyst wall to be composed of a simple epithelium and an underlying connective stroma. Epithelial cells varied in type (columnar, cuboid and flat) (Figs. 8, 9). In all cases, short, irregular projections were observed at the apical border, protruding into cyst lumina. Invaginations and evaginations were evident at the basal border. Desmosome-type contact sites were observed at lateral borders. The nucleus accounted for a large proportion of cell volume in all cases. Columnar epithelium nuclei were ovoid and fairly regular (Fig. 8), becoming less regular as epithelial cells became flatter (Fig. 9). Vacuoles were only evident, in large numbers, in the cytoplasm of columnar cells.





In all cases, a connective stroma was observed below the basement membrane surrounding the epithelial lining; the stroma consisted mainly of amorphous ground substance, numerous bundles of collagen fibres and a cell population consisting largely of undifferentiated elements, mast cells (Fig. 10), spindle cells with elongated nuclei, similar in appearance to fibroblasts, and wandering cells. (Fig. 11).

Discussion

The use of N-Nitrosodiethylamine as a hepatic carcinogen has been reported by various authors (Kelly et al., 1966; Ogawa et al., 1979; Windholz, 1983), as has the role of phenobarbital as promoter (Weisburger et al., 1975; Watanabe and Williams, 1978). Ogawa et al. (1979) induced hepatocarcinomas in Fischer rats by administering a single dose of 200 mg/kg live weight; in the present experiment, 2 doses of that order were administered with a 7-day interval, with the result that after 13 months biliary cystadenomas were found in 100% of experimental animals. It is felt that this neoplasm process may partly be due to the protocol employed. which sought to determine the cumulative effect of the carcinogen, aggravating its effect in the liver.

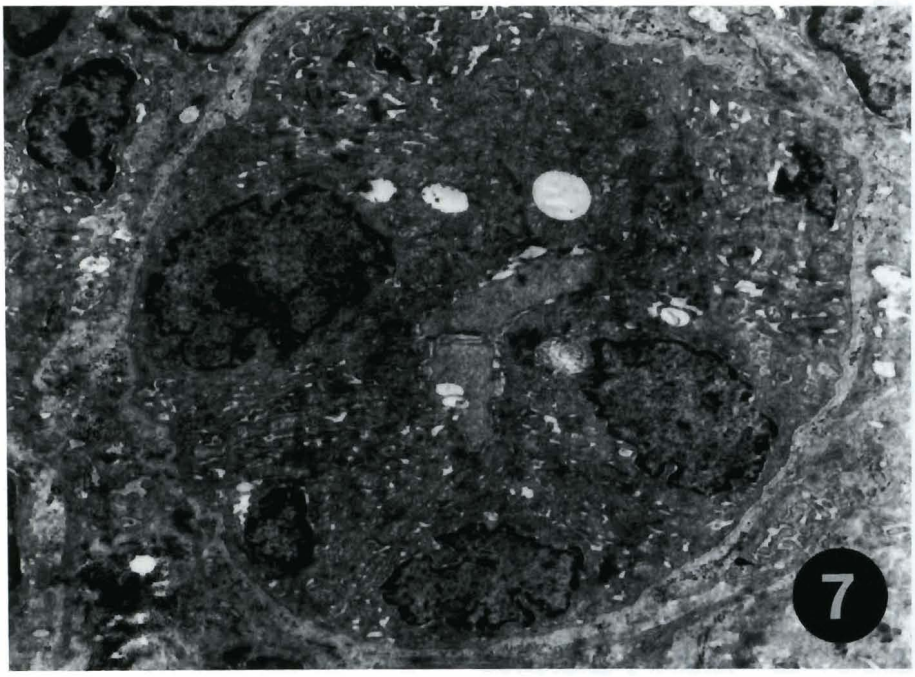
Changes taking place in the hepatic parenchyma were monitored

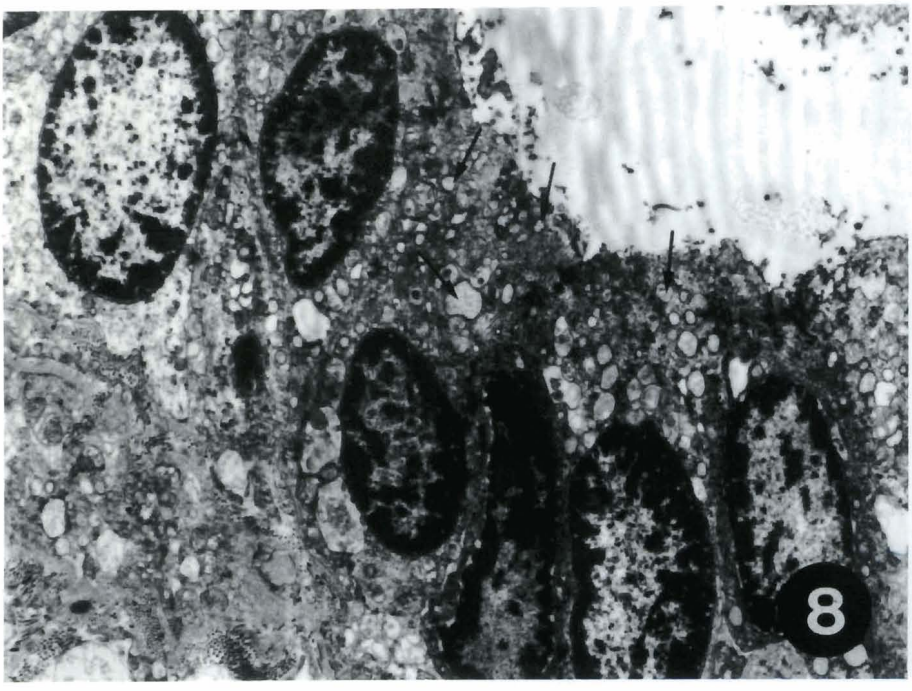
Fig. 5. Group III (11 months). Cyst walls showing two clearly differentiated components: simple epithelium (arrows) and underlying connective stroma (arrow tips). Van Gieson. x 270

Fig. 6. Group III (4 months). Ultrastructural aspect of proliferating cells, showing large nucleus (arrows) of manifestly irregular morphology. x 8,000

at two-month intervals (except for one batch), from the onset of epithelio-proliferative changes up until the development of the cystic lesions characteristic of this type of neoplasm, in order to attempt to establish its origin. Cystadenomas are true cystic neoplasms of the liver; they are biliary in origin, and not related to parasitic lesions (Marcial et al., 1986). The development of this lesion involves a series of preneoplastic and neoplastic changes of a proliferative nature; this type of neoplasm can thus be classified as an epithelial proliferative process, with a cystic or polypoid presentation (Short et al., 1971).

Though it has been reported that human cystadenomas are predominantly located in the right lobe (Ishak et al., 1977; Lewis et al., 1988), no evidence was found in the present study of any preferential location for cysts. In most cases, cysts were distributed randomly throughout the liver, in both the facies diaphragmatica and the facies visceralis. Anatomopathologically, cystic processes were observed to be both uniloculate and multiloculate; these findings coincide with those reported from several sources (Corrin, 1962; Frick and Feinberg, 1982).





Cystadenoma walls comprised two clearly differentiated components: an epithelial lining and a connective stroma. The epithelium was in all cases simple, as reported elsewhere by various authors (Kokal et al., 1983; Edwards et al., 1987; Beretta et al., 1988). No stratified epithelial lining was observed, though Corrin (1962) has reported the simultaneous presence of areas of simple epithelium and areas of stratified epithelium in a case of cystadenoma.

Although Wheeler and Edmondson (1985) report a fair degree of uniformity in epithelial cells, all cells being columnar in appearance, the present findings highlighted the varied morphology and height of epithelial cells. This variability may be linked to the stage of development of the neoplasm (Bannasch and Reiss, 1971; Bannasch and Massner, 1976) and to the amount of fluid contained in cystic cavities, since an increase in fluid-particularly in larger cysts-leads to increased pressure on cyst walls, giving rise to swelling and a consequent flattening of cell components.

Epithelial cell cytoplasm was not found to contain vacuoles staining positive to PAS or Mayer mucicarmine, although they have been reported in moderate quantities by Ishak et al. (1977) and Beretta et al. (1988). The absence of such vacuoles, and the tendency of cells to flatten - as reported earlier - may be

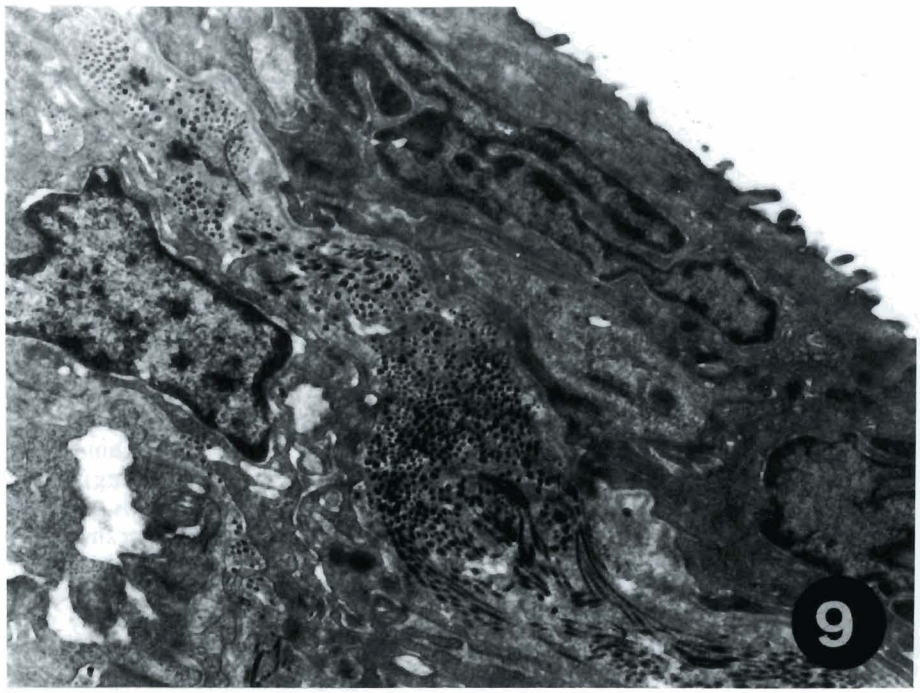
Fig. 7. Group III (4 months). Newly-formed canalicular structure (Detail). x 5,000

Fig. 8. Group III (13 months). Columnar epithelial cells delimiting cyst lumen. Note abundant cytoplasmic vacuoles (arrows). x 6,000

interpreted as indications of the stage of development of the process (Bannasch and Reiss, 1971; Bannasch and Massner, 1976).

The stroma consisted of connective tissue rich in collagen and reticulin fibres, accompanied by a varying number of cells. Collagen fibres have been reported by various authors (Short et al., 1971; Kokal et al., 1983; Wheeler and Edmondson, 1985). No other authors, however, report reticulin fibres in the stroma. The presence of collagen fibres around the cyst epithelium is thought to be due to extensive epithelial proliferation; reticulin fibres appear to derive from the hepatic stroma itself, and their accumulation is due to the growth of cysts, which gives rise to the atrophy through compression of neighbouring hepatocytes, which tend to disappear; reticulin fibres from the hepatic stroma tend to surround and become part of the fibrous cyst wall.

The cell component was composed of elements



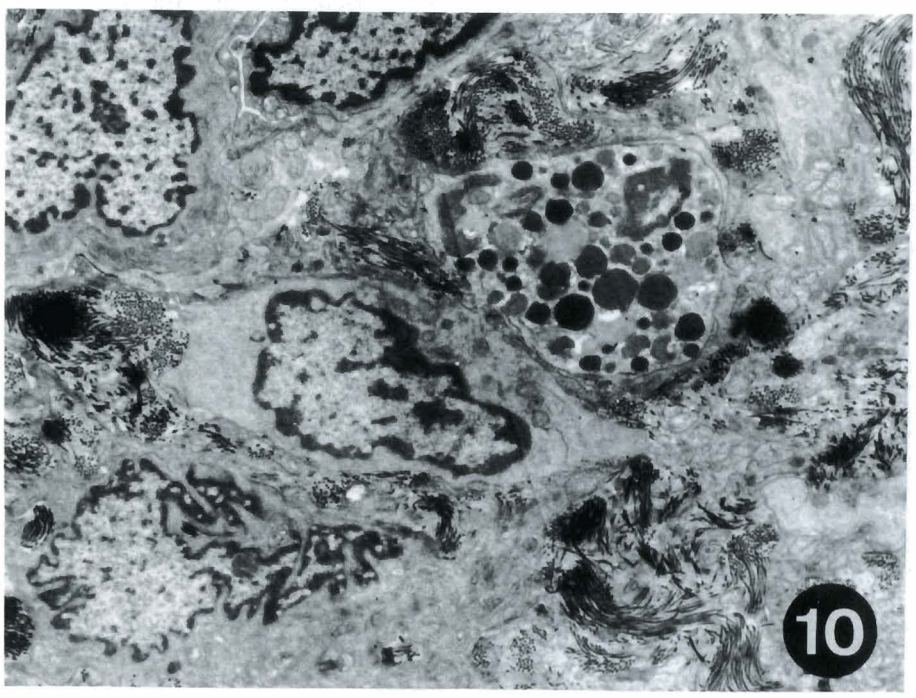


Fig. 9. Group III (10 months). Clear flattening of epithelial lining cells with large irregular-shaped nucleus. x 16,000

Fig. 10. Group III (13 months). Cyst wall connective stroma (Detail). Note undifferentiated cells and mast cells, with numerous bundles of collagen fibres. x 6,000

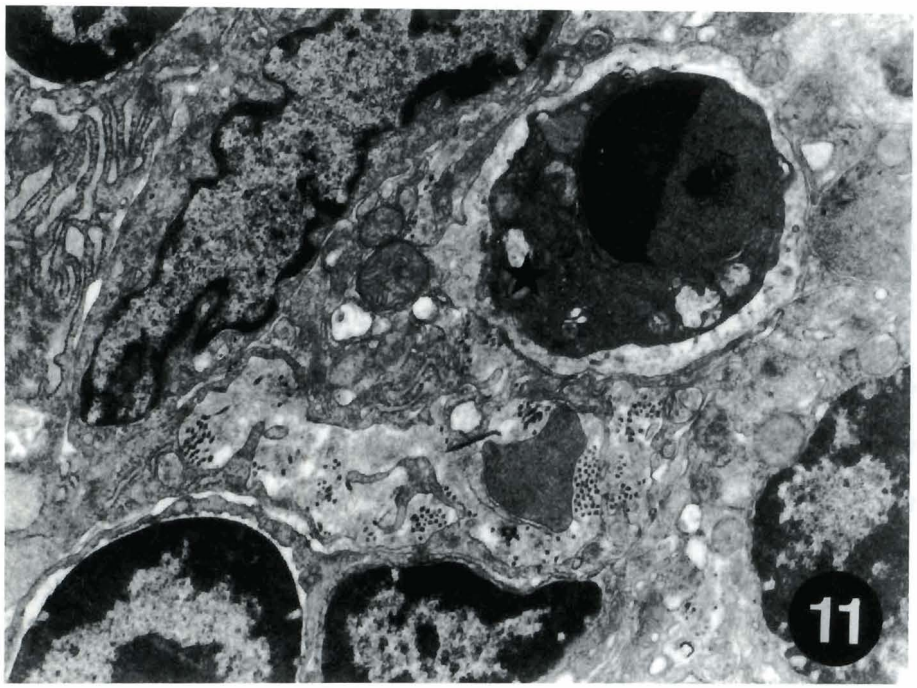


Fig. 11. Group III (13 months). Cells resembling fibroblasts, and cells in apoptosis (star). x 12,000

recognizable as wandering cells, mast cells, spindle cells resembling fibroblasts, and a certain number of hepatocytes, together with cells which were difficult to differentiate and which could be classified as blast cells. Some of these cell elements have been reported by a number of authors (Ishak et al., 1977; Kokal et al., 1983; Goodman, 1987).

Although Coté (1984) has reported a small amount of neocanalicular proliferation in hepatic parenchyma unaffected by cystadenoma, the present study revealed fairly large number of such structures spread over wide areas of the liver. Biliary epithelio-proliferative processes were also observed in areas of the liver alongside the perfectly-established biliary cystadenoma.

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