Morphological, immunohistochemical and ultrastructural changes in dimenthylnitrosamine induced liver injury. Effect of malotilate

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Summary. Dimethylnitrosamine (DMN) induced liver injury in rats with cell necrosis, inflammation, hemorrhages, increased collagen type III synthesis and basement membrane component laminin and collagen IV localization in perisinusoidal sites. Malotilate ingestion during DMN treatment abolished inflammation and decreased interstitial collagen deposits and vascularization. It affected clearly less DMN-caused hemorrhage. When malotilate treatment was started subsequently to development of DMN-injury, it also caused decrease in inflammation, though less, as well as in collagen III, BM and fibronectin deposits. We suggest that the mode of the malotilate effect on reducing the DMN-induced fibrosis of the liver is via inhibiting the inflammation, decreased fibronectin deposition possibly also playing a role.

Key words: Dimethylnitrosamine, Malotilate, Liver injury, Collagens, Fibronectin

Introduction

Cirrhosis of the liver is a complex condition combining several biological, biochemical and clinical characteristics, none of which are unique or diagnostic in itself (Anthony et al., 1978). The morphological characteristics of end stage cirrhosis are well characterized (Popper, 1977, 1981, Popper et al., 1981), but the early lesions and intermediate stages and their significance for the development of cirrhosis are less well known (Lieber, 1978). Early collagen formation causes capillarization, reduces the blood flow and disturbs liver function (Schaffner and Popper, 1963). Specific collagen changes caused by extraneous agents, e.g. ethyl alcohol, have been reported (Ott et al., 1977; Hahn et al., 1980;

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Bianchi, et al., 1984; Yamamoto et al., 1984). Such changes involve the accumulation of collagens I, III and IV as well as types V and VI. Biochemically, increase in the synthesis of type IV, III and I collagen is an early event in the development of cirrhosis (Bianchi et al., 1984; Davis and Madri, 1987). Later on, the excessive collagen deposition is a characteristic feature of cirrhosis. Also other matrix components, such as laminin and fibronectin, are known to accumulate (Ballardini et al., 1985; Niemelä et al., 1985). However, little is known of the reversibility of excessive accumulation of matrix components or their relationship to other biological or morphological changes.

Attempts to produce experimental conditions similar to alcohol induced liver cirrhosis have mostly used repeated applications of carbon tetrachloride (CCl_4) in rats and other animals. A single dose or very few doses will result in liver damage (Aterman, 1954; Rubin et al., 1963; Hartoft, 1964; Pérez-Tamayo, 1983; Martínez-Hernández, 1985). Recent reports, however, have questioned the validity of this model on biochemical and morphological grounds (Pérez-Tamayo, 1983), noting that reversibility is commonly achieved by cessation of treatment (Pérez-Tamayo, 1979). Dimethylnitrosamine (DMN) induced liver injury has been studied mainly with respect to early morphological and biochemical changes and their relationships to later hepatic neoplasms (Druckrey et al., 1967). Less well known is the relationship between hepatocellular injury, stromal changes and fibrosis in this model.

Malotilate (diisopropyl-1.3-dithiol-2-ylidene malonate, Zyma AG, Switzerland) is a new compound suggested for use in the treatment of chronic liver diseases (Siegers et al., 1986). Previously it has been reported to prevent the progression of liver damage in experimental models induced by various extraneous agents (Younes and Siegers, 1985). In addition to its protective effect on liver damage caused by metabolically activated inducers, malotilate also protects against hepatic fibrosis induced by immune reaction in rats

(Dumont et al., 1986). It also opposes the fibrogenic effect of carbon tetrachloride in the lung (Pääkkö et al., 1987). The mechanism of action of malotilate in preventing liver damage and fibrosis is not well understood. There is evidence that malotilate increases the action of the microsomal oxidative system. Thus, the mechanism of the protective effect cannot be the inhibition of the bioactivation of liver toxins (Katoh et al., 1980, 1981; Kawata et al., 1982). In addition, we have recently shown that the prevention of liver fibrosis is not mediated through a direct inhibition of collagen biosynthesis by malotilate (Ala-Kokko et al., 1987).

The purpose of the present work was to study by morphological and immunohistochemical means the presence, distribution and amount of collagen, notably type III, and basement membrane (BM) constituents laminin and collagen IV in DMN induced liver injury, the relationship to cellular changes and the effect of malotilate and its possible mechanism of action in this model.

Materials and methods

Animals

Liver injury was induced in female Sprague-Dawley rats, seven weeks of age. The rats were fed on a commercial diet (Hankkija Oy, Helsinki, Finland) and allowed free access to water. The animals were maintained under 12 h light/dark cycles.

Hepatic fibrosis was induced by injecting DMN intraperitoneally at doses of 1 µl (diluted 1:100 with 0.15 M NaCl)/100 g body weight. Injections were given weekly on the first three consecutive days for one week (group 1), 3 weeks (group 2) and 4 weeks (group 3). No animals receiving DMN alone survived up to day 35. To study the effect of malotilate on hepatic injury malotilate was given orally at doses of 10 mg/100 g body weight five times weekly. The animals received simultaneously DMN and malotilate from the beginning of the experiment, and were killed after 1 week (group 4), 3 weeks (group 5), 4 weeks (group 6) and 5 weeks (group 7). To study the effect of malotilate on pre-induced liver damage, DMN-induction was started at day 0, and continued to the end of the experiment, while malotilate-treatment was started on day 14 and continued for 2 weeks (group 8) and 3 weeks (group 9). Untreated rats served as controls (group 10). Treatments are summarized in Fig. 1.

Samples

The rats were weighed weekly during the experiment. At the end of experiment the rats were anesthetized with diethyl ether, and weighed. The liver biopsy specimens were taken for light (LM) and electron microscopy (EM) studies.

Liver histology

The liver biopsy specimens were fixed in neutral formalin, embedded in paraffin, sectioned serially, and stained with hematoxylin and eosin, periodic acid-Schiff after diastase digestion, van Gieson's stain, or Gomori's reticulin stain as well as other stains when needed.

Antibodies

The antisera were obtained commercially (Institut Pasteur de Lyon, Lyon, France) or kindly provided by Drs. L. and J. Risteli (Collagen Research Unit, Department of Medical Biochemistry, University of Oulu, Finland). Fibronectin was purchased from Dako Corp. (Copenhagen, Denmark). Laminin and collagen type IV was raised in rabbits after immunization with tissue isolated from a murine EHS sarcoma, PIIIP from ascitic fluid of a patient with carcinoma of the pancreas (Niemelä et al., 1985). The antigens were purified before immunization (Grimaud et al. 1980, Risteli et al., 1980, 1981) and the specifications of the antibodies were determined by radioimmunoassay. Crossreaction between the antibodies were less than 1%.

Immunoperoxidase staining

For the immunohistochemical studies the avidin-biotin modification (Stenbäck and Wasenius. 1985) of the peroxidase-antiperoxidase method was used. 5 µm thick paraffin section were deparaffinized and treated with 0.4% pepsin (Merck, Darmstadt, FRG). Endogenous peroxidase were blocked by incubating with H_2O_2. The sections were successively treated with rabbit antibodies, biotinylated anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, Ca.), dilution 1:500, avidin (Vector), dilution 1:1000, and biotinylated horseradish peroxidase complex (Vector). The peroxidase reactions was performed using 3, methyl-2-ethylcarbazole (Sigma Chemical Co., St. Louis, Mo.) as a substrate. Hematoxylin was used as counterstain. Normal rabbit serum and phosphate-buffered saline were used instead of the primary antibody for control stainings.

Electron microscopy

Fresh tissue was fixed in 3% glutaraldehyde in phosphate buffer pH 7.4, postfixed in 1% osmium tetroxide in the same buffer, dehydrated, and embedded in Epon. 1 µm sections were cut and stained with 1% tolouidine blue for orientation by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Jeol JEM 100B electron microscope.

Morphometry

Morphometry to estimate inflammation and hemorrhage was performed according to the principle of Weibel (Weibel and Bolender, 1973) using a grid lattice superimposed on the projected image. Magnification

was 600 ×, and in 10 representative unselected areas the volume fraction of inflammatory cells and hemorrhage was estimated.

Results

The main structural liver alterations after 7 days of DMN treatment (group 1) were on the cellular level. The hepatocytes varied in size and shape and the nuclei were enlarged and hyperchromatic. Ultrastructural analysis showed early signs of cell destruction. The cell membranes were irregular and indistinct, the mitochondria varied in size and shape, and the SER formed multiple small vesicles. Connective tissue changes were limited to some reticulin and PIIIP-positive fibres extending from the portal tract into the parenchyma. The BM proteins laminin and collagen IV were located in the vessel walls in the portal tract and a few scattered sites in the perisinusoidal area, as in untreated animals. The locations of fibronectin was also mainly in the portal area and a few sites in the perisinusoidal space. The distribution was slightly more irregular than in control livers, but the total amount was not distinctly different.

Continuation of DMN treatment for a further two weeks (group 2) elicited severe changes: cellular destruction, sinusoidal hemorrhages and focal inflammation. The morphometric distribution of inflammation and hemorrhages is summarized in Fig. 2. The animals showed extensive hepatocytic necrosis, with
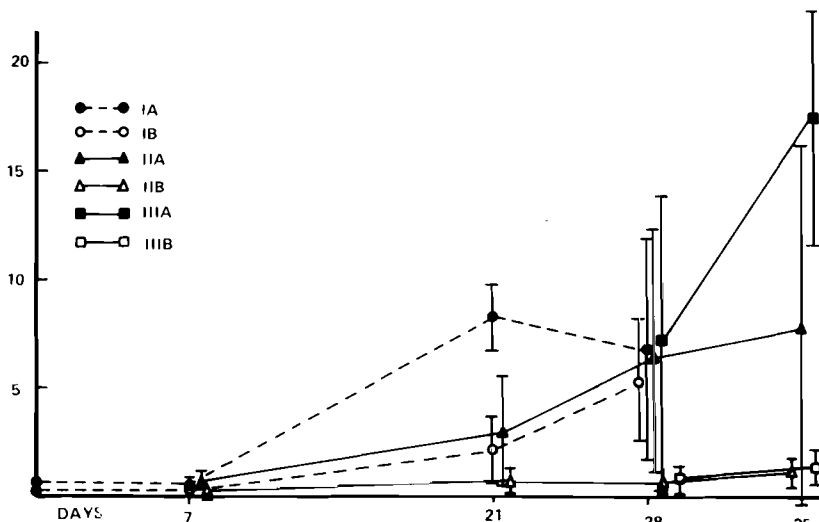
Fig. 2. Percentage of hemorrhages (A) and inflammation (B) by volume after treatment with DMN (I), DMN/malotilate (II) and DMN followed by malotilate (III).

the remaining cells irregular in size and shape and interspersed with inflammatory cells and collagen fibres (Fig. 3). Cell disintegration was clearly visible on EM, with indistinct cell borders, irregular surface extensions, reduced SER, partly saccular Golgi apparatus and rounded or enlogated mitochondria. The focal inflammatory infiltrates extended into the surrounding parenchyma, with disintegration of hepatocytes but not visible increase in BM or PIIIP-positive material in these locations (Fig. 3). The sinusoids were dilated, with cell debris, red blood cell extravasation and signs of collagen fibril formation. Collagen changes were indistinct, and visible mainly as a thickening of the wall of the terminal vein and a slight increase in perisinusoidal BM components. The increase in fibronectin was prominent with thick fibronectin stalks surrounding both the cell trabeculi and individual hepatocytes (Fig. 4), portal staining was also observed.

At the end of experiment, after 4 weeks of DMN treatment (group 3), proliferative nodular changes predominated, the areas between the nodules (Fig. 5) being filled with necrotic cell debris and exudate. The hepatocytes were enlarged, with prominent nucleoli, and cellular disintegration, ballooning and necrosis were distinct. The mitochondria were enlarged and pleomorphic, containing inclusion bodies. SER was vesiculated, RER degranulated. Collagen proliferation was distinct in the sinusoids, and the vessel walls were thickened with reticulin-positive extensions reaching into the surrounding parenchyma (Fig. 6). Early capillarization was also visible, with laminin and collagen type IV forming vascular structures. Thick collagen type IV stalks extended from one portal area to another between the portal areas and central veins. PIIIP-positive fibres had increased, and were found in the fibrous trabeculae and as fine discontinuous strands in the perisinousoidal areas (Fig. 6). Fibronectin was still evident in the perisinusoidal spaces extending from the portal tracts.

TREATMENT; DURATION, DAYS

GROUP	CHEMICAL	0	7	14	21	28	35
1	DMN	⊢——⊣					
2	DMN	⊢————————⊣					
3	DMN	⊢——————————⊣					
4	DMN	⊢——⊣					
	MAL	⊢——⊣					
5	DMN	⊢————————⊣					
	MAL	⊢————————⊣					
6	DMN	⊢——————————⊣					
	MAL	⊢——————————⊣					
7	DMN	⊢————————————⊣					
	MAL	⊢————————————⊣					
8	DMN	⊢——————————⊣					
	MAL	⊢————⊣					
9	DMN	⊢————————————⊣					
	MAL	⊢————————⊣					
10	CONTROLS	I	I	I	I		

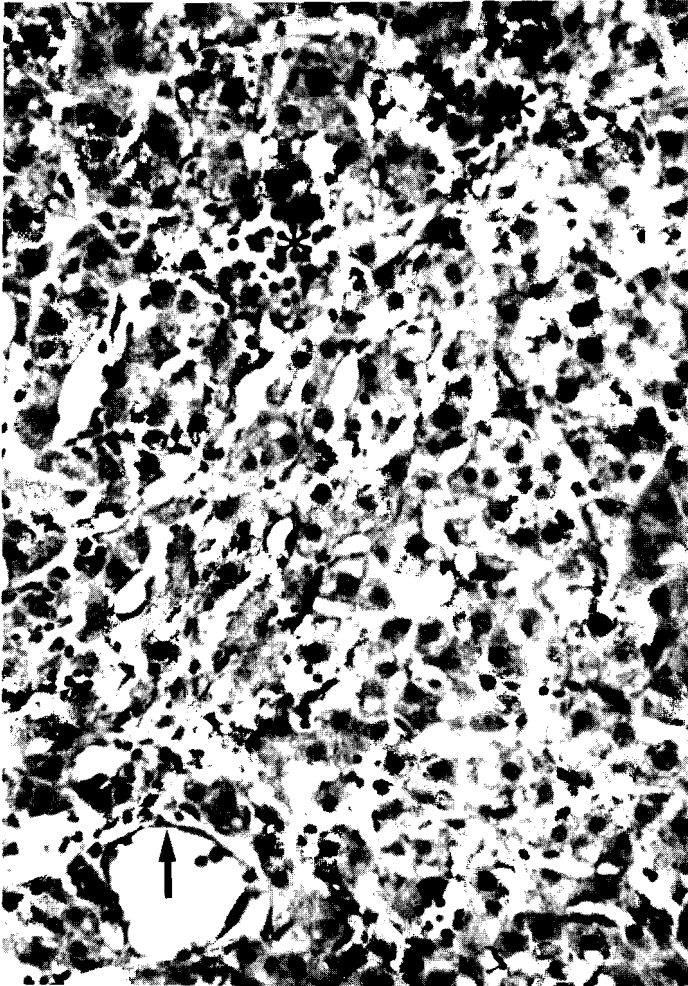**Fig. 1.** Type and duration of treatment in the various groups.

Fig. 3. Inflammatory infiltrates (*) in the liver of a rat treated with DMN 21 days. The vessel walls (→) are poor in BM material. Laminin × 360
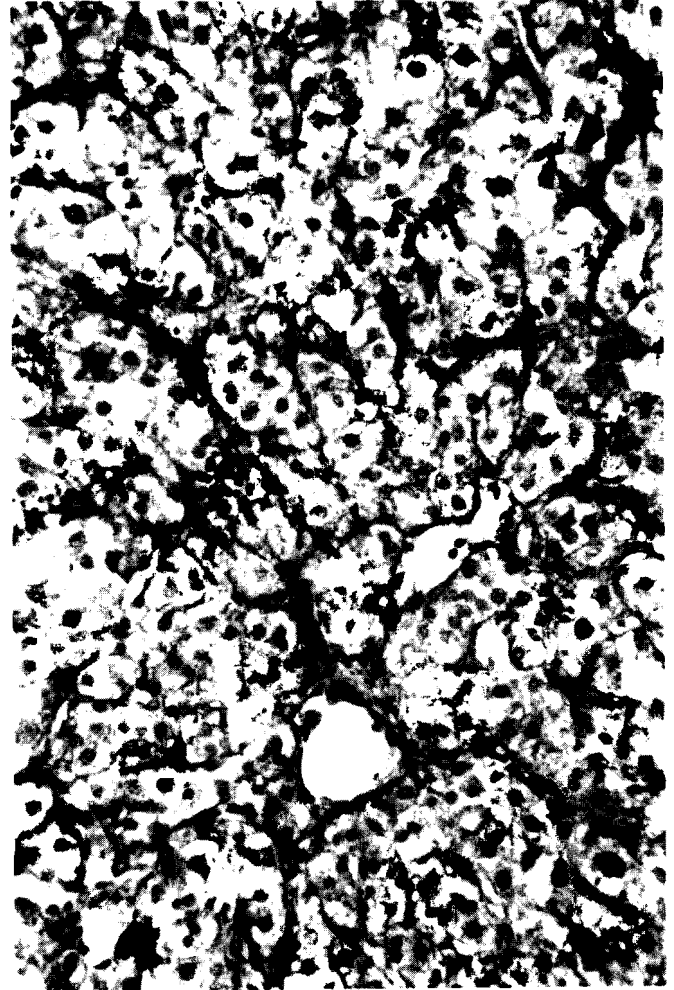

Fig. 4. Abundant fibronectin (→) in the sinusoids of the liver of a rat treated with DMN for 21 days, inflammatory cell infiltrates (*) are also seen. Fibronectin × 360

Malotilate when administered simultaneously with DMN did not cause any distinct differences after 1 week of treatment (group 4) compared to those receiving DMN alone, although some cellular changes were observed on the ultrastructural level and a few collagen fibres between the hepatocytic cells (Fig. 7). Extended treatment with malotilate for 3 weeks (group 5) prevented the effects of DMN alone (group 2) to some extent. The total amount and extent of inflammation markedly decreased when malotilate was given while hemorrhages were unaffected (Fig. 2). Cytological alterations and variations in cell shape and size and in the structure and arrangement of the cytoplasmic organelles were still observed. Fibronectin staining compared to normal controls increased considerably (Fig. 8), but less so than in animals receiving a corresponding amount of DMN alone (group 2). This also applied to the amount of collagen whether of type III or type IV.

When DMN/malotilate treatment was continued for either 4 weeks (group 6) or 5 weeks (group 7), distinct changes in liver structure were seen, but they were clearly less severe than after administration of DMN

alone for 4 weeks (group 3). Alterations in cell shape and size, sinusoidal haemorrhages and oedema were found, but there were clearly fewer inflammatory cells than in the animals receiving DMN alone (Fig. 2). Collagenous proliferation was limited, but occurred in the form of discontinous irregular fibres of BM positive material in a perisinusoidal location (Fig. 9). The capillarization seen after DMN alone was mostly absent, the increase in fibronectin was still evident but less than after DMN alone (group 2).

Malotilate treatment starting 14 days after than DMN and continuing with DMN for an additional 14 days (group 8) reversed cellular necrosis and tissue destruction to some extent compared with 28 days of DMN treatment alone (group 3). The numbers of inflammatory cells present were significantly less than in animals given DMN alone, but haemorrhages were not significantly affected. The hepatic cells and their nuclei were more regular, though the SER was partly saccular and the mitochondria varied in size. Number and size of surface extensions increased markedly, they were, however, slightly irregular in structure (Fig. 10). Some pericellular
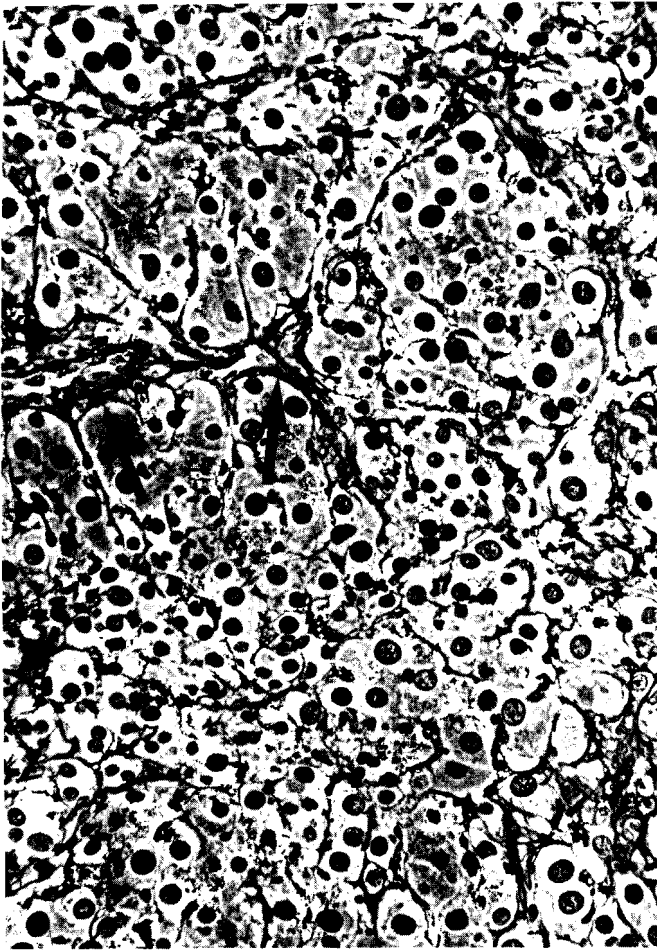
Fig. 5. Nodular arrangements of liver cells in a rat treated with DMN for 28 days, surrounded by reticulin positive fibres (→). Reticulin × 360
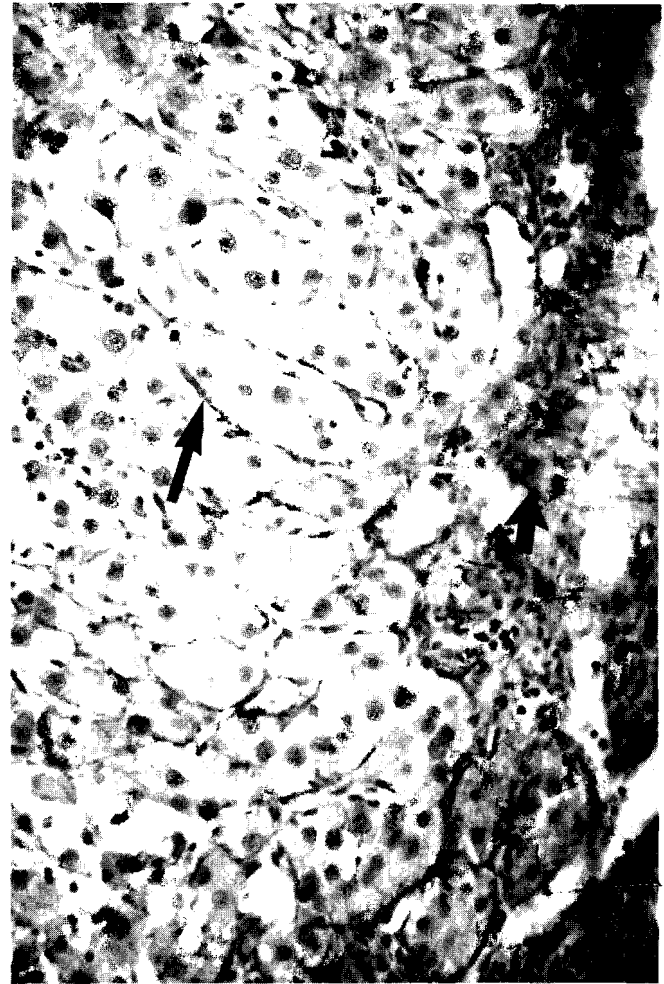

Fig. 6. Collagen type III-positive material (→) in the liver of a rat treated with DMN for 28 days. The material is seen both in a perisinusoidal position and in the septum. Collagen type III × 360

collagen was also observed. The location and amount of collagen type IV, laminin or fibronectin was markedly less altered than in the corresponding group treated with DMN only (group 3).

In the animals receiving malotilate starting 14 days after DMN and continuing together with it for a further 21 days (group 9) distinct morphological changes in the liver with a severe haemorrhagic reaction and some nodule formation was observed. The liver cells were enlarged, with dilated SER and prominent surface extensions. The sinusoidal space contained large haemorrhages (Fig. 2), but there were few inflammatory cell aggregates. Sinusoidal capillarization was distinct, as evidenced by BM stains with widened spaces connected by intricate channels (Fig. 11). Reticulin-positive fibres formed an irregular meshwork extending between the individual cells separating neighbouring nodules. Fibronectin deposits were distinct in the perisinusoidal spaces though in lesser amounts than in the animals given DMN for 28 days (group 3). None of the animals receiving DMN alone survived for 35 days.

Discussion

Repeated DMN treatment induced progressive tissue and cell alterations in the rat liver. Prominent features in this model were inflammation and sinusoidal haemorrhages, with less marked steatosis. Nodular arrangements of liver cells, capillarization and fibrosis, hallmarks of alcoholic cirrhosis, were prominent after extended DMN treatment. Altered cytoplasmic organelles, mitochondrial enlargement and disorganization and dilated SER and RER seen in this study have also been observed in human alcoholic liver disease (Horvath et al., 1973; Grases et al., 1987). Malotilate, administered together with DMN, prevented this process significantly, reducing inflammation and accumulation of connective tissue components as compared with DMN alone, while varying degrees of haemorrhage were still seen in most animals, though less than in those treated with DMN alone.

One week DMN-treatment did not cause any detectable changes in the amount and distribution of BM components laminin or type IV collagen compared with
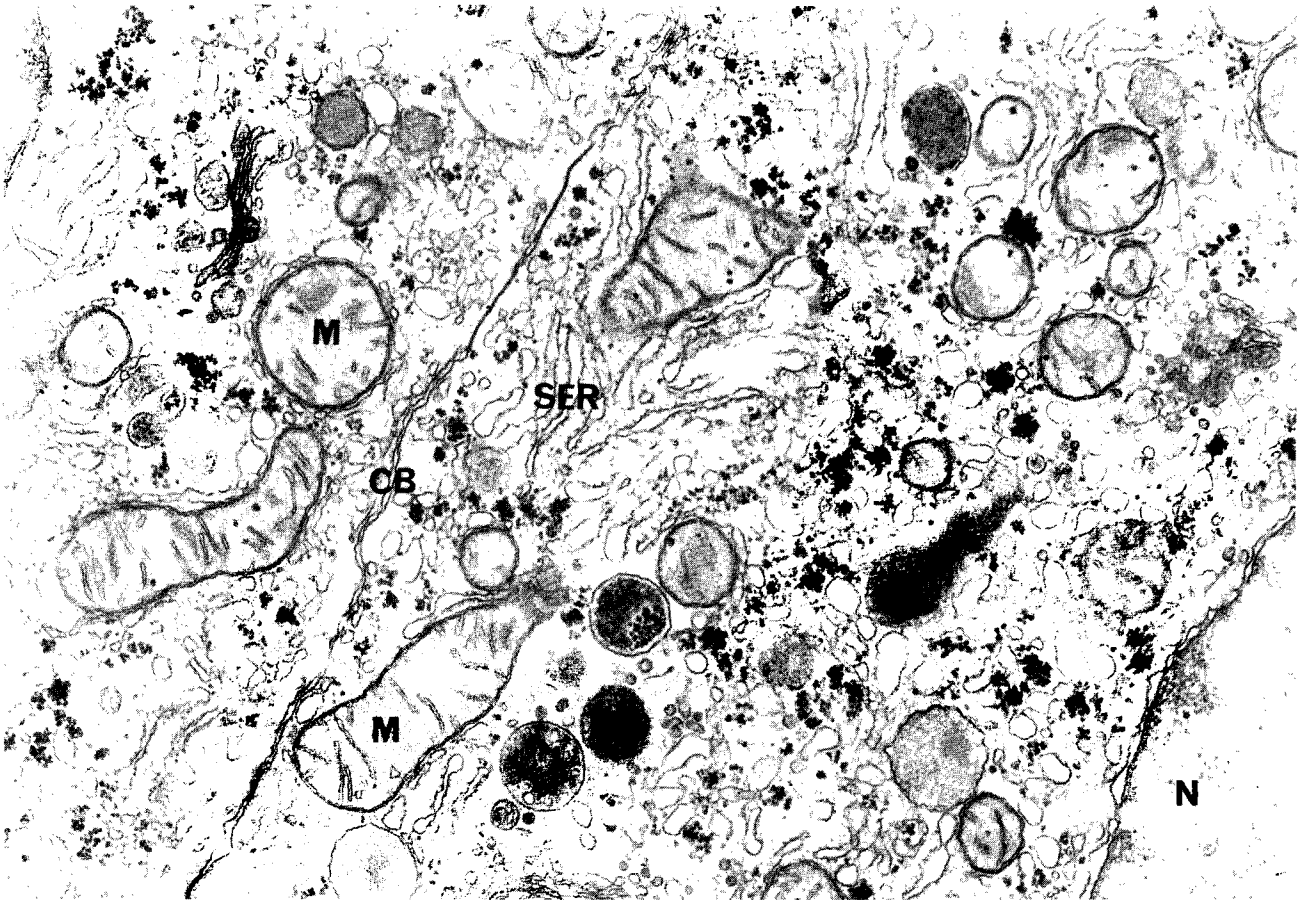
Fig. 7. Irregular mitochondria (M), vesiculated smooth endoplasmic reticulum (SER) and phagocytic bodies (PB) in a liver treated with DMN and malotilate for 7 days, nucleus (N), cell border (CB), Golgi apparatus (G). Uranyl acetate-lead citrate × 10,000
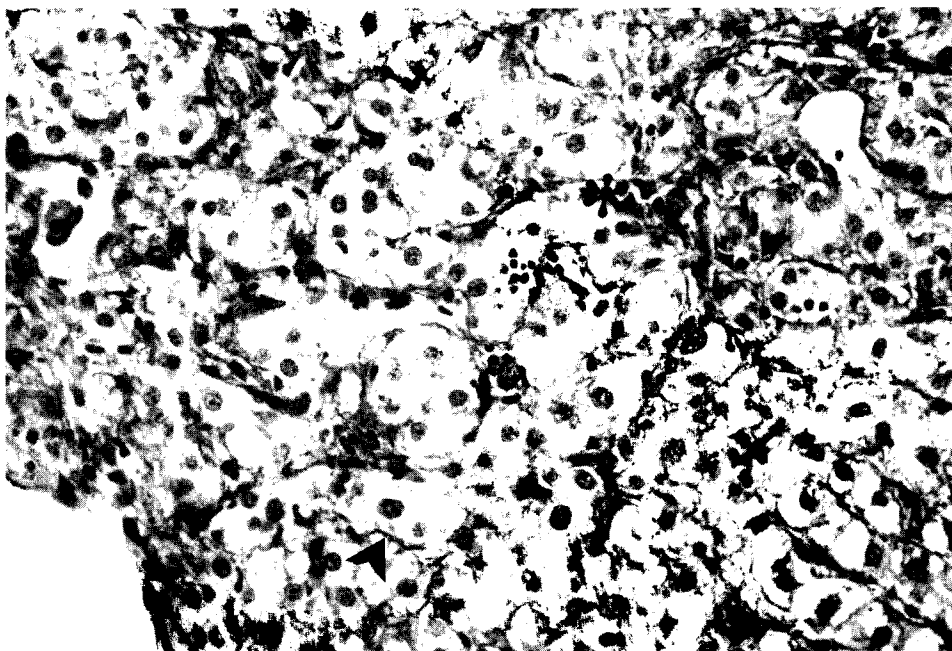

Fig. 8. Cellular irregularities, sinusoidal hemorrhages (*) and strands containing fibronectin (→) in the liver of a rat receiving DMN and malotilate for 21 days. Fibronectin. × 360
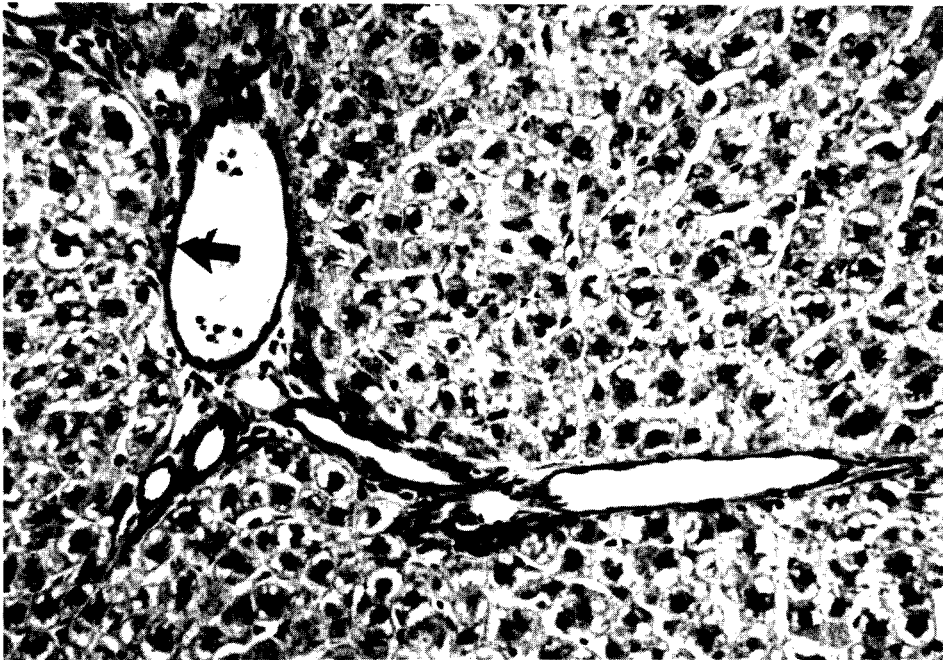
Fig. 9. Distinct BM deposits (➡) in vessels walls and scanty laminin in a perisinusoidal position in the liver of a rat given DMN and malotilate for 35 days. Laminin × 360
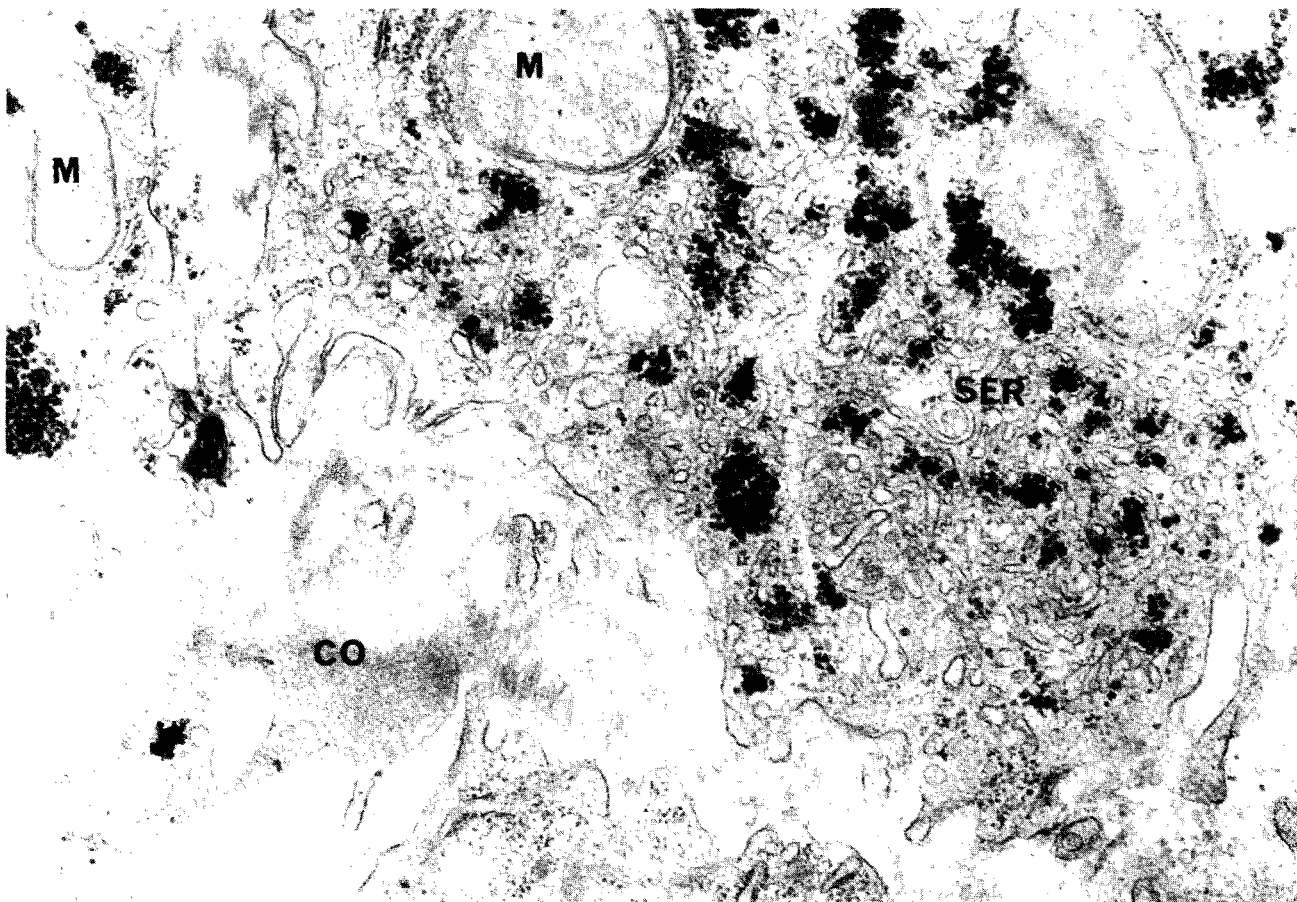

Fig. 10. Dilated smooth endoplasmic reticulum (SER), preserved mitochondria (M) and glycogen (G) in hepatocyte of rat given DMN for 28 days and malotilate for 14 days. Cell extensions (CO) are seen in space of Disse. Uranyl acetate-lead citrate × 10,000
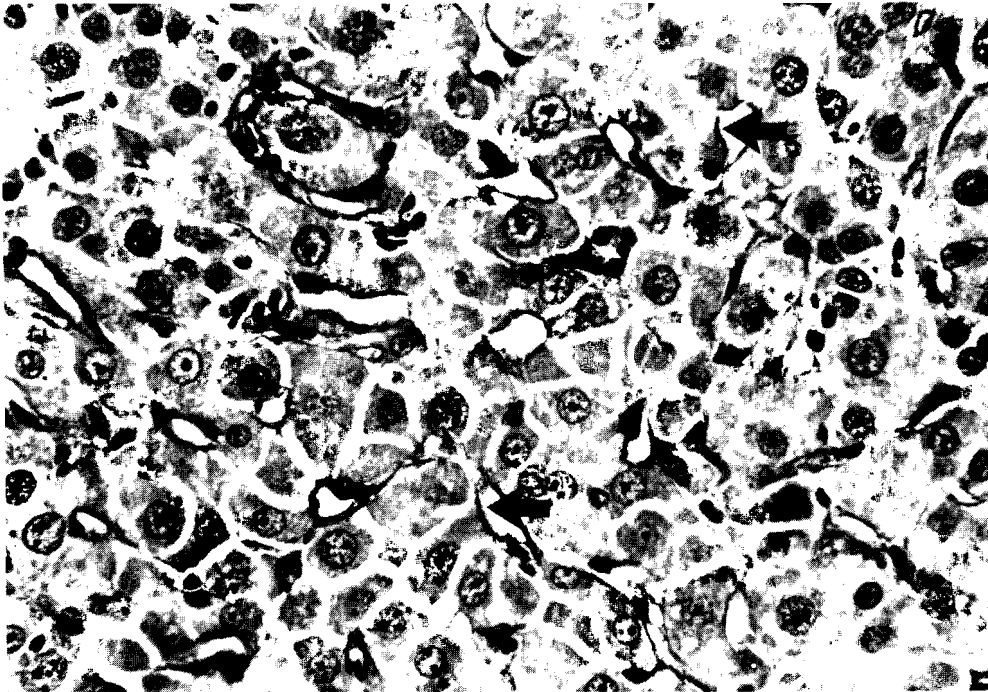
Fig. 11. Distinct BM deposits in vessel walls and scanty laminin in a perisinusoidal position in the liver of a rat given DMN and malotilate for 35 days. Laminin × 360

the untreated animals. After prolonged treatment laminin and collagen IV increased primarily in the sinusoids, less so between the individual hepatocytes. Capillarization of the liver sinusoids, as found in human alcoholic cirrhosis (Schaffner and Popper, 1963; Popper et al., 1981) was also found after 3 weeks of DMN, as seen in CCl₄ (Ala-Kokko et al., 1987) and in serum-induced fibrosis (Linder et al., 1982). BM proteins also increased in the portal triads. Both laminin and collagen type IV appeared simultaneously and in same locations preceding increased accumulation of collagen type III. These findings corresponded to increased mRNA levels, as observed in similarly treated animals (Ala-Kokko et al., 1987).

After extended DMN treatment of four weeks the deposits of type III collagen exceeded those of BM. The increase in collagen type III-positive material occurred in the perisinusoidal space and also in portal areas of the DMN-treated animals. Vessel wall deposits started to increase after one week of DMN, and collagen sprouts extended into the parenchyma by the end of the experiment. Individual hepatocytes in the livers affected by DMN were separated by fine stalks of individual collagen fibres, and increased perisinusoidal interstitial collagen was also found. Reticulin-positive staining was codistributed with collagen type III. Alcohol cirrhosis consists of deposits of type I and III collagens (Rojkind and Martínez-Palomo, 1976; Bianchi et al., 1984; Yamamoto et al., 1984), with proliferation of fibroblasts in the portal tracts and the perihepatocellular and periductular areas (Hahn et al., 1980). Type III collagen was found here in all these areas of the DMN-treated rat liver, indicating that the DMN model resembles human cirrhosis in this respect. The increase in laminin and

collagen type IV as well as collagen type III in all locations in the animals receiving DMN and malotilate was less distinct than in those receiving DMN alone.

Fibronectin is a plasma and stromal protein (Kurkinen et al., 1980; Ruoslahti et al., 1981; Bitterman et al., 1983) with specific binding sites for cell membranes, collagen and proteoglycans. In this study fibronectin deposits increased in DMN treated liver at an early stage being maximal on day 21. The distribution was otherwise similar to that of interstitial collagen, and the increase was less distinct in the animals also receiving malotilate. Fibronectin has been postulated to form a framework for deposits of interstitial collagen (Kurkinen et al., 1980) a notion supported by the present findings.

Malotilate treatment has been reported to reduce tissue injury and collagen formation induced by extraneous agents in the liver (Dumont et al., 1986; Siegers et al., 1986) and lung (Pääkkö et al., 1987), and to lessen the degree of necrosis, steatosis and fibrosis in the liver caused by chemicals i.e. CCl₄ (Ala-Kokko et al., 1987). The mechanism of action for malotilate is, however, not known (Kawata et al., 1982). Malotilate does not inhibit directly collagen biosynthesis (Ala-Kokko et al., 1987). Furthermore, malotilate increases the action of microsomal oxidation systems (Katoh et al., 1981; Kawata et al., 1982), thus the mechanism cannot be the inactivation of toxic metabolities. Malotilate did not reverse DMN-induced changes in liver cell membranes, mitochondria and SER in the present study. Fibrosis caused by heterologous serum occurred in the absence of significant hepatocellular injury, and malotilate was still able to prevent the developing fibrosis (Dumont et al., 1986). Thus, it is unlikely that malotilate acts on these early stages of DMN induced liver damage.

Anoxic-type injury can be postulated from the morphology and distribution of the DMN-induced changes. Focal perivenular fibrosis was observed after a short period of DMN-treatment. Collagen proliferation may obstruct the vascular channels and interfere with the flow of nutrients, but only at a later stage (Orrego et al., 1981). Malotilate-induced improved vascularization is thus probably of secondary importance. Another possibility that fibrogenesis was due to some action of the immune system on collagen metabolism (Sherlock, 1982) was not supported by the morphological appearance of the DMN-induced lesions. Mononuclear cell infiltrates, piecemeal necrosis and cell membrane damage typical of immunological injury (Sherlock, 1982) was not observed. However, the significance of inflammation is supported by the finding that inflammation pararelled subsequent collagen formation and malotilate inhibited inflammation markedly. As collagen formation was not located at the site of inflammation, a mediating substance sensitive to malotilate may also be involved.

The role of fibronectin as a possible mode of action may be supported by the findings in this study. The interaction of fibronectin with fibrin, cells and extracellular matrix makes it well suited to act as a bridge between the early tissue response to injury, the phagocytosis and cell migration phase and the final response, collagen deposition (Martínez-Hernández, 1985). Specifically, fibronectin is shown to be chemotactic for fibroblasts, serves as an attachment site for them and also provides them with signals to replicate (Bitterman et al., 1983). Malotilate reduced fibronectin deposition in this study and also affected deposition of fibronectin in serum induced pig liver injury (Dumont et al., 1986). Whether this is due to an effect on fibronectin synthesis, deposition or distribution remains to be determined.

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