Biochem. J. (1987) 246, 503–509 (Printed in Great Britain)

Preventive effect of malotilate on carbon tetrachloride-induced liver damage and collagen accumulation in the rat

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Malotilate is a new drug suggested for use in chronic liver diseases. It is shown here to prevent liver damage caused by CCl_4. The concomitant administration of malotilate with CCl_4 significantly decreased hydroxyproline accumulation in the liver, liver prolyl 4-hydroxylase and liver and serum galactosylhydroxylysyl glucosyltransferase activities. However, it had no effect on the daily urinary hydroxyproline excretion or the hydroxyproline content of the skin, liver or lungs in normal young growing rats. It also had no specific inhibitory effect on hydroxyproline synthesis or secretion in fibroblast cultures, and did not affect the amount of procollagen-α1(I)-specific mRNAs in these cultures. Thus it seems to have no direct inhibitory effect on collagen metabolism. In addition to inhibition of liver collagen accumulation, malotilate was also able to prevent the development of morphological changes in the liver such as focal necrosis, fatty infiltration and inflammatory changes. It also normalized almost completely the standard liver-function tests. It is possible that malotilate may prevent excessive collagen deposition by inhibiting the inflammation caused by CCl_4-induced liver damage.

INTRODUCTION

Apart from being a passive supportive framework for the hepatic parenchyma, the extracellular matrix is currently considered to have an important role in normal liver function [1–3]. Liver fibrosis involves a distortion of the structural harmony of the extracellular matrix, which is deposited in the liver tissue in an excessive and disordered manner [2,3]. Collagen types I, III, IV, V and VI, all of which are found in the liver [4,5], tend to accumulate in chronic liver disease [2–6], leading to liver fibrosis and ultimately liver cirrhosis. Even though the deposition of collagen is evidently not the primary event in the processes of chronic liver diseases, it does play a central role in disturbing liver function at the advanced stages [2–4,6].

Many attempts have been made to develop treatments for liver cirrhosis, some directed at preventing the inflammatory or necrotic liver changes before actual excessive collagen accumulation, others setting out specifically to inhibit this process [4,7–10]. Thus far, however, there is no effective treatment modality availabile for clinical use.

Malotilate (di-isopropyl 1,3-dithiol-2-ylidene malonate) is a new compound, thought to have 'hepatotrophic' properties and to prevent the progression of chronic liver diseases in experimental models [11,12]. It has thus been suggested for use in treatment of liver cirrhosis, although the mechanism by which it prevents the development of this condition is not known at present. It has been shown previously, however, that malotilate does not inhibit the bioactivation of CCl_4 [13].

The details of collagen metabolism are currently relatively well known. Potentially, there are several steps in this metabolic pathway which are open to pharmacological intervention [4,14,15]. The present experiments provided evidence of a clear preventive effect of malotilate on the excessive collagen accumulation caused by CCl_4 induction in rats, and enabled the possible direct intervention by malotilate in collagen metabolism to be studied in detail.

MATERIALS AND METHODS

Animals

The animals used were 48- or 60-day-old female Sprague–Dawley rats fed on a commercial diet (Hankkija Oy, Helsinki, Finland). After preliminary trials, two main experiments were carried out. The rats were divided into four groups each containing 9–12 animals. The first served as a control group. To investigate the effect of malotilate on collagen metabolism, liver injury was induced by injecting CCl_4 intraperitoneally at doses of 0.1 ml/100 g body wt. (diluted with an equal volume of paraffin oil) twice a week for 40 days. In another experiment CCl_4 was injected subcutaneously at 0.15 ml/100 g body wt. The third group received simultaneously CCl_4 and malotilate, the latter being given orally at doses of 10 mg/100 g body wt. five times weekly for 40 days. The fourth group received only malotilate.

Samples

The rats were weighed during the experiment, and 24 h urine samples were collected for hydroxyproline determination. At the end of the experiment, the rats were anaesthetized with diethyl ether, killed and weighed. Blood samples were taken by heart puncture and the sera separated out. The sera were stored at −20 °C until used.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GGT, galactosylhydroxylysyl glucosyltransferase (EC 2.4.1.66); PH, prolyl 4-hydroxylase (EC 1.14.11.2).

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Liver biopsy specimens were taken for light-microscopic and electron-microscopic studies. The liver, lungs and skin specimens were then rapidly removed, frozen immediately in liquid N_2, weighed and stored at −70 °C until assayed.

For the assays of prolyl 4-hydroxylase (PH) and galactosylhydroxylysyl glucosyltransferase (GGT) activities, the liver specimens were homogenized with 60 strokes of a Teflon/glass homogenizer in 30 mg/ml of a cold (0 °C) solution consisting of 0.2 M-NaCl, 0.1 M-glycine, 50 μM-dithiothreitol, 0.1% (w/v) Triton X-100, 0.01% (w/v) soya-bean trypsin inhibitor and 20 mM-Tris/HCl buffer, pH 7.4 at 4 °C. The homogenates were incubated at 4 °C for 15 min and then centrifuged at 15000 *g* for 30 min at 4 °C. The supernatants were used for the assays.

Assay procedures for tissues and serum

The tissue specimens for the determination of hydroxyproline and total protein were homogenized with an Ultra-Turrax homogenizer at 0 °C in 0.5 M-acetic acid and dialysed against 0.5 M-acetic acid in 4 °C. After hydrolysis in 6 M-HCl at 120 °C overnight, tissue and urinary hydroxyproline was assayed by the method of Kivirikko *et al.* [16], and tissue total protein by the α-amino N method of Rubinstein & Pryce [17]. Liver PH activity was assayed with [^{14}C]proline-labelled substrate [18]. Liver and serum GGT activity was assayed by the method of Myllylä *et al.* [19], as modified for liver [20] and serum [21] specimens. The protein content of the 15000 *g* supernatant was assayed by the method of Lowry *et al.* [22], with bovine serum albumin as a standard.

The assays of the activity of serum alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase [23] were performed in the laboratory of the Department of Clinical Chemistry at the University of Oulu by standard hospital laboratory methods.

Cell culture

Locally established human skin fibroblasts (passages 6–12) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-calf serum, 290 μg of glutamic acid/l, 509 μg of ascorbic acid/ml, 100 units of penicillin/ml and 100 μg of streptomycin/ml. The medium was changed every second day. Confluent cultures were washed three times with phosphate-buffered saline (Gibco Biocult) and incubated for 4 h with various concentrations of malotilate (4 mg/ml, dissolved in dimethyl sulphoxide) in DMEM containing glutamic acid, ascorbic acid, penicillin and streptomycin at the concentrations described above, and 20% dialysed fetal-calf serum. Dimethyl sulphoxide had no toxic effects in the cells in the concentrations used here. The cultures were labelled with 2 μCi of L-[2,3-^{3}H]proline (sp. radioactivity 36 Ci/mmol; Amersham) or L-[3,4,5-^{3}H]leucine (sp. radioactivity 110 Ci/mmol; Amersham)/ml for 20 h. At the end of the labelling, the medium was collected, and proteinase inhibitors were added at final concentrations of 20 mM-EDTA, 10 mM-*N*-ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride. The cells were sonicated in 50 mM-Tris/HCl, pH 7.4, containing 0.2 M-NaCl and the proteinase inhibitors in the same concentrations as above. The cell and medium samples were then dialysed against 0.2 M-NaCl/50 mM-Tris/HCl, pH 7.4.

Assay procedures for cultured cells

The [^{3}H]leucine- and [^{3}H]proline-labelled dialysed cell and medium samples were precipitated with cold (0 °C) 10% (v/v) trichloroacetic acid, and total [^{3}H]leucine and [^{3}H]proline incorporation was measured. The dialysed [^{3}H]proline-labelled cell and medium samples were hydrolysed in 6 M-HCl at 120 °C overnight and assayed for [^{3}H]hydroxyproline by a radiochemical assay [24].

Total cellular RNA was purified as described previously [25]. The amount of DNA was measured by the indole method [26] and RNA by the orcinol procedure [27].

Recombinant-DNA techniques

The recombinant plasmid Hf677 containing cDNA for the human procollagen proα1(I) mRNA [28] was purified and labelled radioactively with [α-^{32}P]dCTP and [α-^{32}P]dGTP (3000 Ci/mmol; Amersham) by nick-translation [29]. The denatured total RNA samples (five dilutions, range 0.1–1.0 μg) were dotted on to nitrocellulose paper with a dot-blot apparatus (Minifold II, Schleicher & Schuell). DNA and RNA hybridization was carried out as described previously [30], and the amount of recombinant plasmids hybridized to the mRNA was quantified by scanning the films (see [6]).

Liver histology

The liver biopsy specimens were fixed in neutral formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin, and other stains when needed, as indicated in the text. For electron microscopy

Table 1. Effect of malotilate on liver weight, liver to total body weight ratio, and liver total protein content in CCl_4-induced injury

Rats (48 days old; initial wt. 162.0 ± 15.9 g) received CCl_4 intraperitoneally (0.10 ml/100 g twice weekly) and malotilate orally (10 mg/100 g five times weekly) for 6 weeks. Values are means ± S.D. Significant differences between the groups were the following: *$P < 0.025$, †$P < 0.0025$ and ‡$P < 0.0005$ compared with the control group; §$P < 0.01$, ‖$P < 0.0005$ compared with the malotilate-treated CCl_4-induced group.

Group	Liver wt. (g)	Liver wt./body wt. ratio (%)	Liver total protein (mg)
Control	10.1 ± 1.2	4.8 ± 0.5	445.6 ± 80.6
Malotilate	10.6 ± 0.6	4.9 ± 0.4	533.8 ± 77.8*
CCl_4	14.0 ± 1.9‡§	7.0 ± 0.5‡‖	520.9 ± 135.1
CCl_4 + malotilate	12.1 ± 1.0†	5.4 ± 0.5*	576.8 ± 96.1*

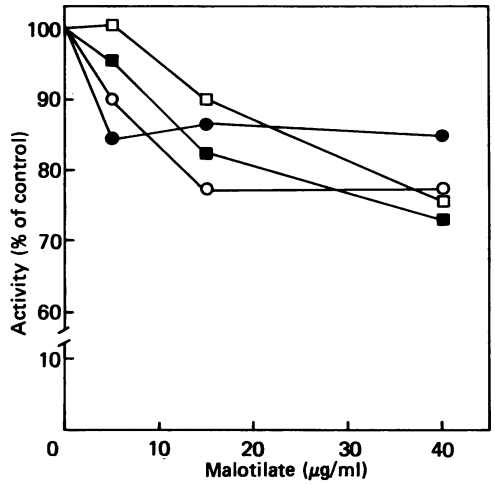


Fig. 1. Effect of malotilate on cultured human skin fibroblasts

Normal human skin fibroblasts were cultured to confluency on cell-culture dishes (diameter 9 cm) in DMEM supplemented with 10% fetal-calf serum and containing 50 μg of ascorbic acid/ml. The medium was then replaced with fresh medium and the cultures were labelled with either [^{3}H]leucine or [^{3}H]proline for 24 h. Parallel cultures were labelled in the presence of malotilate at the concentrations indicated. The total incorporation of radioactive amino acids and the synthesis of radioactive hydroxyproline were measured. Each value is corrected for the DNA concentration of the corresponding cell culture. The secretion of hydroxyproline is calculated as the ratio of the hydroxyproline detected in the medium to the total hydroxyproline content of the cell culture. The results are expressed as percentages of the control cultures. ○, [^{3}H]Proline incorporation; ●, [^{3}H]leucine incorporation; ■, [^{3}H]hydroxyproline synthesized; □, [^{3}H]hydroxyproline secreted.

fresh tissue was fixed in 3% (v/v) glutaraldehyde in phosphate buffer, pH 7.4, post-fixed in 1% OsO_4 in the same buffer, dehydrated, and embedded in Epon. Sections (1 μm) were cut and stained with 1% Toluidine Blue for orientation by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM 100 B electron microscope.

Morphometry to estimate fat deposition was performed by the principle of Weibel, by using a grid lattice superimposed on the projected image [31]. Magnification was ×600, and the density of the fat droplets was estimated in ten representative unselected areas.

RESULTS

Effect of malotilate on animal weight gain, liver to total body weight ratio, and liver total protein content in CCl_4-induced injury

Orally given malotilate had no effect on the weight gain of the 48-day-old female rats (results not shown) or the liver-weight/body-weight ratio, but it did increase the total liver protein content to some extent (Table 1). CCl_4 induction decreased the body-weight gain and increased the liver-weight/body-weight ratio significantly. Malotilate prevented the increase in this ratio (Table 1), but did not restore the body-weight gain (not shown).

Effect of malotilate on CCl_4-induced liver collagen accumulation

No significant differences were found in the hydroxyproline content of the liver between the control and malotilate-treated rats, the values being 2.05 ± 0.34 and 1.81 ± 0.31 mg/liver respectively. The hydroxyproline content was increased significantly ($P < 0.0005$) in CCl_4-induced rats to 3.91 ± 0.48 mg/liver, and malotilate administration partially prevented this collagen accumulation, the value being 2.83 ± 0.48 mg/liver ($P < 0.0025$). Nevertheless, the latter value was significantly higher than that of the controls ($P < 0.0005$).

Effect of malotilate on collagen metabolism in the tissues of young growing normal rats and in cultured human skin fibroblasts

To examine whether the decrease in liver hydroxyproline by malotilate in the CCl_4-induced rats was due to a direct effect on collagen metabolism, two types of experiment were carried out. First, malotilate was given to young growing rats having rapid collagen turn-over. No effect on relative hydroxyproline content in the skin, liver or lungs was noted, and their daily urinary hydroxyproline excretion was also unchanged (results not shown).

Secondly, the effect of malotilate on procollagen

Table 2. Effect of malotilate on liver PH and GGT and serum GGT activities and standard liver-function serum tests in CCl_4-induced injury

Female rats (60 days old) received CCl_4 subcutaneously (0.15 ml/100 g twice weekly) and malotilate orally (10 mg/100 g five times weekly) for 6 weeks. Values are means ± S.D. ($n = 6$). Significant differences were: *$P < 0.0005$ and †$P < 0.005$ between the CCl_4-induced and control groups, and ‡$P < 0.05$, §$P < 0.01$, ‖$P < 0.0025$ and ¶$P < 0.0005$ between the CCl_4-induced, malotilate-treated and the CCl_4-induced groups.

Group	10^{-6} × Liver PH activity (d.p.m./liver)	10^{-6} × Liver GGT activity (d.p.m./liver)	10^{-6} × Serum GGT activity (d.p.m./l)	Serum alkaline phosphatase (units/l)	Serum alkaline amino-transferase (units/l)	Serum aspartate amino-transferase (units/l)
Control	20.3 ± 3.0	5.5 ± 0.7	62.0 ± 5.1	620.4 ± 86.4	59.9 ± 6.1	165.4 ± 28.3
Malotilate	26.3 ± 5.3	7.3 ± 0.8	64.1 ± 6.5	547.8 ± 81.3	57.4 ± 13.1	155.7 ± 64.1
CCl_4	73.0 ± 21.8*	11.3 ± 2.1*	103.2 ± 20.4*	1220.3 ± 401.2*	218.0 ± 131.0†	506.1 ± 191.1*
CCl_4 + malotilate	48.2 ± 20.0‡	8.6 ± 2.1‡	72.7 ± 34.3‖	823.6 ± 183.3§	82.2 ± 16.1‖	209.3 ± 33.6¶

metabolism was studied in fibroblast cultures established from human skin. The confluent cell cultures were incubated for 24 h with various concentrations of malotilate, which exhibited a slight toxic effect on the fibroblasts, the DNA and total protein contents of the cells in culture being 75% and 78% of the control values, respectively, with a malotilate concentration of 50 μg/ml. [^{3}H]Proline and [^{3}H]leucine incorporation into the trichloroacetic acid-precipitable fraction, reflecting total protein synthesis, was decreased by malotilate (40 μg/ml) to 76% and 85% of the control values respectively (Fig. 1). Similarly the synthesis and secretion of [^{3}H]hydroxyproline was apparently decreased in a concentration-dependent manner. The magnitudes of the decreases in total incorporation of radioactivity, hydroxyproline synthesis and secretion were about the same (Fig. 1). Also, the total RNA from the cell cultures of a similar experiment was extracted and the amount of proα1(I) mRNA estimated by dot-blot hybridization by using recombinant plasmid Hf677, which contains a cDNA insert for the human proα1(I) mRNA. Malotilate in concentrations ranging from 5 to 40 μg/ml had no significant effect on the concentration of proα1(I) mRNA, the values ranging between 101 and 110% of the control mean (results not shown).

Effect of malotilate on liver PH, liver and serum GGT activities and standard liver-function serum tests

To examine further the mechanism by which malotilate prevents liver collagen accumulation, another experiment was performed in which acute liver damage was induced by injecting CCl_4 subcutaneously (0.15 ml/100 g) into 60-day-old female rats for 6 weeks. Some of the test animals simultaneously received malotilate (10 mg/100 g). Liver PH and GGT and serum GGT were measured at the end of the experiment. Standard liver-function tests were also carried out, and samples were taken for liver histology. CCl_4 induction significantly increased liver PH and GGT activities and serum GGT, alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase activities (Table 2).

All these values were significantly lower in the malotilate-treated CCl_4-induced group (Table 2).

Effect of malotilate on liver histology

Malotilate alone did not cause any morphologically detectable changes relative to the untreated control, whereas CCl_4 caused severe fatty infiltration, focal necrosis and inflammation (Fig. 2*a*). The hepatic parenchymal cells were slightly enlarged, with an increase in the smooth endoplasmic reticulum and a decrease in the rough endoplasmic reticulum. Changes were also observed in the mitochondria, lysosomes and cell membranes. The sinusoids were dilated, with Kupffer-cell proliferation, focal congestion and an increase in collagen fibres (Fig. 2*c*). Some mononuclear infiltrates were found in the periportal areas. Fat droplets predominated in the cytoplasm, ranging in size from minimal ones observed only by electron microscopy to droplets filling the entire cytoplasm and displacing the nuclei. The fatty droplets were most numerous in pericentral locations (Figs. 2*a* and 2*c*).

When malotilate was given simultaneously with CCl_4, a distinct decrease in the morphological alterations was observed. Cell size decreased, and the changes in smooth and rough endoplasmic reticulum reverted almost to normal, as did the other cytoplasmic components. The sinusoids were narrow, necrosis and fatty changes were slight, as was connective-tissue proliferation, and inflammatory changes were virtually non-existent (Figs. 2*b* and 2*d*).

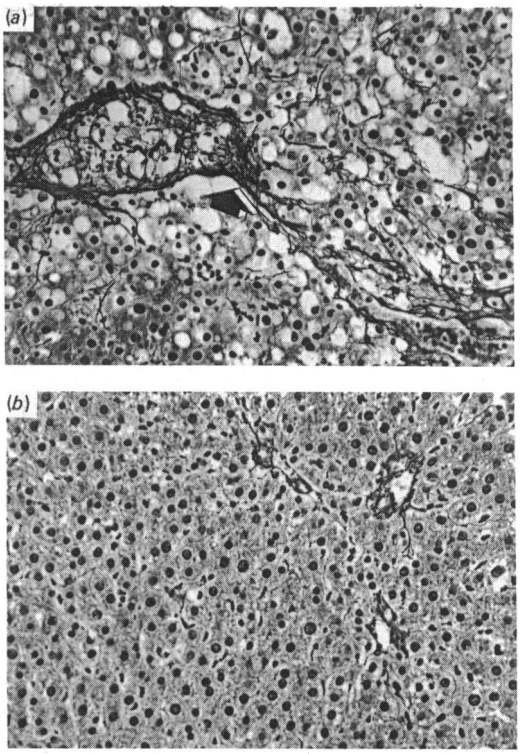
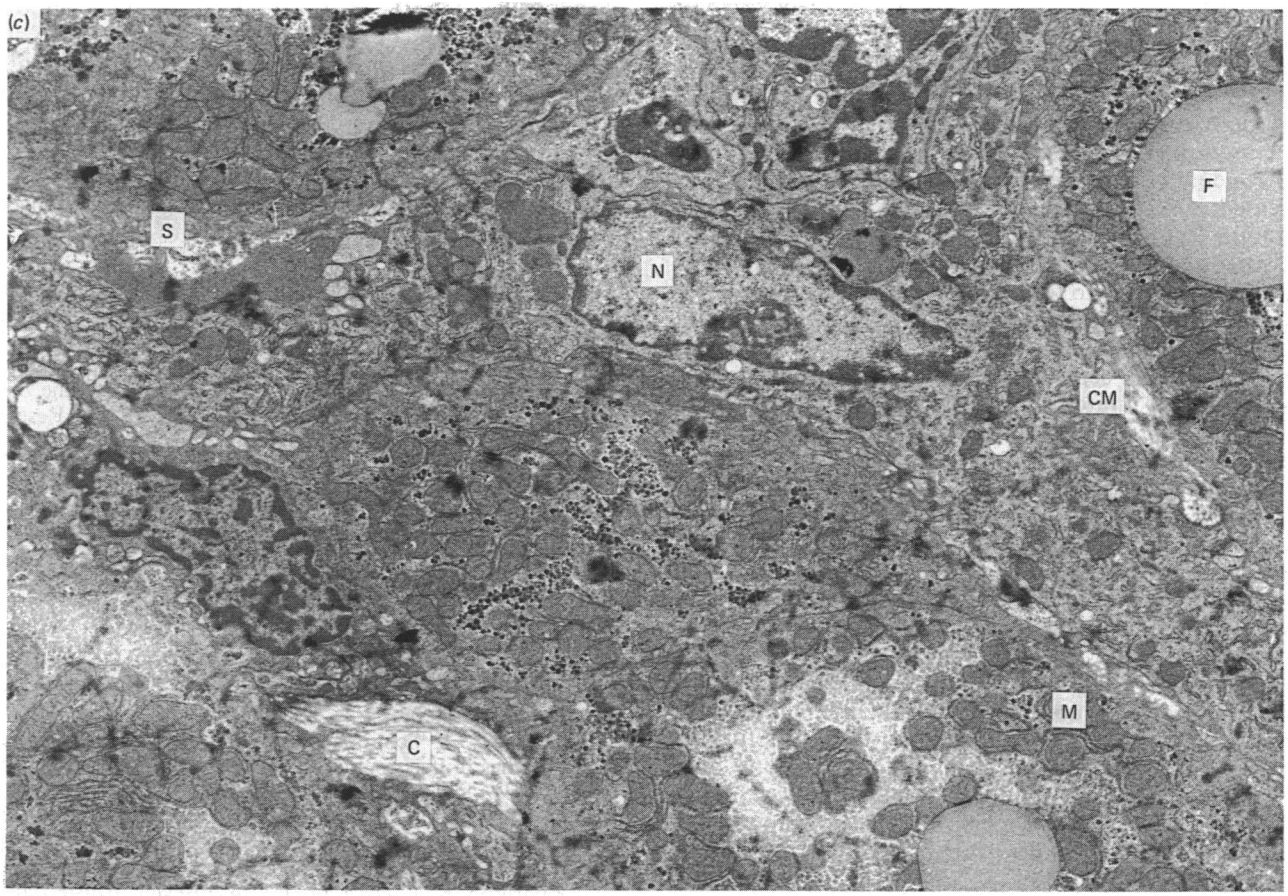
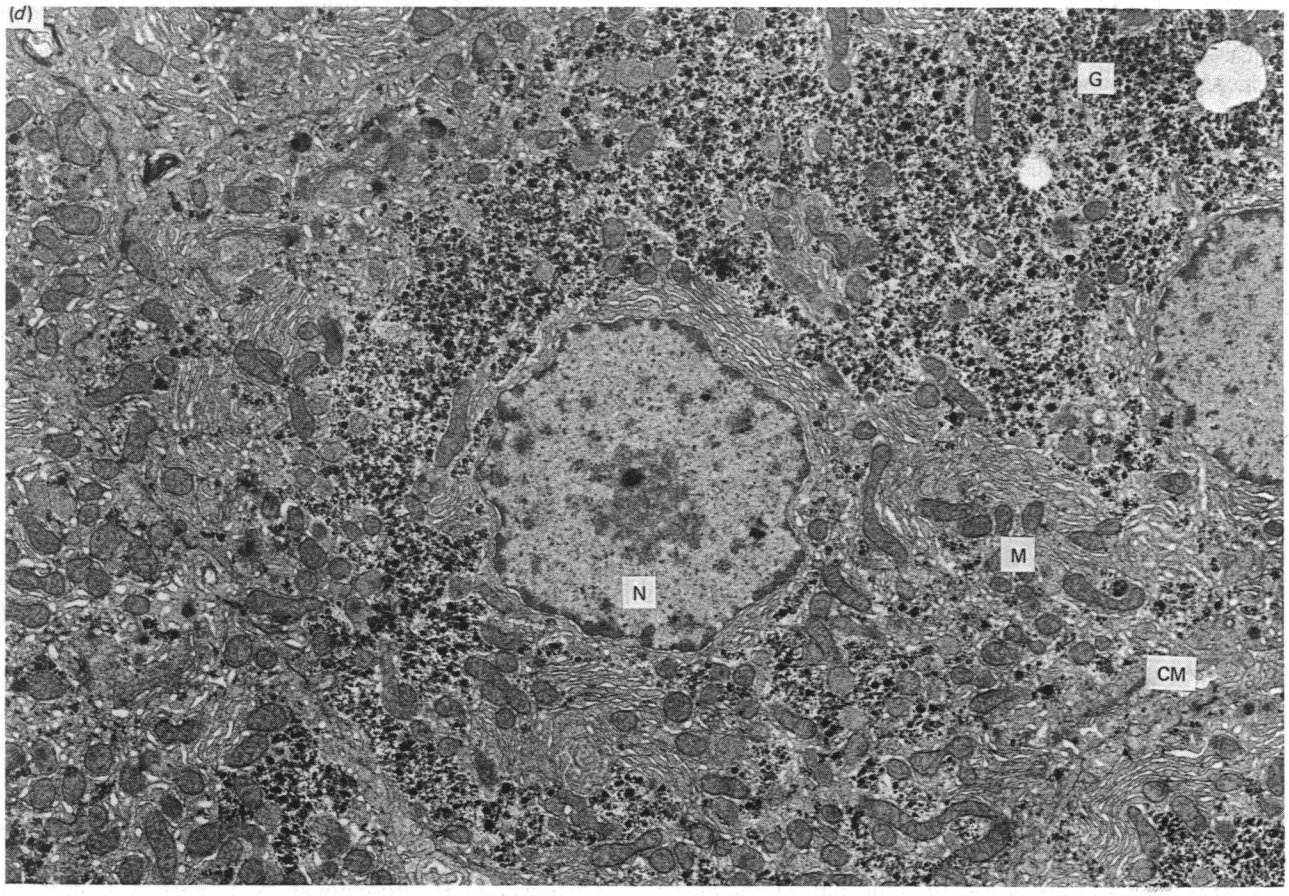


Fig. 2. Effect of malotilate on liver histology

Liver damage was induced in 60-day-old female rats by CCl_4, as described in the Materials and methods section and in Table 2. Some of the animals received malotilate (10 mg/100 g) simultaneously. (*a*) The liver of a CCl_4-induced rat showing severe morphological alterations with fatty droplets and increase in collagen fibres (⬆). Reticulin stain; magnification × 360. (*c*) The liver of a CCl_4-induced rat with cells containing irregular cell membranes (CM) and nuclei (N), pleomorphic mitochondria (M), abundant fatty droplets (F) of various sizes, and increase in collagen fibres (C) in sinusoids (S). Uranyl acetate/lead citrate stain; magnification × 4800. (*b*) The liver of a malotilate-treated CCl_4-induced rat with preserved architecture, except for some perinuclear vacuolization. Reticulin stain; magnification × 360. (*d*) The liver of a malotilate-treated CCl_4-induced rat showing regular hepatocytes with few fatty droplets, preserved cell membranes (CM), mitochondria (M), regular nuclei (N) and abundant glycogen (G). Uranyl acetate/lead citrate stain, magnification × 4800.

Morphometry performed on the liver histology showed all of the animals in the CCl_4-induced group to have fatty changes in the liver, one of them mild (fatty changes < 5%), four moderate (fatty changes 5–10%) and seven severe (fatty changes 10–20%), the total being 12. By contrast, the malotilate-treated CCl_4-induced group included five livers without fatty changes, two with mild and three with moderate changes, and none falling into the severe category (the total was 10). The average densities of fat by volume in these groups were 11% and 3.1% respectively.


(c)
S
N
F
CM
M
C


(d)
G
M
N
CM

DISCUSSION

Experimental liver fibrosis and cirrhosis can be induced by a variety of techniques. In the CCl_4 model used here, early liver damage is followed by an increase in connective tissue [32]. The pathogenesis of this experimentally induced condition involves progressive repeated episodes of focal hepatocyte necrosis, fibrosis resulting from the collapse of the pre-existing collagen network, and collagen synthesis *de novo*, regeneration of surviving liver cells to form nodules, and development of broad connective-tissue septa [32]. The initial hepatocytic lesion is centrilobular, as also shown here, and connective-tissue septa radiate progressively from this and eventually unite [33,34]. A clear increase in total hydroxyproline content was noted in the CCl_4-induced liver, reflecting synthesis *de novo* of liver collagen and an increase in its amount, as well as focal necrosis, fatty infiltration and signs of inflammation. Furthermore, such markers of collagen metabolism as the activities of liver PH and serum and liver GGT were also significantly above the control values.

CCl_4 induction increased the liver-weight/body-weight ratio significantly, whereas malotilate given simultaneously with CCl_4 was able to normalize this ratio almost entirely. In contrast, the increase in the total protein content of the liver caused by CCl_4 was further accentuated by malotilate, and malotilate alone also somewhat increased the liver protein content. This observation is in agreement with previous reports on malotilate stimulating liver protein synthesis, referred to as a 'hepatotrophic' effect [35,36].

Malotilate appeared to have a protective effect against the liver damage caused by CCl_4. It prevented the increase in the total hydroxyproline content of the liver and decreased the morphological changes. The activities of liver PH and serum and liver GGT were also decreased, as well as the values obtained in the standard serum liver function tests.

When malotilate was given to normal growing young rats, it did not affect the hydroxyproline content of various tissues or urinary hydroxyproline excretion. Similarly, in skin fibroblast cultures no specific effect was found on the synthesis or secretion of hydroxyproline or the content of the proα1(I)-specific mRNA species. Thus malotilate seems to have no direct effect on the metabolism of collagen.

A considerable body of evidence has now accumulated indicating that immune and inflammatory cells modulate collagen metabolism. Lymphocytes, macrophages and monocytes affect fibroblast metabolism through the secretion of peptide and protein factors capable of either stimulating or inhibiting fibroblast proliferation, fibroblast movement, or collagen and collagenase production [2,4,5,37,38]. In addition, collagen-synthesis-stimulating factors have been identified in CCl_4- or thioacetamide-induced liver injury in mice and rats, and in cirrhotic human liver [4,38]. Thus it has been suggested that liver fibrosis and cirrhosis, and also other tissue fibroses, may be at least partly caused by an inflammation, in which the accumulated inflammatory cells trigger the fibrosis by secreting the above-mentioned fibroblast-stimulating factors [2,4,37,38]. We observed in the present study a distinctive decrease in CCl_4-induced inflammatory changes by the administration of malotilate, concomitantly with the decrease in collagen accumulation, and we thus suggest that the preventive effect of this compound with respect to CCl_4-induced fibrotic changes in liver may be inhibition of the inflammatory response in the liver. Malotilate also clearly had other favourable effects on CCl_4-induced liver damage, and the elucidation of the primary mechanism by which it exerts this protective action warrants further investigation. Malotilate is certainly a potential new drug for use in treating chronic liver diseases.

We thank Professor Kari I. Kivirikko for his valuable criticism, and Miss Arja Oja for her expert technical assistance. The recombinant plasmid Hf677 was kindly given by Dr. M.-L. Chu, Dr J. C. Myers and Dr F. Ramirez. This study was supported financially by Zyma A.G. (Switzerland), the Sigrid Jusélius Foundation (Finland), and the Medical Research Council of the Academy of Finland.

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Received 16 January 1987/11 May 1987; accepted 26 May 1987