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EFFECTS OF NOVEL DITHIOL MALONATE DERIVATIVES ON LIVER LIPID PEROXIDATION AND ON MICROSOMAL ELECTRON TRANSPORT SYSTEM

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ABSTRACT: *The effects of 5 novel hepatotrophic agents, dithiol malonate derivatives (DMDs; DMD1-DMD5), on the liver microsomal lipid peroxidation induced by carbon tetrachloride (CCl_4) and the correlations with the changes of microsomal electron transport system were investigated. All DMDs were found to inhibit the lipid peroxidation induced by CCl_4 in mice and rats as well in vitro liver microsomal system. Therefore, each DMD seemed to have direct mode of action on liver microsomes to inhibit the lipid peroxidation. As an ex vivo study, the induced lipid peroxidation by CCl_4 and the changes in electron transport system were determined with liver microsomes obtained from rats chronically treated with DMDs for 7 days. The induced lipid peroxide contents in liver microsomal system were lower in DMD1, DMD2 and DMD3 treated group, but higher in DMD4 and DMD5 group when compared to the control group. Cyt. p-450 contents in the microsomes were decreased by the treatment with DMD1, DMD2 and DMD3, but increased significantly by DMD4 with great extent and by DMD5 with less extent. The cyt. p-450 isozymes induced by treatment of DMD4 and DMD5 were identified as 3-methylcholanthrene (MC) type. The NADPH cyt. -C reductase activities of the microsomes treated with DMD1, DMD2, DMD4 and DMD5 were increased in the range of around 20% to 50%, but decreased with DMD3, All DMDs increased cyt. $-b_5$ content and did not alter NADH-cyt, $-b_5$ reductase activities in the microsomes. In summary, the 5 novel hepatotrophic agents (DMDs) markedly protected against lipid peroxidation induced by CCl_4 in vivo* and *in vitro* possibly through the mechanism of direct action on the liver microsomes. The degree

Abbreviations: DMD1, isopropyl 2-(1,3-dithiol-2-ylidene)-2-(N-(thiazol-2-yl) carbamoyl) acetate; DMD2, isopropyl 2-(1,3-dithol-2-ylidene)-2-(N-(4-methylthiazol-2-yl) carbamoyl) acetate; DMD3, isopropyl 2-(1,3-dithiol-2-ylidene)-2-(N-(2-pyridyl) carbamoyl) acetate; DMD4, isopropyl 2-(1,3-dithiol-2-ylidene)- 2-N-(p-trifluoromethylphenyl) carbamoyl) acetate; DMD5, isopropyl 2-(1,3-dithiol-2-ylidene)-2-(N-(1,3,4-thiadia-zol-2-yl) carbamoyl) acetate.

of inhibition produced by DMDs on lipid peroxidation induced by CCl_4 seemed to coincide rather with cyt. p-450 contents than with other components of liver microsomal electron transport system including NADPH-cyt, -C reductase.

INTRODUCTION

Hapatotoxicity of carbon tetrachloride (CCl_4) is one of great importance to medical science because it provides models for the study of potential hepatotrophic or hepatotoxic agents. CCl_4, the prototype of direct hapatotoxicants, produces lipid peroxidation, chemical alterations in the plasma membranes (1), and denaturation of proteins (2).

It has been known that the liver microsomal electron transport system is involved in liver toxicity of CCl_4 because the activation of CCl_4 through metabolism elicits the damage of microsomal membranes and eventual liver damages (3). The first step in the metabolic activation is the formation of reactive species, trichloromethyl free radical, which can become covalently bound to components of microsomes (4). In the process, a chain reaction of peroxidation of the membrane polyunsaturated fatty acids is initiated. This lipid peroxidation occurs throughout all membrane lipid in an autocatalytic fashion producing extensive membrane damage and cell necrosis (5).

Recently, a chemically unique hepatotrophic agent, diisopropyl-1,3-dithiol-2-ylidene malonate(malotilate), has been developed as a drug for chronic hepatitis and liver cirrhosis. It has been reported to alter the activities of liver microsomal electron transport system, and NADPH-dependent lipid peroxidation. They pointed out that its inhibitory effects against liver toxicity might be due to the increased contents of cyt b_5 in liver microsomes (6,7).

Five novel dithiol malonate derivatives (DMDs; DMD1-DMD5) had been synthesized in our laboratories and they revealed to have more potent protective activities than malotilate against liver damage caused by CCl_4 previously (8). In the present study, their protective and therapeutic effect against lipid peroxidation and fatty liver induced with CCl_4 were investigated. The effects of DMDs on the activities of microsomal electron thansport system including cyt p-450, cyt b_5, NADPH cyt $-C$ reductase and NADH cyt b_5 reductase were also examined with the intention of examining the potential correlations with their inhibitory effect upon the lipid peroxidation.

MATERIALS AND METHODS

1. Protective effect of DMDs against lipid peroxidation in mice

Male ICR strain mice weighing 18-22g were used. DMDs suspended in 0.2% (w/v) carboxymethyl cellulose sodium(CMC) solutions were administered orally at the dose of 250 mg/kg or 100 mg/kg. CCl_4 solution (0.2% v/v, 0.5ml/20g) in olive oil was

administered orally 6 hr after the DMDs treatment. All animals were sacrificed 16 hr after CCl_4 injection. Livers were removed and homogenized in 4 volumes of 1.15% KCl solution. The lipid peroxide content of liver homogenate was measured according to Ohkawa *et al* (9).

2. Therapeutic effect of DMDs against fatty liver in rats

Male Sprague-Dawely strain rats weighing 180-220g were used. The rats were pretreated intraperitoneally with CCl_4 solution (50% v/v, 0.2ml/100g) in olive oil daily for 3 days. DMDs were administered orally at the dose of 25mg/kg concomitantly with the CCl_4 treatment for successive 4 days. All animals were sacrificed 24 hr after the last treatment. Livers were perfused with ice-cold saline, minced in ice-cold 1.15% KCl solution and homogenized in 4 volumes of the KCl solution. The lipid peroxide content of the liver homogenate was measured (9). Triglyceride and total cholesterol of homogenate were determined by enzymatic methods using commercial test kits (Eiken Chemical Co., Tokyo, Japan) and total lipid was determined by sulfophosphovanillin reaction using commercial test kits (E. Merck, Darmstadt, West Germany).

3. Inhibition of microsomal lipid peroxidation in vitro

Male Sprague-Dawely strain rats weighing 180-200g were used. Liver from rats previously fasted for 16 hr were perfused with ice-cold saline, pooled and homogenized in 4 volumes of 1.15% KCl solution. The homogenate was cerntrifuged 10,000g for 20 min in a refrigerated centrifuge. The resulting supernatant was further centrifuged 102,000g for 60 min to harvest microsomal fraction. After washing with 1.15% KCl solution, the pellet was suspended in 0.1 M potassium phosphate buffer (pH 7.4) and immediately frozen at −80°C for later experiments. Protein concentration in microsome was measured by Lowry method using bovine serum albumin as a standard (10).

Microsomal lipid peroxidation was determined by the method of Abe *et al* with slight modification (11). The reaction mixture containing 4 mg of microsomal protein and various concentrations of DMDs ($1 \times 10^{-3} - 5 \times 10^{-6}$ M) in 0.1 M phosphate buffer (pH 7.4) was preincubated for 5 min at 37°C. The reaction was started by the addition of 0.4 mg NADPH and 10ul CCl_4 and the mixture incubated for 40 min at 37°C. The reaction was stopped by the addition of 0.2 ml of 15% TCA solution and the mixture centrifuged at 10,000 rpm for 10 min. The lipid peroxide content of the supernatant fraction was determined.

4. Effect of DMDs on microsomal electron transport systm

Male Sprague-Dawely stain rats weighing 180-200 g were used. DMDs suspended in 0.2% CMC solution were administered orally at a dose of 250 mg/kg for 7 days. All animals were sacrificed 24 hr after the final administration of DMDs. Liver microsomes were prepared as described above. The degree of lipid peroxidation induced by CCl_4 in the microsomes was assessed as descrbed above (11).

The contents of cyt p-450 and cyt h_5 in the microsomes were determined spectrophotometrically using extinction coefficients of 91 and 185mM^{-1} cm^{-1}, respectively, as described by Omura *et al* (12). The activity of NADPH cyt. C reductase was determined by the method of Philips *et al* (13) and the enzyme activity was calculated as nmole of cytochrome C reduced per min per mg protein using extinction coefficient of 19.6 mM^{-1} cm^{-1}. The activity of NADH cyt b_5 reductase was determined by the method of Takesue *et al* (14) and its activity was calculated as umole of ferricyanide reduced per min per mg protein using extinction coefficient of 1.02 mM^{-1} cm^{-1}.

5. Identification of major form of induced cyt. p-450 isozymes in microsomes from DMDs treated rats

The major form of liver microsomal cyt. p-450 induced with DMDs was identified with PB-cyt. p-450 (MAb-PB)or MC-cyt. p-450 specific monoclonal antibodies (MAb-MC). [^{35}S]-labeled anti-mouse IgG(k) was used as a second antibody for the detection of MAbs bound to cyt. p-450 isozymes in solid phase (35,000 cpm/3.7ug/well) (15). Liver microsomal proteins from rats treated with DMDs were seperated by SDS polyacrylamide gel electrophoresis by the method of Laemmli (16). The separated microsomal proteins were electrophoretically transfered to a nitrocellulose sheet (17) and antigenic components reactive to MAb-PB or MAb-MC were immunostained using goat antimouse IgG conjugated with alkaline phosphatase and BCIP/NBT phosphatase substrate system (Kirkegard and Perry Lab., Rockvile, MD, US) (18).

Aryl hydrocarbon hydroxylase (AHH) activity was determined by measuring the amount of benzo(a)pyrene (BP) conversion to phenolic products equivalent to 3-OH-BP by the method of Nebert *et al* (19). 7-Ethoxycoumarin O-deethylase (ECDE) activity was determined by the method of Greenlee *et al* (20). For the analysis of enzyme inhibition by MAbs, 50ug of microsomal protein was preincubated with 200ug of MAbs in a final volume of 0.5m*l* PBS for 15 min at room temperature, and then the mixture was assayed for AHH or ECDE activity.

RESULTS

1. Protective effect of DMDs against lipid peroxidation in mice

Lipid peroxide content of liver homogenate from CCl_4 treated animals was increased to 3.5 times that of normal group. Pretreatment of animals with DMDs significantly prevented the increase of lipid peroxidation induced with CCl_4. As shown in Fig. 1, the inhibitory activities of DMD1 to DMD5 against the lipid peroxidation are 97%, 82%, 35%, 69% and 57%, respectively, at the dose of 250mg/kg, and 36%, 39%, 29%, 49% and 28%, respectively, at the dose of 100mg/kg.

2. Therapeutic effect of DMDs against fatty liver in rats

The contents of lipid peroxide and hepatic lipids in the liver homogenates of animals chronically treated with CCl_4 for 7 days were significantly higher than those of normal

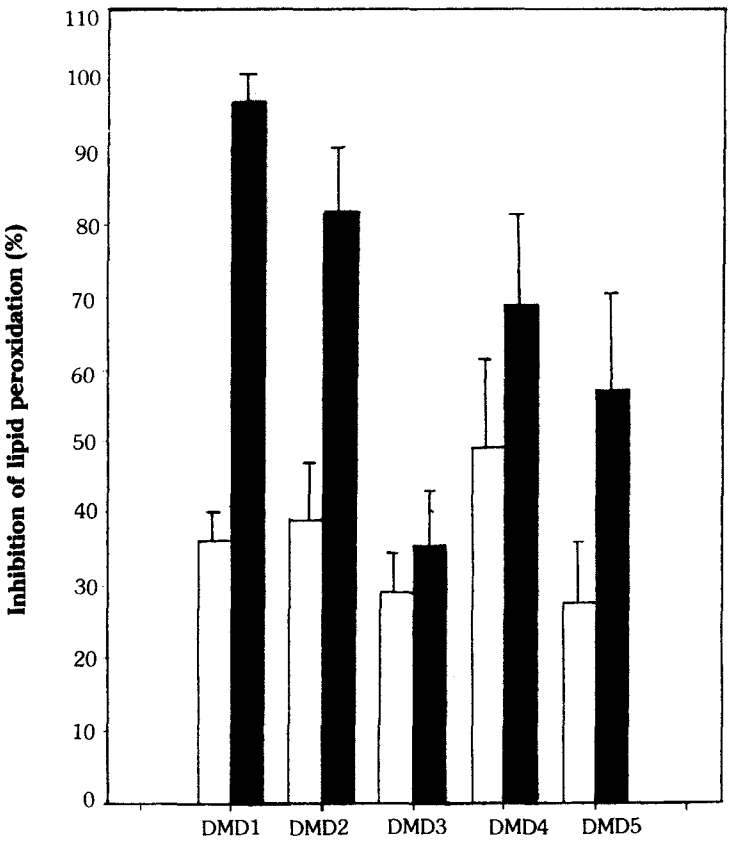

Fig. 1. Protective effect of dithiol malonate derivatives against lipid peroxidation in mice. The lipid peroxide contents were determined as described in the text. All values represent inhibition percentages of DMDs against lipid peroxidation induced by CCl_4. Open column denotes the DMDs-treated group at the dose of 100mg/kg and closed column at 250mg/kg. The bar on the column indicates the standard error.

group. Concomitant treatment of animals with DMDs for the last 4 days suppressed the increase of lipid peroxidation and hepatic lipid contents. As shown in Table 1, the inhibitory activities of DMD1 to DMD5 against lipid peroxidation are 80%, 75%, 55%, 59% and 56%, against triglyceride content, 48%, 43%, 46%, 37% and 30%, against total cholesterol, 46%, 9%, 18%, 36% and -18% and against total lipid, 46%, 30%, 42%, 38% and 22%, respectively.

3. Inhibition of microsomal lipid peroxidation in vitro

DMDs inhibited lipid peroxidation induced with CCl_4 not only *in vivo* but also *in vitro*. When microsomal protein obtained from normal rats was preincubated with various concentrations of DMDs before adding CCl_4 and NADPH to the reaction mixture *in vitro*, lipid peroxide formed during the incubation period was decreased in a concentration dependent manner. The order of inhibitory activities of DMDs on lipid peroxidation is DMD2 > DMD3 > DMD1 > DMD5 > DMD4 (Fig. 2).

Table 1. Therapeutic effect of dithiol malonate derivatives against fatty liver induced by CCl_4

Treatment	Lipid peroxide (nmole/g liver)	Triglyceride (mg/g liver)	Cholesterol (mg/g liver)	Total lipid (mg/g liver)
Control	437 ± 24	11.9 ± 0.3	2.4 ± 0.1	40.5 ± 1.7
CCl_4	1130 ± 206**	56.7 ± 1.0***	3.5 ± 0.2***	146.6 ± 8.7***
DMD1	575 ± 93#	35.2 ± 5.9**##	3.0 ± 0.1**#	97.4 ± 14.2**#
DMD2	612 ± 80#	37.4 ± 6.1**#	3.4 ± 0.1***	114.7 ± 6.5***#
DMD3	752 ± 44***	36.0 ± 4.0***##	3.3 ± 0.1***	102.6 ± 5.7***##
DMD4	719 ± 59**	40.3 ± 3.6***##	3.1 ± 0.1***#	106.1 ± 4.4***##
DMD5	742 ± 41***	43.2 ± 4.7***#	3.7 ± 0.1***	122.9 ± 15.1**

Data represent mean ± SE of six animals.
Statistical significance vs control group assessed by one way ANOVA, *;P<0.05, **;P<0.01, ***; P<0.001.
Statistical significance vs CCl_4 treated group assessed by one way ANOVA, #;P<0.05, ##;P<0.01, ###;P<0.001.
The rats were pretreated with CCl_4 solution (50% v/v, 0.1ml/100g i.p.) in olive oil once daily for 3 days.
DMDs (25mg/kg, p.o.) were administered concomitantly, with the CCl_4 treatment for successive 4 days.

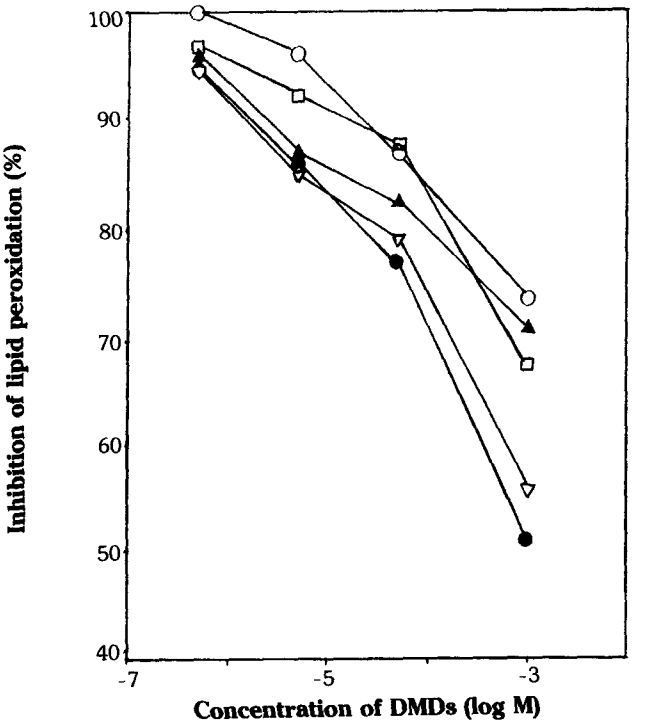

Fig. 2. Inhibition of dithiol malonate derivatives against microsomal lipid peroxidation in vitro. The microsomes obtained from normal rats were preincubated with various concentrations (1×10^{-3} -5×10^{-6}) of DMDs before adding CCl_4 and incubation mixtures were subjected to TBA reaction for measuring lipid peroxide contents. All values represent inhibition percentage of lipid peroxidation determined by addition of CCl_4. Open circle denotes DMD5, closed triangle denotes DMD4, open square denotes DMD1, open triangle DMD3 and closed circle denotes DMD2. Each values represents the mean values obtained from triplicate determinations.

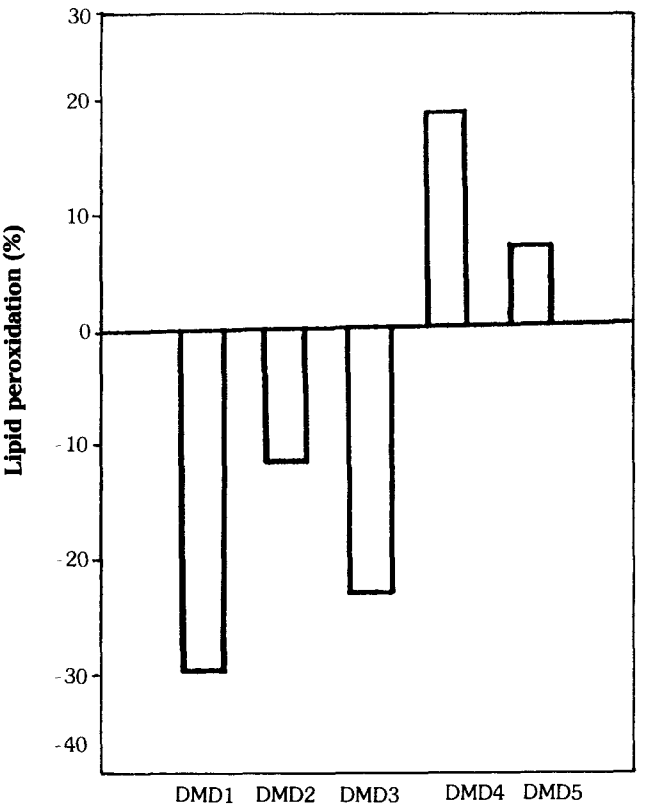

Fig. 3. Determination of lipid peroxidation in liver microsomal system from rats chronically treated with dithiol malonate derivatives ex vivo. DMDs were administered orally to rats at the dose of 250mg/kg for 7 days. Lipid peroxidations were assessed as described in the text. All values are expressed as percent changes of lipid peroxidation from liver microsomes of control rats. Each value represents the mean value obtained from duplicate determinations with pooled microsomes.

Table 2. Effects of dithiol malonate derivatives on liver microsomal electron transport system

Treatment	Cyt. P-450 content (nmole/mg protein)	Cyt. b_5 content (nmole/mg protein)	NADPH-cyt. c reductase (nmole/mg/min)	NADH-cyt. b_5 reductase (μmole/mg/min)
Control	0.59 ± 0.01	0.28 ± 0.02	69.8 ± 4.4	2.8 ± 0.2
DMD1	0.51 ± 0.03*	0.36 ± 0.01**	85.9 ± 3.7*	2.8 ± 0.2
DMD2	0.52 ± 0.02*	0.36 ± 0.01**	74.8 ± 4.7	2.8 ± 0.2
DMD3	0.40 ± 0.07*	0.38 ± 0.04*	66.8 ± 8.2	2.6 ± 0.2
DMD4	1.01 ± 0.08***	0.45 ± 0.05***	94.1 ± 5.5***	2.8 ± 0.1
DMD5	0.69 ± 0.02***	92.9 ± 6.8*	3.0 ± 0.2	

Data represent mean + SE of five animals.

Statistical significance vs control group assessed by one way ANOVA, *; $P<0.05$, **; $P<0.01$, ***; $P<0.001$.

Dithiol malonate derivatives were administered orally to rats at a dose of 250mg1kg for 7 days.

NADPH cyt C reductase activity was calculated as nmole of cytochrome c reduced per min per mg of microsomal protein.

NADH cyt. b_5 reductase activity was calculated as umole of ferricyanide reduced per min per mg of microsomal protein.

Table 3. Characteristics of liver microsomal cyt. p-450 from rats treated with dithiol malonate derivatives.

Treatment	Monoclonal antibodies	Binding to cyt. p-450 RIA (cpm)	Immunoblotting
Control	NBS	2070	–
	MAb-PB	2423	–
	Mab-MC	2530	–
DMD 1	NBS	1978	–
	MAb-PB	2062	–
	MAb-MC	2937	–
DMD 2	NBS	2331	–
	MAb-PB	2051	–
	MAb-MC	3692	–
DMD 3	NBS	2452	+
	MAb-PB	2243	–
	MAb-MC	3462	–
DMD 4	NBS	2330	–
	MAb-PB	1863	–
	MAb-MC	5929	+
DMD 5	NBS	2110	–
	MAb-PB	2262	–
	MAb-MC	4860	+

NBS (normal BALB1c mouse spleen cell hybridoma) was used as a control MAb.
MAb-PB and MAb-MC were specific MAb for purified cyt. p-450 in liver microsome from rats treated with PB and MC, respectively.

In the solid phase RIA, microsome (100ug) was precoated on at 4°C overnight and incubated with MAbs at 37°C for 2hr. The bound antibodies were detected with [^{35}S]-methionine-labeled rat anti-mouse IgG (k) (35,000 cpm/ 3.7μg/well).

4. Effects of DMDs on microsomal electron transport system

The contents of lipid peroxide formed by CCl_4 in liver microsomes from rats of control and treated with DMD1 to DMD5 were 4.35 (100%), 3.06 (70%), 3.85 (89%), 3.35 (77%), 5.18 (119%), and 4.66 (107%) nmole MDA/mg protein, respectively, (Fig. 3). As shown in Table 2, the microsomal contents of cyt. p-450 were significantly increased in DMD4 (172%) and DMD5 (118%) treated group, but decreased in DMD1 (87%), DMD2 (89%) and DMD3 (68%) treated groups. The microsomal contents of cyt. b_5 were significantly increased to about 129%, 127%, 135%, 162% and 160% of the control level in DMD1 to DMD5 treated groups, respectively. The activities of NADPH-cyt. C reductase were significantly increased in DMD1 (123%), DMD4 (135%) and DMD5 (123%) treated group, but not significantly changed in DMD2 and DMD3 treated groups. The activities of NADH cyt. b_5 reductase were not significantly changed in all of DMDs treated groups.

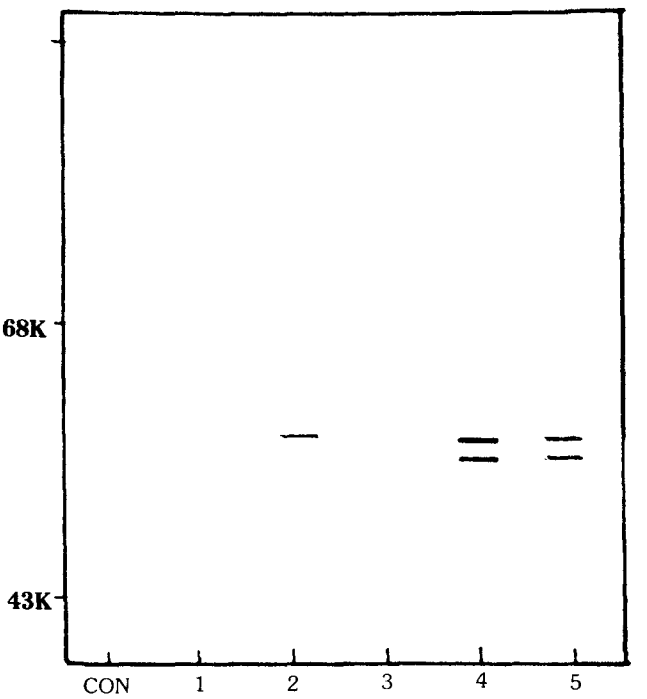

Fig. 4. Immuno-Western blots of microsomal cyt. p-450 isozymes from rats treated with dithiol malonate derivatives. Liver microsomes were obtained from rats treated DMDs (250mg/kg, p.o.) for 7 days. After SDS-polyacrylamide gel electrophoresis of liver microsomes (10 μg protein), the cyt. p-450 components were detected using MAb-MC. Expression of lanes: con. control; 1 to 5, DMD1 to DMD5.

5. Identification of major form of induced cyt. p-450 isozymes in microsomes from DMDs treated rats

As shown in Table 3, the microsomes from DMD2, DMD4 and DMD5 treated animals reacted positively with MAb-MC in RIA. The specific bindings of MAbs for cyt. p-450 isozymes in microsome treated with DMDs were further examined by immunoblotting. Nonspecific MAb and MAb-PB did not bind any microsomal protein from animals treated with DMDs. MAb-MC binded the microsomal protein from animals treated with DMD2, DMD4 or DMD5. The liver microsomes from DMD4 or DMD5 treated rats exhibited two apparent bands at the position of cyt. p-448L (M.W. 54,000) and cyt. p-448H (M.W. 56,000) while liver microsome from DMD2 treated rats exhibited a clear band of cyt. p-448L and an obscure band of cyt. p-448H (Fig. 4).

As shown in Table 4, the microsomal ECDE activities were higher in DMD1 (148%), DMD2 (165%), DMD4 (1,075%), and DMD5 (511%) treated groups, but lower in DMD3 (83%) treated group when compared to the control group. The AHH activities were higher in DMD1 (127%), DMD2 (133%), DMD4 (537%), and DMD5 (330%) treated groups, but lower in DMD3 (83%) treated group. MAb-PB showed no inhibitory effect on ECDE activities in liver microsomes of rats treated with DMD1 to DMD5. MAb-MC inhibited the microsomal ECDE activities in the rats treated with DMD1-DMD5 by up to 13%, 38%, 64%, 19%, 76% and 73%, respectively. MAb

Table 4. Effect of monoclonalantibodies on AHH and ECDE activities of liver microsome from rats treated with dithiol malonate derivatives.

Treatment	Monoclonal antibodies	ECDE activity (7-OH-C pmole/ mg/min)	% changes	AHH activity (3-OH-BP pmole1 mg/min)	% changes
Control	None (control)	530	0	494	0
	NBS	658	24	609	23
	MAb-PB	701	32	609	23
	MAb-MC	445	−16	461	−7
DMD 1	None (control)	786	0	626	0
	NBS	744	−5	609	−3
	MAb-PB	829	5	774	24
	MAb-MC	488	−38	362	−42
DMD 2	None (control)	872	0	659	0
	NBS	1256	44	873	33
	MAb-PB	1467	64	939	43
	MAb-MC	317	−64	395	−40
DMD 3	None (control)	445	0	412	0
	NBS	658	48	494	20
	MAb-PB	573	29	428	4
	MAb-MC	359	−19	263	−36
DMD 4	None (control)	5696	0	2653	0
	NBS	5995	5	3345	26
	MAb-PB	6806	20	2884	9
	MAb-MC	1384	−76	906	−66
DMD 5	None (control)	2707	0	1631	0
	NBS	2878	6	2060	26
	MAb-PB	2920	8	1302	−20
	MAb-MC	734	−73	708	−57

NBS (normal BALB/c mouse spleen cell hybridoma) were used as a control MAb.
MAb-PB and MAb-MC were specific MAb for purified cyt. p-450 in liver microsome from rats treated with PB and MC, respectively.

Microsome (50μg) and antibodies (200μg for ECDE and AHH) were preincubated in a final volume of 0.5ml PBS at room temperature for 15 min and subjected to ECDE and AHH assay at 37°C for 10 min.

-PB showed no inhibitory effect on AHH activities in liver microsomes of rats treated with DMDs except DMD5, where, the MAb-PB inhibited the AHH activities of liver microsomes of rats treated with DMD5 by about 20%. MAb-MC inhibited the microsomal AHH activities in the rats treated DMD1 to DMD5 by 42%, 40%, 36%, 66% and 57%, respectively.

DISCUSSION

There is a wealth of information regarding initial steps in hepatotoxin metabolism

and end-stage structural and functional pathological changes as the eventual result of the action of hepatotoxin on the hepatocytes (3,5). The panorama of the pathological changes includes early breakup of ER with loss of associated enzymatic function, fat accumulation, dispersal of polyribosomes with loss of protein synthetic capacity, malfunction of some mitochondria functions, breakdown of the cell membrane with leakage of cytosolic components to extracellular fluid and eventual necrosis. However, the intermediate pathological processes standing between initial metabolism and end-stage pathological conditions have not been clearly known. But the liver injury caused by CCl_4 stands out as a somewhat exceptional case. It is now generally accepted that lipid peroxidation may play a key role in the intermediate pathological conditions. The basic sequence of events involves initial generation of trichloromethyl radical by the microsomal electron transport systems. These initial events are accompanied by covalent binding of the CCl_4 cleavage product largely to lipids and proteins of cellular ER, and by the initiation of lipid peroxidation. The destructive character of lipid peroxidation in itself is assumed to constitute a sufficient cause for the manifold pathological effects (21). In the present study, CCl_4 treated animals were chosen as an animal model system of hepatic injury upon which the inhibitory effects of DMDs on lipid peroxidation and the changes of liver microsomal electron reansport systems by DMDs were investigated.

All DMDs exerted protective and therapeutic effects upon hepatic lipid peroxidations induced by CCl_4 in rats and mice. Other hepatoprotective agents has been also reported to suppress the enhanced lipid peroxidation in the liver through different mechanisms. For example, (+)-cyanidanol-3 and diethyl dithiocarbamate (dithiocarb) may act as antioxidants and radical scavengers (22); dithiocarb, in addition, is a strong inhibitor of microsomal mixed function oxidases (23). N-acetylcysteine like reduced glutathione evoked its action by removing both hydrogen peroxide and the hydroperoxides already formed (24). Although the mode of action of DMDs is not clear yet, our *in vivo* results together with the results *in vitro*, where all DMDs also inhibited the lipid peroxidation induced by CCl_4 in liver microsomal system suggested that each DMD itself or possibly its metabolites seems to act directly on liver microsome to inhibit the lipid peroxidation and thus attenuate the pathological symptoms of liver injury such as cellular accumulation of triglyceride, cholesterol and total lipid induced by CCl_4.

The activity of the microsomal electron transport system in the liver seems to be important in the process of CCl_4 poisoning, because prior stimulation of the system with phenobarbital or DDT increased the loss of cyt. p-450 and the extent of liver damage (25,26), whereas prior suppression of the system with aminotriazole or SKF-525A reversed the effects (27,28). Although it is generally accepted that CCl_4 is metabolized in the membranes of the endoplasmic reticulum, still there is disagreement on the exact site where the activation to the trichloromethyl radical takes place whether it is at cyt. p-450 locus or NADPH-cyt. c reductase (29,30). In our ex vivo study, the lipid peroxidations in the liver microsomal systems were increased with induced cyt. p-450 contents (DMD4, DMD5), but decreased with reduced cyt. p-450 contents (DMD1, DMD2, DMD3). The degree of lipid peroxidation did not show a consistent correlation with the changes of NADPH-cyt. c reductase activities. These results coincide with the

report that the activating site of CCl_4 would be microsomal cyt. p-450 locus (29).

The forms of cyt. p-450 induced by DMDs were further assessed by means of immunological methods such as RIA, immunoblotting and inhibition of cyt. p-450 catalyzed enzymes using MAb specific for PB or MC cyt. p-450. MC induced at least two forms of cyt. p-448, a high spin form (cyt. p-448H) and a low spin form (cyt. p-448L) (31). DMD4 and DMD5 markedly induced cyt. p-448L and cyt. p-448H in the liver microsomes, but DMD2 slightly induced cyt. p-448 forms. DMD1 and DMD3 did not induce any cyt. p-448 isozymes. As a result, DMD4 and DMD5 can be added as a new MC type cyt. p-450 inducer. The microsomal cyt. b_5 has been reported to be involved in the desaturation and the elongation of long chain fatty acid, thus participating in an arrangenment of fatty acids in the membrane phospholipids (32). The increase in cyt. b_5 content in microsomes from rats treated with all DMDs was not consistent with the degree of lipid peroxidation in our study. Therefore microsomal cyt. b_5 may not play a key role in inhibition of DMDs against lipid peroxidation induced by CCl_4.

In conclusion, the 5 novel hepatotrophic agents (DMDs) markedly protected lipid peroxidation induced by CCl_4 *in vivo* and *in vitro*. The mechanism of inhibitory effects of DMDs seemed to be through the direct action on the liver microsomal system. The degree of inhibition of lipid peroxidation seemed to coincide rather with cyt. p-450 contents than with NADPH-cyt. c reductase activities or cyt. b_5 contents in the liver microsomes.

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