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Effect of 3-Amino-1,2,4-triazole on Carbon Tetrachloride-induced Necrosis[1)]

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Various enzyme activities in the rat serum and liver were determined and the effect of 3-amino-1,2,4-triazole (AT) on CCl_4-induced necrosis was studied. Serum transaminase (GOT and GPT) activities were markedly increased by the injection of CCl_4 (0.1 ml/100 g, *i.p.*). The increase fell about 60 per cent with both the pre- and post-treatment of AT (100 mg/100 g, *i.p.*). It was demonstrated by biochemical and histological technique that the treatment with AT also repressed the fatty liver which occurrs along with liver necrosis. The effect of CCl_4 and AT on subcellular distribution of acid phosphatase was examined. AT repressed the release of enzyme from light mitochondrial fraction to supernatant fraction caused by CCl_4. AT significantly depressed the release of enzyme from the liver lysosomes by freezing and thawing in both the control- and CCl_4-treated rat. These results indicate that AT shows a depressing effect on fatty liver, a stabilizing effect on lysosomes, and a repression on liver necrosis.

Keywords——aminotriazole; CCl_4-induced necrosis; liver; triglyceride; transaminase; lysosome; lysosomal membrane

It has been reported that 3-amino-1,2,4-triazole (AT) is a herbicide which shows several effects on mammals, such as the inhibition of catalase activity in the liver and kidney,[3)] the inhibition of δ-aminolevulinic acid dehydratase,[4)] the antithyroid effect,[5)] and the repression of phenobarbital-induced change in liver microsomes.[6)] Our laboratory has reported that AT markedly decreased triglyceride (TG) level in rat liver[1)] and significantly depressed CCl_4-, ethanol- and ethionine-induced fatty liver.[7)] Furthermore, it has been reported that AT repressed the histological change in liver necrosis caused by CCl_4[8)] and decreased the motality.[6b)]

On the other hand, it is understood that lysosomes play an important role in liver necrosis caused by CCl_4.[9)] In the present study, we have studied the effect of AT on CCl_4-induced necrosis by examining the change in serum transaminase (GOT and GPT) levels. In addition, in order to study on the effect of AT on the stability of lysosomal membrane, subcellular distribution and the solubilization by freezing and thawing of lysosomal enzyme (acid phosphatase) were examined.

Materials and Methods

Treatment of Animals——Male Wistar rats weighing 120—150 g were used. CCl_4 was injected intraperitoneally to the rats at a dose of 0.1 ml per 100 g of body weight with 50% (v/v) solution in olive oil. The

1) Previous paper: H. Ishii, T. Suga, and S. Niinobe, *Biochem. Pharmacol.*, **25**, 1438 (1976).
2) Location: *1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.*
3) W.G. Heim, D. Appleman, and H.T. Pyform, *Science*, **122**, 693 (1955).
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control rats were injected olive oil in a volume of 0.1 ml per 100 g of body weight. AT was injected intraperitoneally to the rats at a dose of 100 mg per 100 g of body weight with 10% (w/v) in saline. The control rats were injected a corresponding volume of saline. In the AT pretreatment experiment, AT was injected 30 min before the injection of CCl_4, and the rats were sacrificed 12 hr later. In the AT-post-treatment experiment, AT was injected 24 to 36 hr after the injection of CCl_4 and the rats were sacrificed 48 hr later.

Assay of Serum Enzymes——Animals were sacrificed by decapitation and serum was obtained from the blood by the centrifugation. Transaminases (GOT and GPT) were determined by the modified method of Reitman and Frankel,[10] and alkaline phosphatase (ALPase) was determined by the modified method of Kind and King.[11]

Subcellular Fractionation——Rats were sacrificed by decapitation, and the livers were removed, minced and homogenized with ice-cold 0.25 M sucrose in a Potter–Elvehjem homogenizer. The homogenates were fractionated by differential centrifugation according to the procedure of de Duve, *et al.*[12] The fractions were suspended in 0.25 M sucrose.

Release of Acid Phosphatase from Light Mitochondrial Fraction by Freezing and Thawing——The light mitochondrial fraction of the liver was isolated as described above, freezed in dry ice-acetone and thawed in an incubator at 37° with gentle agitation. After centrifugation of the suspension at $12500 \times g$ for 20 min, enzyme activity of the precipitated fraction was determined.

Histological Analysis——Rats were sacrificed by decapitation. Livers were removed and immersed into 10% (v/v) formalin solution (pH 7.4). The livers were freezly sectioned and stained by Sudan Black.

Assay Methods——Acid phosphatase activity was determined by using β-glycerophosphate as a substrate, and the inorganic phosphate liberated was measured according to the method of Lindberg and Ernster.[13] Enzyme preparations used for determination of the activity were treated with Triton X-100 as a final concentration of 0.5% before incubation. Determination of protein content was carried out by the method of Lowry, *et al.*[14] with bovine serum albumin as a standard. The triglyceride content was determined by the method described in the previous paper.[1]

Results

The Effect of 3-Amino-1,2,4-triazole (AT) on Fatty Liver

The effect of AT on CCl_4-induced fatty liver is shown in Table I. TG level in liver decreased about 50 per cent by the injection of AT. At 12 hr after CCl_4 injection, liver TG level

Table I. Effect of Aminotriazole on CCl_4-induced Fatty Liver

	Number of rats	Triglyceride in liver	
		mg/g[a]	p[b]
Exp. 1			
Control	4	6.5± 1.2	
AT	4	3.4± 0.6	<0.01 *vs.* Control
CCl_4	4	33.4± 6.2	<0.01 *vs.* Control
AT–CCl_4	4	12.4± 4.3	<0.01 *vs.* CCl_4 alone
Exp. 2			
Control	7	4.2± 1.5	
AT	7	2.9± 1.0	<0.05 *vs.* Control
CCl_4	5	106.4±17.8	<0.01 *vs.* Control
CCl_4–AT	4	72.8± 6.4	<0.02 *vs.* CCl_4 alone

Male rats were used. In the Exp. 1, CCl_4 (0.1 ml/100 g, *i.p.*) was injected to the rats 12 hr before the sacrifice. AT (100 mg/100 g, *i.p.*) was injected to the rats 30 min before CCl_4. In the Exp. 2, CCl_4 was injected to the rats 48 hr before the sacrifice. AT was injected to the rats 24 to 36 hr after the CCl_4. All animals were fasted for 12 hr before the sacrifice.

a) The results represent the means ± S.D.

b) Significantly different from each group.

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increased about 5 folds higher than that of the control. However, the CCl_4-induced increase in TG level was depressed about 60 per cent by the pretreatment with AT. This result corresponds to the result reported in the previous paper.[7] Furthermore, the increase in liver TG level caused by CCl_4 was also depressed by the post-treatment of AT. At 48 hr after CCl_4 injection, liver TG level was increased about 25 folds. On the other hand, although the percentage of repression was lower in the AT-post-treated rats than in the AT-pretreated rats, The TG level in AT-post-treated rats was decreased about 30 per cent when compared to the CCl_4 group. Histological observations are shown in Fig. 1. In the liver cells, at 12 hr after

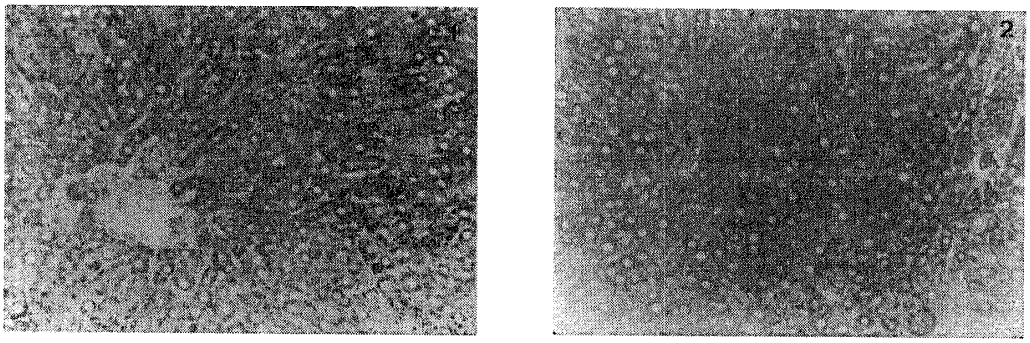

Fig. 1. Light Microscopy of Rat Liver treated with CCl_4 and CCl_4–AT
Sections were stained with Sudan Black (×200)

1. CCl_4 was injected to the rats 12 hr before sacrifice.
2. AT was injected to the rats 30 min before CCl_4.

Table II. Effect of Aminotriazole (AT)–Pretreatment on Serum Enzyme Levels in CCl_4-treated Rat

	Number of rats	GOT U/ml[a]	GOT p	GPT U/ml[a]	GPT p
Control	4	141± 9.8		30.3± 2.6	
AT	4	163± 5.1	<0.02[b]	42.0± 5.0	<0.02[b]
CCl_4	4	1087±285	<0.01[b]	277.0±96.0	<0.01[b]
AT–CCl_4	4	583± 99.8	<0.05[c]	130.0±40.9	N.S[c,d]

Male rats were used. All animals were fasted for 24 hr prior to the sacrifice. CCl_4 (0.1 ml/100 g, *i.p.*) was injected to the rats 12 hr before sacrifice. AT (100 mg, *i.p.*) was injected to the rats 30 min before CCl_4.
a) The results represent the means ± S.D.
b) Comparison of treatment with control by t-test.
c) Comparison of AT treatment with CCl_4 by t-test.
d) p values larger than 0.05 were considered not significant (N.S).

Table III. Effect of Aminotriazole (AT) Post-Treatment on Serum Enzyme Levels in CCl_4-treated Rat

	Number of rats	GOT U/ml[a]	GOT p	GPT U/ml[a]	GPT p	ALPase U/dl[a]	ALPase p
Control	7	144± 20		30.3± 5.8		12.0±6.6	
AT	7	159± 22	N.S[b,d]	33.0± 7.7	N.S[b,d]	14.0±3.5	N.S[b,d]
CCl_4	5	3620±432	<0.01[b]	494.0±10.2	<0.01[b]	43.6±5.7	<0.01[b]
CCl_4–AT	4	2510±679	<0.05[c]	371.0±66.8	<0.01[c]	32.0±5.1	<0.05[c]

Male rats were used. All animals were fasted for 60 hr prior to the sacrifice. CCl_4 (0.1 ml/100 g, *i.p.*) was injected 48 hr before sacrifice. AT (100 mg/100 g, *i.p.*) was injected 24 to 36 hr after the CCl_4.
a) The results represent the means ± S.D.
b) Comparison of treatment with control by t-test.
c) Comparison of treatment with CCl_4 by t-test.
d) p values larger than 0.05 were considered not significant (N.S).

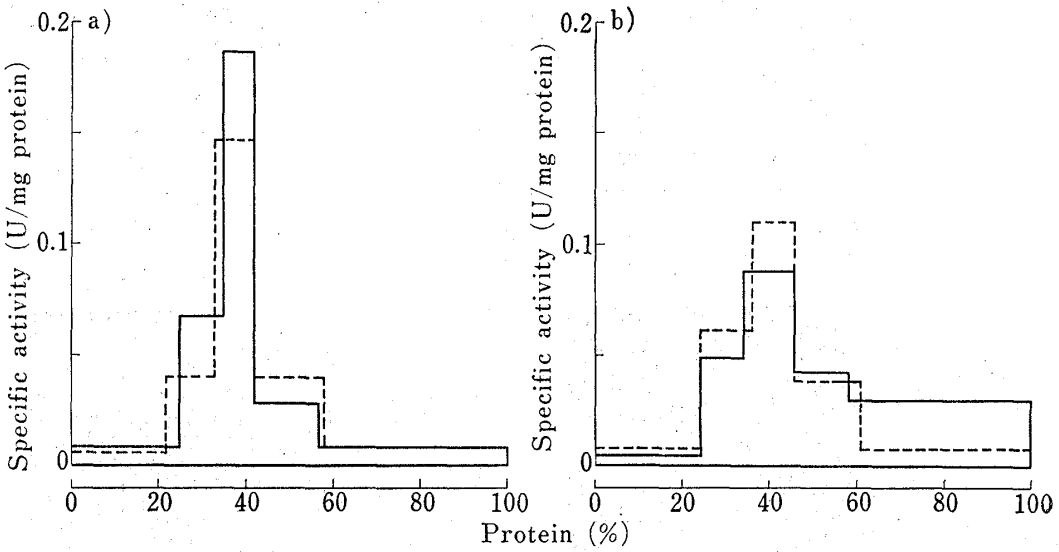

Fig. 2. Effect of Aminotriazole (AT) on Subcellular Distribution of Acid Phosphatase in the CCl_4-induced Necrosis

CCl_4 (0.1 ml/100 g, *i.p.*) was injected 12 hr before the sacrifice. AT (100 mg/100 g, *i.p.*) was injected 30 min before CCl_4. The liver homogenates were centrifuged according to the method of de Duve, *et al.*[12]

a) The solid and dotted lines represent the patterns of acid phosphatase in livers of control and AT treatment respectively.

b) The solid and dotted lines represent the patterns of acid phosphatase in livers treated with CCl_4 and AT-CCl_4 respectively.

the injection of CCl_4, many lipid droplets were seen in the intermediate area of the sinusoid, and disorder of cells around the central vein was observed. On the other hand, a marked decrease in the lipid droplets was observed in the liver of the AT-pretreated rat. Only a very small number of lipid droplets were found around the intermediate area and the central vein. Repression of cell disorder was not found by AT pretreatment in this microscopic observation.

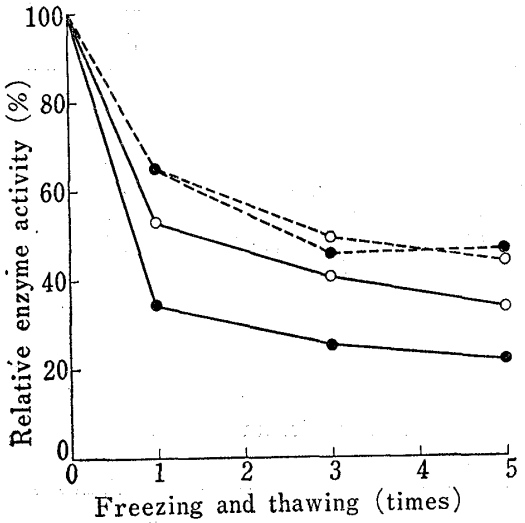

Fig. 3. Effect of Aminotriazole (AT) on Solubilization of Acid Phosphatase by Freezing and Thawing

CCl_4 (0.1 ml/100 g, *i.p.*) was injected 12 hr before the sacrifice. AT (100 mg/100 g, *i.p.*) was injected 30 min before CCl_4. Light mitochondrial fraction from 10% (w/v) liver homogenate were freezed in dry ice-acetone and thawed in an incubator at 37° with gentle agitation. After centrifugation of suspension at 12500 × *g* for 20 min acid phosphatase activity of precipitated were determined. Ordinate represents relative enzyme activity remaining in the pellet.

○—○, Control; ●—●, CCl_4; ○---○, AT; ●---●, AT-CCl_4.

Effect of AT on Liver Necrosis

The effect of AT on CCl_4-induced necrosis was studied by measuring serum enzyme levels (Tables II and III). The transaminase (GOT and GPT) levels were increased slightly by the injection of AT. At 12 hr after the CCl_4 injection, the GOT and GPT levels were increased to about 8 and 9 folds, respectively, when compared with those of the control. However, this increase caused by CCl_4 was depressed about 50 per cent by the pretreatment with AT (Table II). The effect of AT post-treatment on CCl_4-induced necrosis is shown in Table III. GOT and GPT levels showed a tendency to increase by AT injection, although this change was not significant when compared to those of the control. At 48 hr after the injection of CCl_4, the serum enzyme levels were increased as follows; GOT, 23 folds; GPT, 13 folds; and ALPase, 4 folds. On the other hand, the enzyme levels of AT post-treated

rats were decreased 30 per cent although the repression was lower than that in AT pretreated rats.

Effect of AT on Lysosomes

The effect of AT on subcellular distribution of acid phosphatase in CCl_4-treated liver is shown in Fig. 2. AT alone did not affect the distribution of acid phosphatase. On the other hand, the enzyme activity in light mitochondrial fraction was decreased and the activity in supernatant fraction was increased by 3 folds by the injection of CCl_4 when compared with control. However, the pre-treatment with AT repressed the release of enzyme into the supernatant. The effect of AT treatment on the solubilization of acid phosphatase in light mitochondrial fraction by freezing and thawing is shown in Fig. 3. Acid phosphatase in lysosomes was decreased after being solubilized by freezing and thawing. The solubilization of the enzyme was markedly increased in the liver lysosomes of CCl_4-treated rat. However, the solubilization of the enzyme in the lysosomes of AT-treated rat was decreased about 50 per cent when compared with that of the CCl_4. AT also repressed the solubilization of the enzyme in the control.

Discussion

It has previously been reported that AT markedly decreased triglyceride (TG) level in the liver,[1] and also the pretreatment with AT repressed CCl_4-, ethanol- and ethionine–induced fatty liver.[7] In this study, we have investigated the effect of AT post-treatment on CCl_4–induced fatty liver along with the effect of AT pretreatment by means of biochemical and histological techniques.

CCl_4–induced fatty liver was depressed in both cases of the pre- and post-treatment with AT, that is, AT was found to depress not only fatty liver formation but also a direct effect on the extent of fatty liver. This result demonstrates that AT has a strong depressing action on liver TG. In our present experiment, the depressing action of AT on fatty liver was made clear by histological observation. Accumulation of lipid droplets in the liver caused by CCl_4 appeared clearly in the intermediate area of the sinusoid. However, AT pre-treatment markedly decreased the lipid droplets to the degree that only few droplets around the intermediate area and the central vein could be observed.

It is well known that serum transaminase (GOT and GPT) activities are increased in liver necrosis. The increase of the activities shown here resulted from the release of the enzymes from the liver into the blood stream. The GOT and GPT activities are remarkably increased after CCl_4 injection. However, the pre- or post-treatment with AT repressed the increase of these enzyme levels caused by CCl_4. From these results it is likely that AT significantly repressed the CCl_4-induced liver necrosis. Through histological technique, D'Acosta, *et al.*[8] reported that the pretreatment of AT markedly repressed CCl_4–induced necrosis. Our results support their findings by biochemical techniques, in addition, our studies show that AT was effective even after the CCl_4 injection.

The preventing effect of AT on CCl_4-induced necrosis was further studied on the cell level of the liver. It was proposed by Beaufay, *et al.*[9] that the injury of hepatic lysosomes plays an important role in the initiation of cell necrosis. In addition, it was ascertained by Slater, *et al.*[15] that the ratio of free/bound of the lysosomal enzyme was increased by the administration of CCl_4. The subcellular distribution of acid phosphatase in the rat treated with AT alone did not show any significant difference between treated- and control animals, whereas the release of the enzyme from lysosomes to supernatant fraction occurred by the injection of CCl_4, and the ratio of free/bound increased about 3 folds when compared with that of the

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control. However, this increase in the ratio caused by CCl_4 was repressed by the pretreatment with AT, and the distribution pattern of this enzyme was similar to that of the control. Thus, it has been made clear that AT possesses the preventing effect on the damage of lysosomes caused by CCl_4. Therefore, in order to study the effect of AT on the stability of lysosomal membrane, the solubilization of acid phosphatase by freezing and thawing was determined with or without AT-treatment to the rat. The decrease of acid phosphatase in lysosomes which was caused by freezing and thawing became more remarkable by the treatment of CCl_4. This result indicates that the treatment of CCl_4 changes the condition of the lysosomal membrane and makes it more destructible. On the other hand, it was recognized that AT treatment stabilizes the lysosomal membrane and prevents the membrane from the damage caused by CCl_4. Thus, stabilization effect of AT on lysosomal membrane might be major one of reasons why the CCl_4-induced necrosis is prevented by AT.

Further works are needed to study the relationship between the protecting effect of AT on lysosomes and the repressing effect of AT on TG accumulation.

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