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Protection by 3-Amino-1,2,4-Triazole against the Lowered Uptake of *p*-Biphenylmethyl-(*dl*-Tropyl-α-Tropinium) Bromide into the Hepatic Lysosomes of Carbon Tetrachloride-treated Mice

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The effects of 3-amino-1,2,4-triazole (AT) on the uptake of tritiated *p*-biphenylmethyl-(*dl*-tropyl-α-tropinium) bromide (^{3}H-BTTB) into the subcellular fractions of livers of carbon tetrachloride (CCl_4)-treated mice were studied. The uptake of ^{3}H-BTTB into the liver of CCl_4-treated mice was decreased to 50% of that in the control group. Pretreatment with AT resulted in an effective restoration of the decrease caused by CCl_4 treatment up to the AT group's level, though AT itself lowered the uptake of ^{3}H-BTTB to 61% of that of the control group. The subcellular distribution of ^{3}H-BTTB in the liver of CCl_4-treated mice was different from that of the control: the ratio of the radioactivity in the lysosomal fraction to that of the supernatant fraction (the L/S ratio) was decreased by CCl_4 treatment from 1.85 to 1.66. This decrease was almost completely reversed to the control value by the pretreatment with AT and there was a similar change in the distribution of acid phosphatase. Furthermore, the cross-points of the lysosomes of the liver in mice which had been treated with CCl_4, AT and AT-CCl_4 were determined by means of cross-partition in aqueous polymer two-phase systems. CCl_4 made the surface charge of the lysosomal membrane more acidic, though AT reversed this change. There may be an important relationship between the uptake of BTTB into lysosomes and the characteristics of the lysosomal membranes.

Keywords——carbon tetrachloride; 3-amino-1,2,4-triazole; BTTB; acid phosphatase; subcellular distribution; cross-point

It is known that the quaternary ammonium derivatives of tropane alkaloids have an affinity for lysosomes and that, when administered to animals, they are specifically incorporated into the lysosomes of the liver.[1–3] Among them, *p*-biphenylmethyl-(*dl*-tropyl-α-tropinium) bromide (BTTB) was found to be the most specific to the lysosomes and thus to be the most suitable tool for binding experiments.

A single dose of CCl_4 administered to rats results in severe centrilobular necrosis and fatty degeneration of the liver.[4] Slater *et al.*[5] have indicated that changes of lysosomal enzyme activities occur in the later stage of CCl_4 administration and that the lysosomes play an important role in the scavenging process of injured cells. In addition, it was suggested from both morphological[6] and biochemical[7] studies that 3-amino-1,2,4-triazole (AT), a herbicide, stabilized the lysosomal membranes and protected the membranes from damage by CCl_4.

To test this hypothesis, the effect of AT, given 30 min before administration of CCl_4, on the uptake of BTTB into lysosomes of mouse liver was studied after the administration of ^{3}H-BTTB as a lysosomotropic drug and the results obtained were compared to the change in the behavior of the lysosomes.

Methods

Animals and Drugs——Male mice of the ddY strain weighing 20—25 g were used throughout these experiments. Liver injury was induced by intraperitoneal injection of CCl_4 at a dose of 1 ml/kg of body weight and mice were killed 12 h later. Mice were starved for 24 h before sacrifice. AT was injected intraperitoneally into mice at a dose of 1 g/kg of body weight as a 10% (w/v) solution in saline, 30 min before the injection of CCl_4. The control mice were injected with a corresponding volume of saline. ^{3}H-BTTB (345 μCi/mg) was injected intraperitoneally into all mice at a dose of 0.17 mg/kg of body weight 1 h before sacrifice.

Cell Fractionation of Liver——Mice were killed by decapitation and the livers were removed immediately, rinsed in cold 0.25 M sucrose, and weighed. The liver homogenates (10%, w/v) were prepared in an ice-cold 0.25 M sucrose solution with three strokes in a Potter-Elvehjem homogenizer with a Teflon pestle. An aliquot of the homogenate was used for the determination of acid phosphatase activity and radioactivity. The remainder was subjected to differential centrifugation, according to the method of de Duve *et al.*[8] to obtain 5 fractions, *i.e.*, nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and supernatant fraction (S). The washing procedure for the nuclear sediment was not performed because of loss of radioactivity. The light mitochondrial fraction was partitioned into two phases as described below. Each fraction was used for the determination of radioactivity and enzyme activity.

Partition in Aqueous Polymer Two-Phase Systems——The final phase systems used had the following compositions.[9]

System I: 6% (w/w) Dextran T 500, 6% (w/w) polyethyleneglycol 4000, 250 mmol of sucrose/kg, 50 mmol of NaCl/kg and 5 mmol of citrate–phosphate buffer/kg.

System II: The same composition as system I but with 100 mmol of Na_2SO_4/kg instead of 50 mmol of NaCl/kg.

The citrate–phosphate buffer was used to obtain pH values between 4.0 and 7.5. The system was allowed to equilibrate in a cold room until it was thoroughly separated. Systems I and II (10 g each) were prepared in test tubes, and 0.1 ml of sample was added to each. The solutions were thoroughly mixed by inverting the test tubes 10 times at 4°C, and allowed to settle for 60 min, then 2.5 ml of the top phase was carefully removed and the acid phosphatase activity in each fraction was measured.

Biochemical Assays——Acid phosphatase (EC 3.1.3.2) activity was determined according to the method of Appelmans *et al.*[10] by using sodium β-glycerophosphate as a substrate and the liberated inorganic phosphate was measured by the method of Lindberg and Ernster.[11] In the case of the two-phase systems, acid phosphatase activity was determined according to the method of Bessey *et al.*[12] with *p*-nitrophenylphosphate as the substrate. Total acid phosphatase activity was determined after treatment of the fraction with 0.5% Triton X-100. ^{3}H-BTTB was determined in a scintillation counter (Aloka LSC-502) using a hydrophilic scintillator. Determination of protein content was carried out by the method of Lowry *et al.*[13] with bovine serum albumin as the standard.

Statistical Method——Results were analyzed for statistical significance by analysis of variance and by the use of Student's *t* test.

Results

Effects of CCl_4 and AT on the Uptake of ^{3}H-BTTB into Mouse Liver

The uptake of ^{3}H-BTTB in the liver of CCl_4 treated mice (CCl_4 group) was decreased to 50% of the control level as reported previously[14] (Table I).

Table I. Effect of Aminotriazole (AT) Pretreatment on the Uptake of ^{3}H-BTTB into the Liver of Carbon Tetrachloride-treated Mice[a]

Group	Radioactivity (dpm × 10^{-5}/g liver) (%)	Acid phosphatase (μmol Pi/min per g liver) (%)
Control	3.6±0.3 (100)	1.08±0.08 (100)
AT	2.2±0.2 (61.1)	1.02±0.06 (94.4)
CCl_4	1.8±0.1 (50.0)	0.94±0.04 (87.0)
AT-CCl_4	2.3±0.1[b] (63.9)	1.06±0.03[c] (98.1)

a) Values are given as means±standard deviations of five mice. Numbers in parentheses represent per cent of the control value.
b) $p<0.01$ as compared with the controls or CCl_4.
c) N.S. as compared with the controls or CCl_4. (analysis of variance; one-way classification).

The radioactivity in the liver was decreased to about 61% of the control value by a single injection of AT (AT group). The pretreatment with AT (AT-CCl_4 group) significantly reversed the decrease in ^{3}H-BTTB uptake due to CCl_4 treatment to 64% of the control. Total activity of acid phosphatase in the liver did not show any significant change in the CCl_4 or AT group when compared with the control.

Subcellular Distribution of ^{3}H-BTTB

As shown in Fig. 1, the highest specific activity of ^{3}H-BTTB in the control group was found in the light mitochondrial fractions as described previously.[1]

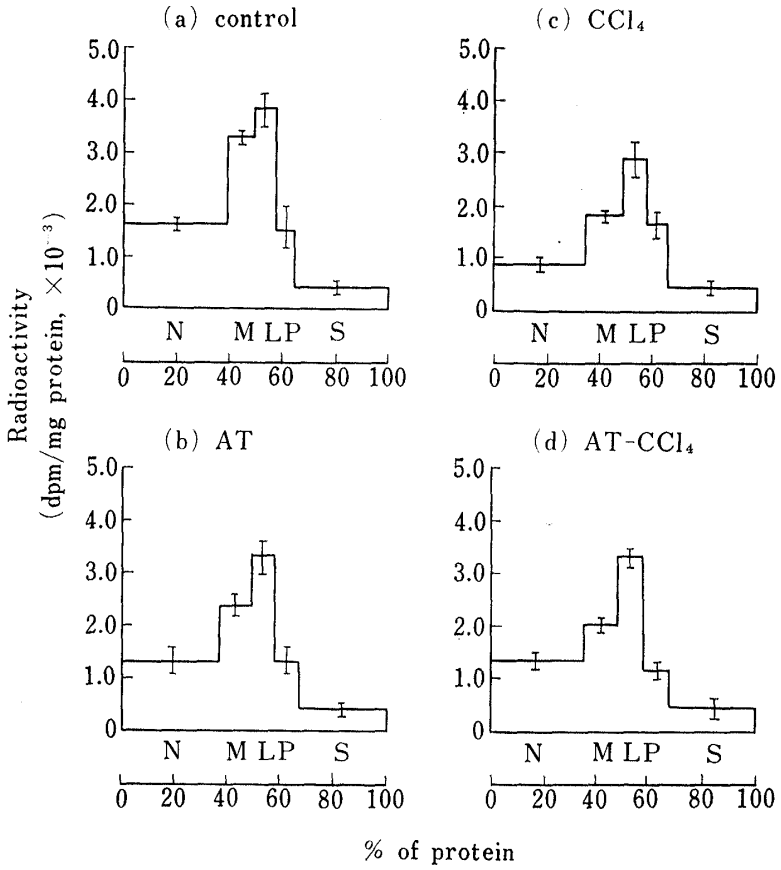

Fig. 1. Subcellular Distributions of the Radioactivity of ^{3}H-BTTB in the Liver of Mice under Various Conditions

Male mice were treated as described in "Methods." The liver homogenates were centrifuged according to the method of de Duve *et al.*[8] The ordinate shows the specific radioactivity of each fraction, and the abscissa shows the protein content expressed as a percentage of total recovered protein. Each value represents the mean of three animals. Vertical bars show the standard deviations.

The amount of ^{3}H-BTTB in the AT group was decreased to 77% in the heavy mitochondrial fractions as compared with the control. The amount of ^{3}H-BTTB in the light mitochondrial fractions after the injection of CCl_4 was decreased to 68% of the control level (Fig. 1c). The distribution of ^{3}H-BTTB in the light mitochondrial fraction from the AT group was slightly lower than that of the control (80% of control). The pretreatment with AT prevented the decrease of ^{3}H-BTTB radioactivity in the light mitochondrial fraction caused by the CCl_4 treatment. The effect of AT on the subcellular distribution of acid phosphatase in the liver of CCl_4-treated mice is shown in Fig. 2.

AT alone did not alter the distribution of acid phosphatase. Acid phosphatase in the lysosomes of CCl_4-treated mice was markedly decreased by 52% as compared with the control, but a 1.5-fold increase of the enzyme was also observed in the supernatant fraction of CCl_4-treated mice. The specific activity of acid phosphatase in the light mitochondrial fraction of the AT-CCl_4 group was increased 1.7-fold when compared with the CCl_4 group, but the value in the supernatant fraction was significantly decreased to 54% of that of the latter.

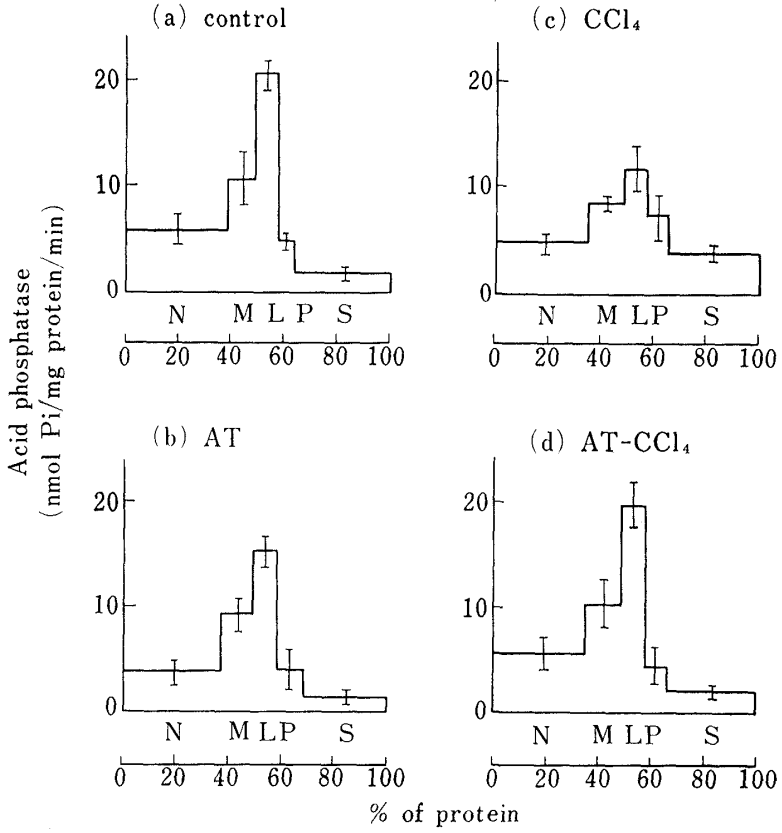

Fig. 2. Subcellular Distributions of Acid Phosphatase Activity in the Liver of Mice under Various Conditions

The procedure was as described in the legend to Fig. 1.

Effects of CCl_4 and AT on the Subcellular Distribution of Radioactivity after the Administration of ^{3}H-BTTB

The radioactivity incorporated into the lysosomal and supernatant fractions and the activity of acid phosphatase in these fractions are shown in Table II.

The incorporation of radioactive BTTB into liver lysosomal fractions was apparently lowered by the treatment with CCl_4 and that in the supernatant fraction was somewhat decreased when compared with the control though this was not statistically significant. AT itself did not produce any significant change in the uptake of the radioactivity or the acid

Table II. Effect of Aminotriazole (AT) Pretreatment on the Uptake of ^{3}H-BTTB into the Subcellular Fractions of the Liver of Carbon Tetrachloride-treated Mice[a]

Group	Radioactivity (dpm/g liver $\times 10^{-5}$)			Acid phosphatase (μmol Pi/min per g liver)		
	Lysosomes	Supernatant	L/S[b]	Lysosomes	Supernatant	L/S[b]
Control	0.56±0.05	0.31±0.03	1.85	0.31±0.02	0.13±0.01	2.46
AT	0.54±0.05	0.28±0.03	1.95[c]	0.26±0.02	0.10±0.01	2.44[c]
CCl_4	0.38±0.04	0.23±0.02	1.66[d]	0.16±0.02	0.20±0.01	0.80[e]
AT-CCl_4	0.56±0.02	0.26±0.02	2.15[f]	0.33±0.04	0.12±0.01	2.75[g]

a) Values are given as means±standard deviations of five mice.
b) The L/S ratios are expressed as the ratio of activity in the lysosomal fraction to that in the supernatant fraction.
c) N.S.
d) $p<0.05$ as compared with the control.
e) $p<0.01$ as compared with the control.
f) $p<0.05$ as compared with CCl_4 group.
g) $p<0.01$ as compared with CCl_4 group.

phosphatase activity in either the lysosomal or the supernatant fraction. In addition, the lowered uptake of radioactivity induced by CCl_4 was restored in both fractions by the pretreatment with AT, almost to the control levels. On the other hand, the acid phosphatase activity in the lysosomal fraction was significantly decreased by the treatment with CCl_4 and the activity in the supernatant fraction showed a significant increase above the control value. The activity of acid phosphatase in these fractions in the livers after the injection of AT alone into mice was not different from that of the controls. When AT was injected 30 min before administration of CCl_4, the activities of acid phosphatase in the lysosomal and supernatant fractions were restored to those of the controls.

Effects of CCl_4 and AT on the Cross-Points of Mouse Liver Lysosomes

Figure 3 shows the cross-points determined by means of cross-partition of the liver lysosomes of mice after the drug treatments.

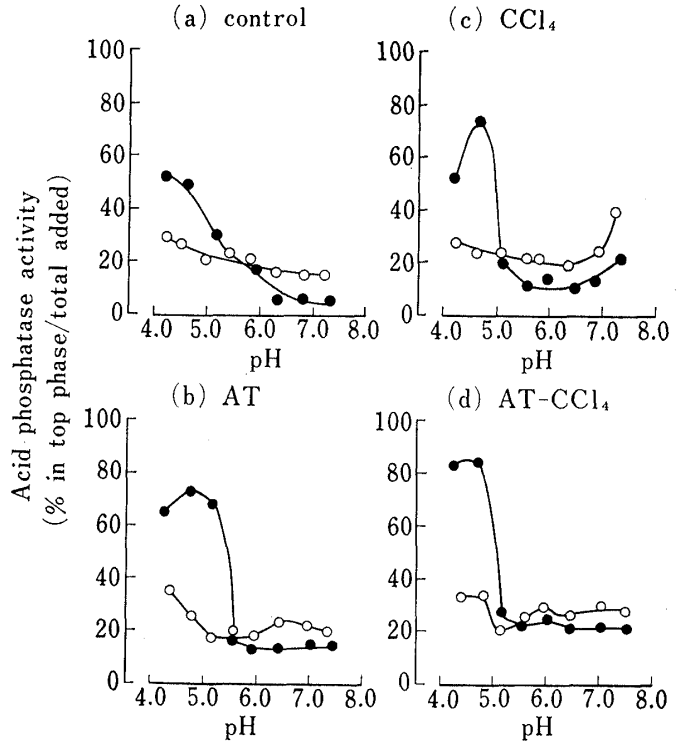

Fig. 3. Effect of Aminotriazole (AT) Pretreatment on the Cross-Points of Lysosomes from the Liver of CCl_4-treated Mice

Male mice were treated as described in "Methods." Mice were killed by decapitation and the light mitochondrial fractions of the liver were obtained according to the method of de Duve *et al.*[8] The method of determination of cross-points is given in "Methods." The abscissa shows the final pH in each fraction. The ordinate is the percent recovery in the top phase of total enzyme activity added. The curves show the means of three separate determinations. The cross-point of the AT-CCl_4 group is significantly different from that of the CCl_4 group ($p<0.01$). The value of the latter is also significantly different from that of the control ($p<0.01$).
●—●: system I, ○—○: system II.

As shown in Fig. 3a, the cross-point of acid phosphatase obtained in the control mice was pH 5.6. When mice were treated with AT alone, the cross-point was pH 5.5 (Fig. 3b). The value was somewhat shifted to the acidic side as compared with that of the control. In the case of CCl_4-treated mice, the cross-point was pH 5.1 (Fig. 3c). Thus, the cross-point of the lysosomes of CCl_4-treated mice was shifted to the acidic side as compared with that of the control. On the other hand, in the case of the AT-CCl_4 group, the cross-point was pH

5.4 as shown in Fig. 3d. This value is intermediate between the control and CCl_4 group values. The result for the CCl_4 group is significantly different from the control or the AT-CCl_4 group at $p<0.01$ (Student's t test).

Discussion

In the present study, the effect of AT, given 30 min before administration of CCl_4, on the uptake of ^{3}H-BTTB into whole mouse liver and subcellular fractions was investigated. In the case of AT alone, the uptake of ^{3}H-BTTB into mouse liver was lowered, but the effect was not statistically significant. It may be considered that AT affects the plasma membrane or competes with BTTB uptake. Pretreatment with AT restored the lowered uptake of ^{3}H-BTTB caused by CCl_4 to a level similar to that seen with AT alone. These experimental results suggest that the decreased uptake of ^{3}H-BTTB into liver cells is due to the damage caused to liver parenchymal cells by CCl_4 and that AT restored both the damage to hepatic parenchymal cells and the decrease in the uptake of ^{3}H-BTTB into the liver cells.

This protective effect of AT on the lowered uptake of ^{3}H-BTTB due to CCl_4 treatment was further studied at the subcellular level of the liver. The ratio of the radioactivity in the lysosomal fractions to that in the supernatants (the L/S ratio) of acid phosphatase in CCl_4-treated mice was also lowered, in agreement with the report described by Ugazio *et al.*[15] The decrease in the L/S ratio of the radioactivity in CCl_4-treated liver may indicate lowered uptake of the drug into the lysosomes in addition to lowered uptake in the whole liver, though the extent is less than that in the case of acid phosphatase activity. This may further indicate that a part of ^{3}H-BTTB once incorporated is bound to some insoluble component of the lysosomes even after the lysosomal membranes are destroyed. In other word, it may indicate that some part of BTTB binds to the insoluble lysosomal components such as membranes. Pretreatment with AT markedly inhibited the increase of liver triglyceride caused by CCl_4.[16]

Cross-partition in aqueous polymer two-phase systems can be applied for the determination of the isoelectric point not only of protein[17,18] but also of subcellular organelles.[19] The cross-points of the lysosomes of the livers of mice which had been treated with AT, CCl_4, and AT-CCl_4 were thus determined by means of cross-partition in aqueous polymer two-phase systems. This method was useful for the present experiment because the polymers used here have a protective effect on the membranes of organelles.[20] From the cross-points of the lysosomes under various conditions, it appears that there may be an important relationship between the uptake of BTTB into lysosomes and the properties of the lysosomal membranes.

It has been reported that rat liver lysosomal membranes are heterogeneous.[21] Although it is considered that the cross-points obtained here represent mean values of populations of different states of lysosomes, it remains unclear what surface substance is involved in the partition of the lysosomal membranes. It seems that the isoelectric points of the lysosomal membranes can be made to vary by changing the surface charge of the membranes, and this in turn modifies the binding properties of basic drugs such as BTTB.

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