

## Protection against Alloxan-Induced Diabetes by Diethyldithiocarbamate and Disulfiram in Mice

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**ABSTRACT**—Diethyldithiocarbamate (DEDIC, 0.25–2.00 mmol/kg) injected into mice at 0.5 hr prior to alloxan administration dose-dependently protected the mice against the diabetogenic actions of 75 mg/kg alloxan. Disulfiram (DS, 0.50–2.00 mmol/kg), a corresponding disulfide form, also exhibited similar protection. The maximum effect of DEDIC was found by dosing at 0.5 hr prior to alloxan, and the effect afforded by DEDIC pretreatment persisted up to 3 hr, whereas the effect of DS was exhibited when the compound was given 0.5 hr prior to alloxan. Of the metabolites of DEDIC, diethylamine and carbon disulfide had no effect. At 0.5 hr after injection, DEDIC alone had a potent increasing ability on blood glucose in a dose-dependent manner, but DS was less potent. Mannoheptulose, an antagonist of glucose action at pancreatic  $\beta$ -cells, when given 24 min after DEDIC and 6 min before alloxan, eliminated the DEDIC-induced protection. Fasted mice did not exhibit hyperglycemia at 0.5 hr after DEDIC injection, and alloxan given at that time produced diabetes. These findings indicate that DEDIC itself protected mice from alloxan-induced diabetes by the indirect mechanism of producing hyperglycemia at the time of alloxan administration. The anti-diabetogenic action of low doses of DS and DEDIC, in animals lacking hyperglycemia at the time of alloxan injection, is likely based on a mechanism other than one involving hyperglycemia.

**Keywords:** Diethyldithiocarbamate, Disulfiram, Diabetes (alloxan-induced)

Diethyldithiocarbamate (DEDIC) and disulfiram (DS) were shown to suppress lipid peroxidation in various systems (1–4), which was thought to play a role in promoting cellular damage. However, their anti-oxidative mechanism has not yet been clarified, although the high reactivity of DEDIC with hydrogen peroxide has been demonstrated (5).

Alloxan-induced diabetes in experimental animals is thought to be produced through selective destruction of pancreatic  $\beta$ -cells by active oxygen-containing radicals formed by the reduction-oxidation cycle of alloxan and its reduced form, dialuric acid (6). Various aliphatic alcohols and urea derivatives with scavenging capacity for hydroxyl radicals were reported to protect animals from alloxan-induced diabetes (7–10). There has been no information on the influence of DEDIC and DS on alloxan toxicity.

In the present study, to evaluate the anti-oxidative activity of DEDIC and DS, we investigated their influence on the diabetogenic action of alloxan in mice.

### MATERIALS AND METHODS

#### *Chemicals and animals*

DEDIC, DS, diethylamine, carbon disulfide and alloxan monohydrate were obtained from Wako Pure Chemical Industries, Osaka. Mannoheptulose was from Sigma Chemical Company, St. Louis, MO, USA. Other reagents used were of analytical grade. The animals used were male ddY mice weighing about 30 g (6-week-old) and were obtained from Japan SLC, Inc., Hamamatsu.

#### *Alloxan-induced diabetes*

Food and water were withheld from the mice 3–4 hr prior to alloxan injection. Alloxan monohydrate at 75 mg/kg was injected intravenously (tail vein) into the mouse; the alloxan was prepared in cold isotonic saline and kept on ice prior to injection. One hr after alloxan, the mice were given free access to food and water. The extent of hyperglycemia at 72 hr after alloxan injection was used as an index of alloxan-induced damage. Pretreatment with DEDIC and other compounds (dissolved or suspended in 1.0% CMC Na, sesame oil or saline) was given

intraperitoneally 0.5 hr before alloxan unless specified otherwise. Blood was collected by decapitation between 14:00–15:00, and blood glucose was measured by a glucose-oxidase method (Blood Sugar-GOD-Perid-test, Boehringer Mannheim Yamanouchi, Tokyo).

#### Assay of superoxide dismutase (SOD)

Mouse pancreas was rapidly removed after decapitation of the animal and then homogenized in 9 volumes of 0.05 M sodium phosphate buffer at pH 7.4 containing  $10^{-4}$  M EDTA. The homogenates were centrifuged at  $700 \times g$  for 10 min, and the supernatant was used for the SOD assay. SOD was measured by the method of Heikkila and Cabbat (11), which was based on the inhibitory action of SOD on the rate of the autoxidation of 6-hydroxydopamine.

#### Assay of hydrogen peroxide

In the glucose assay method, enzyme reagent containing glucose oxidase, peroxidase and chromogen was used to measure hydrogen peroxide. To assess the effect of DEDC and DS on the stability of hydrogen peroxide, each compound was incubated with  $10^{-3}$  M hydrogen peroxide at 37°C in 0.05 M phosphate buffer, pH 7.4. After 5 min, aliquots (0.1 ml) were mixed with the above enzyme reagent (4.0 ml) for determination of hydrogen peroxide. Thirty min later, the absorbance at 620 nm was determined.

#### Statistical analysis

Data were shown as the mean  $\pm$  S.E.M. Comparisons

of the mean values were made by analysis of variance followed by Duncan's multiple range test.

## RESULTS

Alloxan injection to mice at 75 mg/kg caused a large increase in blood glucose at 72 hr (Table 1). Mice pretreated with DEDC (0.25–2.00 mmol/kg, i.p.) 0.5 hr prior to alloxan were protected dose-dependently against the diabetogenic action of alloxan. When a blood glucose level of over 200 mg/dl was employed to indicate the onset of the diabetogenic action of alloxan, DEDC pretreatment also protected against the onset of diabetes. DS (0.50–2.00 mmol/kg) also had a similar effect.

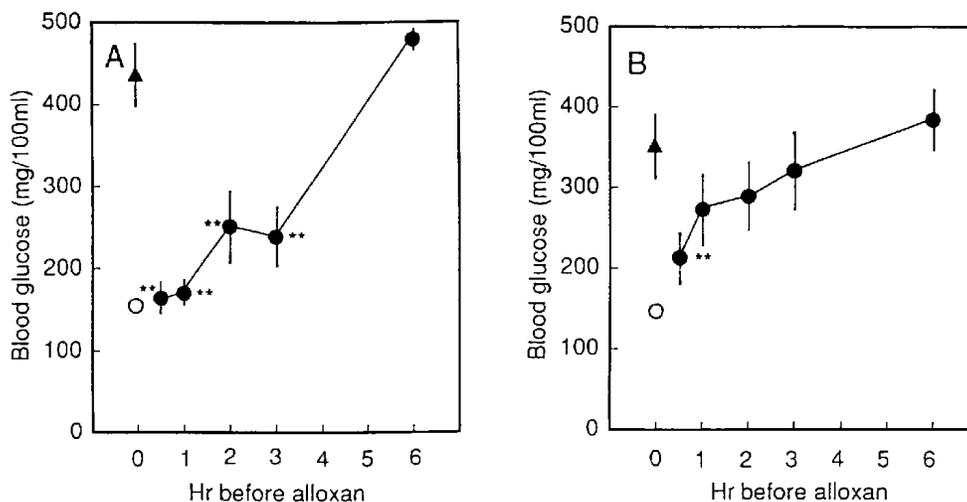
Figure 1 shows the influence of the time interval between the dose of DEDC or DS and the dose of alloxan on the diabetogenic action of alloxan. The maximum effect of DEDC was found at 0.5 hr after pretreatment of this drug and the effect afforded by DEDC pretreatment lasted 3 hr. The effect of DS was merely found in mice treated at 0.5 hr prior to alloxan. DS was reported to be rapidly metabolized to DEDC. Thereafter, DEDC was known to be metabolized reductively into diethylamine and carbon disulfide (12–14). Thus, the effects of these metabolites were examined (Table 2), but each compound had no effect. Therefore, although we can not exclude the possibility that DS itself may be involved in its anti-diabetogenic action, the effect of DS is likely to be partly developed via the metabolic conversion into DEDC.

While blood glucose was elevated at 0.5–2 hr in mice receiving DEDC and DS alone (no alloxan), the increased

**Table 1.** Protection of DEDC and DS against the diabetogenic action of alloxan in mice

Treatment	Blood glucose (mg/100 ml)	Incidence <sup>#</sup>
1.0% CMC + Saline	140.9 $\pm$ 5.7 (10)**	0/10
1.0% CMC + Alloxan	386.8 $\pm$ 19.6 (10)	10/10
DEDC		
0.25 mmol/kg + Alloxan	267.5 $\pm$ 48.9 (10)**	5/10
0.50 + Alloxan	250.0 $\pm$ 33.6 (10)**	6/10
1.00 + Alloxan	189.0 $\pm$ 23.2 (10)**	2/10
2.00 + Alloxan	155.4 $\pm$ 21.1 (10)**	1/10
DS		
0.25 + Alloxan	312.1 $\pm$ 34.3 (10)	8/10
0.50 + Alloxan	211.2 $\pm$ 32.9 (10)**	3/10
1.00 + Alloxan	179.0 $\pm$ 33.6 (10)**	1/10
2.00 + Alloxan	171.5 $\pm$ 33.2 (10)**	2/10

Each compound was injected intraperitoneally 30 min before alloxan (75 mg/kg, i.v.). Blood glucose was measured at 72 hr after alloxan. <sup>#</sup>: Positive for over 200 mg/100 ml of blood glucose. Significantly different from the alloxan control (\*\*P < 0.01). Each value represents the mean  $\pm$  S.E.M. of the results of 10 mice.



**Fig. 1.** Effects of the time interval between the dose of DEDC (A) or DS (B) and the dose of alloxan on the diabetogenic action of alloxan in mice. ○ Normal control; ▲ alloxan control; A: ● DEDC, 2.00 mmol/kg, i.p., B: ● DS, 1.00 mmol/kg, i.p. Points and bars represent the mean  $\pm$  S.E.M. of the results of 10 mice. Significantly different from the alloxan control group (\*\* $P < 0.01$ ).

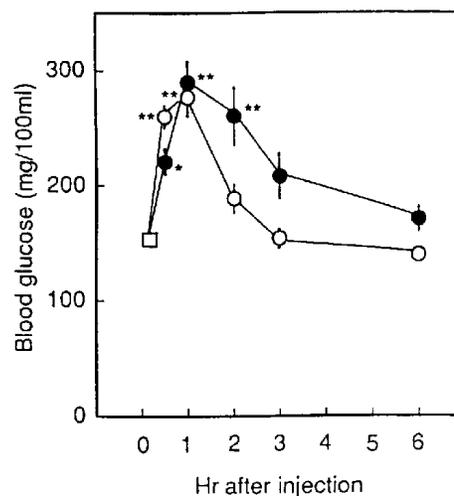
blood glucose recovered to a normal level by 3 hr (Fig. 2). Furthermore, under the same conditions, there was no rise in blood glucose at 72 hr. In the case of DEDC, the blood glucose at 0.5 hr after injection was found to rise dose-dependently, but DS was less potent (Table 3). These results showed that, in particular, mice pretreated with DEDC exhibited hyperglycemia at the time of the alloxan injection. The administration of glucose prior to alloxan injection was known to protect against the diabetogenic action of alloxan (15, 16). Furthermore, the protection from alloxan-induced diabetes afforded by glucose was reported to be eliminated by mannoheptulose, an

antagonist of glucose action at pancreatic  $\beta$ -cells (15–17). If the hyperglycemia resulting from DEDC injection protected the mice from alloxan, administration of mannoheptulose should eliminate this protective effect. To examine this possibility, mannoheptulose (4.0 g/kg, i.v.) was given 24 min after DEDC and 6 min before alloxan (Table 4). Mannoheptulose intensified the diabetogenic action of alloxan in the control group, and it completely eliminated the protection against alloxan-induced dia-

**Table 2.** Effects of diethylamine and carbon disulfide on the diabetogenic action of alloxan in mice

Treatment	Blood glucose (mg/100 ml)
Exp. 1	
Saline + Alloxan	405.2 $\pm$ 20.0 (10)
Diethylamine	
0.50 mmol/kg + Alloxan	428.4 $\pm$ 11.6 (10)
1.00 + Alloxan	375.7 $\pm$ 32.8 (10)
Exp. 2	
Sesame oil + Alloxan	420.2 $\pm$ 22.6 (10)
Carbon disulfide	
0.50 mmol/kg + Alloxan	440.5 $\pm$ 14.3 (10)
1.00 + Alloxan	442.7 $\pm$ 12.3 (10)

Each compound was injected intraperitoneally 30 min before alloxan. Blood glucose was measured at 72 hr after alloxan. Each value represents the mean  $\pm$  S.E.M. of the results of 10 mice.



**Fig. 2.** Time course changes of blood glucose in mice injected with DEDC and DS. □ Normal control; ○ DEDC, 2.00 mmol/kg, i.p.; ● DS, 1.00 mmol/kg, i.p. Points and bars represent the mean  $\pm$  S.E.M. of the results of 8 mice. Significantly different from the normal control group (\* $P < 0.05$ , \*\* $P < 0.01$ ).

**Table 3.** Effects of DEDC and DS on blood glucose in mice

Treatment	Blood glucose (mg/100 ml)
1.0% CMC	141.6 ± 4.2 (7)
DEDC 0.25 mmol/kg	170.7 ± 10.2 (7)
0.50	197.3 ± 10.0 (7)**
1.00	208.9 ± 14.5 (7)**
2.00	252.3 ± 14.5 (7)**
DS 0.25	150.1 ± 3.7 (7)
0.50	160.4 ± 8.5 (7)
1.00	160.7 ± 8.8 (7)
2.00	224.4 ± 12.6 (7)**

Blood glucose was measured at 30 min after intraperitoneal injection of DEDC or DS. Significantly different from the control group (\*\*P < 0.01). Each value represents the mean ± S.E.M. of the results of 7 mice.

betes in the DEDC-pretreated group. In contrast, the coadministration of DEDC with mannoheptulose in the absence of alloxan did not alter blood glucose at 72 hr.

Other experiments were carried out to examine whether the protection provided by DEDC was due to hyperglycemia at the time of alloxan injection. It was possible to eliminate DEDC-induced hyperglycemia by fasting mice for 24 hr prior to DEDC injection. The fasted mice given DEDC at 1.00 mmol/kg, i.p. did not exhibit hyperglycemia at 0.5 hr, the usual time for alloxan injection [control: 65.7 ± 3.6 (N=8) vs. DEDC: 64.7 ± 5.6 mg/100 ml (N=8)]. When DEDC was given to fasted mice 0.5 hr prior to alloxan, DEDC had no protective effect (Table 5). These findings indicate that the anti-diabetogenic action of DEDC is based on the DEDC-induced hyperglycemia at the time of alloxan injection. Similar approaches were not carried out in the case of DS, which was less potent in increasing blood glucose, but the same may be true for the protective effect of the high dose of DS (2.00

**Table 5.** Influence of 24-hr starvation on the protection by DEDC against alloxan-induced diabetes

Treatment	Blood glucose (mg/100 ml)
1.0% CMC + Alloxan	488.1 ± 18.0 (10)
DEDC	
0.50 mmol/kg + Alloxan	493.1 ± 20.1 (10)
1.00 + Alloxan	533.3 ± 39.3 (10)

DEDC was injected intraperitoneally 30 min before alloxan. Blood glucose was measured at 72 hr after alloxan. Each value represents the mean ± S.E.M. of the results of 10 mice.

**Table 6.** Effects of DEDC and DS on pancreatic SOD in mice

Treatment	SOD (µg/g tissue)
1.0% CMC	114.0 ± 10.7 (6)
DEDC 0.50 mmol/kg	123.3 ± 7.4 (6)
1.00	93.7 ± 7.8 (6)
2.00	65.0 ± 6.5 (6)**
DS 0.50	111.6 ± 9.2 (6)
1.00	100.3 ± 7.6 (6)
2.00	103.6 ± 10.3 (6)

Mice were decapitated 30 min after intraperitoneal injection of each compound. Significantly different from the control (\*\*P < 0.01). Each value represents the mean ± S.E.M. of the results of 6 mice.

mmol/kg) that increased blood glucose.

Since DEDC is known to be a potent chelator of copper and to inhibit various copper-containing enzymes such as SOD (18), the effects of DEDC and DS on pancreatic SOD activity were examined (Table 6). DEDC at 2.00 mmol/kg decreased the SOD activity, whereas its lower doses did not. DS was found to have no effect.

When DEDC was incubated with 10<sup>-3</sup> M hydrogen

**Table 4.** Influence of mannoheptulose (MH) on the protection by DEDC against alloxan-induced diabetes

Treatment	Blood glucose (mg/100 ml)	Incidence <sup>f</sup>
1.0% CMC + Saline + Saline	143.6 ± 3.2**	0/10
1.0% CMC + Saline + Alloxan	405.7 ± 31.5	9/10
1.0% CMC + MH + Alloxan	484.8 ± 17.2	10/10
DEDC + MH + Saline	158.6 ± 3.7**	0/10
DEDC + MH + Alloxan	473.9 ± 76.7	10/10
DEDC + Saline + Alloxan	150.1 ± 21.3**	2/10

MH (4.0 g/kg) was injected intravenously 24 min after DEDC (2.00 mmol/kg, i.p.) and 6 min before alloxan. Blood glucose was measured at 72 hr after alloxan. <sup>f</sup>: Positive for over 200 mg/100 ml of blood glucose. Significantly different from the alloxan control (\*\*P < 0.01). Each value represents the mean ± S.E.M. of the results of 10 mice.

**Table 7.** Scavenging activity of hydrogen peroxide by DEDC and DS in vitro

Compound	mM	Scavenging of H <sub>2</sub> O <sub>2</sub> (%)
DEDC	0.1	9.8
	0.2	21.5
	0.5	83.3
DS	0.1	1.1
	0.2	1.6
	0.5	3.6

Each value represents the mean of duplicate experiments.

peroxide for 5 min at 37°C, hydrogen peroxide was markedly scavenged depending on the concentration of DEDC (Table 7). However, DS did not cause any loss of hydrogen peroxide. On the other hand, neither DEDC nor DS at a concentration of 10<sup>-4</sup> M possessed any SOD-like activity in vitro (data not shown).

## DISCUSSION

The present findings indicate that DEDC prevents alloxan-induced diabetes through an indirect mechanism. Administration of glucose prior to alloxan is known to prevent alloxan-induced diabetes. Since the cytotoxic action of alloxan is initiated within minutes of its injection, the protective levels of glucose need only be present for a very short time. DEDC dose-dependently raised blood glucose at 0.5 hr after injection. Hyperglycemia caused by DEDC, which was present at the time of alloxan injection, is thought to protect mice from alloxan-induced diabetes. The idea that DEDC-induced hyperglycemia protects mice from alloxan-induced diabetes was ascertained by two experiments performed in the present studies. Mannoheptulose, an agent that antagonizes the action of glucose at pancreatic  $\beta$ -cells, has been shown to eliminate the protection from alloxan provided by glucose injection. In the present study, mannoheptulose completely eliminated the protection afforded by DEDC, suggesting that the hyperglycemia produced by DEDC is responsible for its protective effect. Furthermore, DEDC-induced protection against alloxan toxicity was not found in the fasted mice in which DEDC could not cause hyperglycemia.

The protection against alloxan-induced diabetes by DEDC and DS did not necessarily coincide with the hyperglycemia present at the time of alloxan injection. The hyperglycemia at 1–2 hr after DS injection could not protect mice from alloxan-induced diabetes. On the other hand, the protection seen when DEDC was given 2 and 3 hr prior to alloxan was not accompanied by hyperglycemia at the time of alloxan injection. Additionally, protec-

tions against alloxan toxicity were also found in the mice given the low doses of DEDC and DS that did not increase blood glucose at 0.5 hr after injection (Tables 1 and 3). These results suggest that an alternative mechanism, not involving hyperglycemia, may participate in the protection against alloxan-induced diabetes by DEDC and DS.

The cytotoxicity of alloxan is mediated through the generation of reactive oxygen-containing radicals in a cascade of chemical reactions, initiated by the reduction-oxidation cycle of alloxan and its reduced form, dialuric acid. The autoxidation of the latter has been shown to generate superoxide anion and hydrogen peroxide and to be catalyzed by copper, iron and manganese (19). Hydroxyl radicals are generated from superoxide anion and hydrogen peroxide by the iron-catalyzed Haber-Weiss reaction and is proposed to be ultimately responsible for the pancreatic damage (6). In the present study, while superoxide anion could not be scavenged by 10<sup>-4</sup> M DEDC and DS, DEDC markedly caused scavenging of hydrogen peroxide, but DS did not (Table 7). In the DNA cleavage by Fenton's reagent (hydrogen peroxide/FeCl<sub>2</sub>), DS was assumed to inhibit either hydroxyl radical formation from hydrogen peroxide or at a later step of the reaction (20). However, there is no evidence to indicate the direct interaction of DEDC and DS with hydroxyl radical.

In the present study, it may be supposed that the amount of hydroxyl radicals formed in the iron-catalyzed Haber-Weiss reaction is decreased, if either the elimination of hydrogen peroxide or the suppression of the Haber-Weiss reaction and autoxidation of dialuric acid by metal-chelation may occur in mice pretreated with DEDC or DS. Thus, it is conceivable that DEDC and DS prevent mice from alloxan-induced diabetes through either an action on the generation system of reactive oxygen-containing radicals or a direct scavenging action on hydrogen peroxide. Also, since SOD is known to suppress dialuric acid autoxidation, the Haber-Weiss reaction and alloxan-induced diabetes (6, 19), the decrease of pancreatic SOD is expected to aggravate the diabetogenic action of alloxan. However, in this study, the DEDC-induced protection against alloxan toxicity was not influenced by 2.00 mmol/kg of DEDC, which decreased pancreatic SOD. Thereafter, to clarify the above possibility, it is necessary to assess the influences of DEDC and DS on the isolated pancreatic islets exposed to alloxan.

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