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The Partial Protective Effect of the Hydroxyl Radical Scavenger Dimethyl Urea on Streptozotocin-Induced Diabetes in the Mouse in Vivo and in Vitro

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Summary. The protective effect on streptozotocin-induced diabetes of dimethyl urea, a hydroxyl radical scavenger, has been evaluated in vivo and in vitro. Pretreatment with dimethyl urea before a single diabetogenic dose of streptozotocin partially protected NMRI mice from hyperglycaemia, whereas the serum glucose of C57BL/KsJ mice increased during week 2 of observation. When the pancreases of these latter mice were examined histologically, insulitis was found in 15 out of 22 animals. The protective effect of dimethyl urea in the NMRI mice was not due to short-term hyperglycaemia induced by the drug, since pretreatment with glucose did not protect from streptozotocin but potentiated its diabetogenic effect. Dimethyl urea reduced the inhibition caused by streptozotocin on proinsulin biosynthesis of NMRI islets in vitro. It is suggested that streptozotocin-induced diabetes in mice may involve generation of hydroxyl radicals which are toxic to islet B cells. If this immediate cytotoxicity is reduced by a scavenger, a more slowly developing hyperglycaemia and an accompanying insulitis may occur in particularly susceptible animals.

Key words: Streptozotocin, dimethyl urea, NMRI mice, C57BL/KsJ mice, proinsulin biosynthesis, insulitis.

The mechanisms by which alloxan and streptozotocin (SZ) exert their diabetogenic actions on the pancreatic B cells are still not clearly understood, but recent evidence suggests that their toxicity may be mediated by free oxygen radicals. The radicals are generated in a sequential process during oxygen reduction, finally resulting in the highly reactive and noxious hydroxyl radical [1]. Cohen and Heikkila [2] have demonstrated that such radicals can be generated in a cell free system from dialuric acid, the reduced form of alloxan. It has been demonstrated also, both in vivo and in vitro, that substances which are known to scavenge free radicals either directly or enzymically, can prevent the cytotoxic effects of alloxan on islet B cells [3–8].

Evidence in support of a free radical mediated toxicity by SZ is sparse. Slonim et al. [9] demonstrated free radical production by SZ in vitro in rat pancreatic microsomes and Robbins et al. [10] and Marklund and Grankvist [11] have shown that the superoxide anion scavenging enzyme, superoxide dismutase, partially protects the islets when administrated before SZ in vivo. On the other hand, Gold et al. [12] were unable to demonstrate any protection from SZ action by superoxide dismutase in vitro measuring islet insulin release. In the present study we examined the effect of a hydroxyl radical scavenger, dimethyl urea (DMU), on SZ-induced hyperglycaemia in vivo and on SZ-inhibited islet proinsulin biosynthesis in vitro. This particular scavenger was chosen since it has been shown to protect against alloxan-induced diabetes [4, 5, 8] and to be a potent hydroxyl scavenger in vitro [13].

Materials and Methods

Animals

Adult male NMRI mice (Anticimex, Sollentuna, Sweden) and inbred C57BL/KsJ mice originally obtained from Jackson Laboratories, Bar Harbor, Maine, USA, aged 8–12 weeks, were used. The animals were allowed free access to water and laboratory chow (Ewos-Anticimex, Type R3; Ewos, Södertälje, Sweden) during the experimental period.

Chemicals

Streptozotocin (lot 60, 273-3 and 5) was a gift of Dr. W. E. Dulin, Upjohn Company, Kalamazoo, Michigan, USA. Collagenase was from Worthington Biochemicals, Freehold, New Jersey, USA and bovine albumin (fraction V) was from Miles Laboratories, Slough, Bucks, UK. Hanks' solution was supplied by Statens Bakteriologiska Laboratorium, Stockholm, Sweden. L-[4,5-^{3}H] leucine was obtained from Amersham International, Bucks, UK. CNBr-activated Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden. Econofluor was purchased from New England Nuclear, Boston, Massachusetts, USA

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Table 1. Serum glucose concentrations in NMRI and C57BL/KsJ mice pretreated with either dimethyl urea or saline before a single dose of streptozotocin

Mouse strain	Serum glucose concentrations (mmol/l)			
	NMRI		C57BL/KsJ	
Treatment	Saline + SZ (n = 15)	DMU + SZ (n = 15)	Saline + SZ (n = 18)	DMU + SZ (n = 22)
Before pretreatment	8.5 ± 0.5	8.9 ± 0.5	8.6 ± 0.3	8.9 ± 0.2
30 min after pretreatment	10.6 ± 0.2	16.3 ± 0.8[c]	9.4 ± 0.4	11.7 ± 0.6[c]
3 days after SZ	17.2 ± 2.4	11.0 ± 0.9[a]	19.7 ± 2.2	10.0 ± 0.9[a]
7 days after SZ	21.6 ± 2.5	13.3 ± 0.9[b]	25.2 ± 2.4	13.8 ± 1.6[b]
14 days after SZ	23.9 ± 2.6	14.4 ± 1.8[b]	32.3 ± 2.9	22.2 ± 1.9[b]

Results expressed as mean ± SEM. Male NMRI or C57BL/KsJ mice were given DMU (4 g/kg body-weight, IP) or an equal volume of saline (9 g/l) 30 min before SZ (160 mg/kg body-weight, IV). [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$: significance of difference for each strain between the DMU and saline pretreated groups

and Soluene from Packard Instruments, Downers Grove, Illinois, USA. Hepes and 1,1-dimethyl urea were supplied by Sigma Chemicals, St Louis, Missouri, USA. Other chemicals of analytical grade were provided by E. Merck, Darmstadt, FRG.

Experiments in Vivo

NMRI or C57BL/KsJ mice were pretreated IP either with DMU at a dosage of 4 g/kg body-weight, or with an equal volume of saline (9 g/l, 500 µl), 30 min before IV administration of a diabetogenic dose of SZ (160 mg/kg body-weight). In a separate series of experiments, NMRI mice were given glucose solution (300 g/l, 250 µl) IP 30 min before SZ; control mice received saline (250 µl) IP. Blood samples were collected by retro-orbital sinus puncture and assayed using an automated glucose analyser (Beckman Instruments, Fullerton, California, USA). Serum glucose samples were taken without anaesthesia before pretreatment, 30 min after pretreatment at the time of SZ administration and on days 3, 7 and 14, the mice being killed after the last sampling.

The mice were killed by cervical distension and the pancreases were removed quickly, fixed in Bouin's solution and embedded in paraffin. Sections (7 µm thick) were cut, stained with hematoxylin-eosin and examined on a blind basis for the general histology and especially for the occurrence of round cell infiltration of the islets, i. e. insulitis.

Experiments in Vitro

NMRI mice were used exclusively in this series of experiments. Pancreatic islets were isolated by collagenase digestion [14]. The islets were incubated in groups of 50 in a bicarbonate buffer (1 ml) [15] supplemented with Hepes (10 mmol/l), albumin (2 mg/ml) and glucose (5.5 mmol/l). SZ was dissolved in cold citrate buffer (pH 4.5) immediately before use and 5–10 µl of this solution were added to the islets to obtain the required SZ concentrations. The incubations were performed in air at room temperature (30 min) and terminated by addition of the bicarbonate buffered medium (3 ml). In each experiment, control islets were treated with citrate solution (5–10 µl). To test the effects of DMU, it was added to the albumin and Hepes supplemented bicarbonate buffer medium (final concentration 6 mg/ml) and preincubated with islets for 30 min at 37 °C to allow penetration of the cells before exposure to SZ.

Immediately after the SZ incubations, the islets in two groups of 20 from each incubation vial were transferred to the same bicarbonate buffered medium as above (100 µl) containing L-[4,5-^{3}H]leucine (50 µCi/ml), albumin (2 mg/ml) and glucose (16.7 mmol/l). After 2 h incubation at 37 °C (air + 5% CO_2), the islets were washed and sonicated for 10 s in redistilled water (200 µl). A fraction of the aqueous homogenate was subsequently incubated with antibovine insulin serum coupled to Sepharose 4B beads to separate labelled proinsulin from other labelled islet proteins [16]. Control values obtained by incubation of the same homogenate with normal guinea-pig serum proteins coupled to Sepharose beads were subtracted from the experimental values. Soluene (0.5 ml) and Econofluor (5 ml) were added to each sample and the antibody-bound and trichloroacetic acid precipitable radioactivity (total protein biosynthesis) was determined in a liquid scintillation spectrometer (Packard Tri-Carb, model 3255). The corresponding mean c. p. m. values per 20 islets from each experiment were calculated. The rate of proinsulin biosynthesis of the experimental group was expressed as a percentage of the control group.

Statistical Analyses

Groups of data were compared using the unpaired Student's t-test, or Fisher's exact test for 2 × 2 tables [17].

Results

Effects of Dimethyl Urea on the Diabetogenic Effects of Streptozotocin in Vivo

There was a continuous increase in the serum glucose concentration of NMRI mice given saline before SZ administration (Table 1). Thus, 12 out of 15 mice were hyperglycaemic with a serum glucose > 12.5 mmol/l after 2 weeks. In contrast, only five of the 15 DMU-pretreated NMRI mice were hyperglycaemic 2 weeks following SZ injection. At all times after the SZ injection serum glucose was lower in the DMU-pretreated group. DMU in itself produced a significant serum glucose elevation 30-min post-injection ($p < 0.001$). i. e. at the time when SZ was administered.

When the same experiments were performed in C57BL/KsJ mice, DMU protected against hyperglycaemia during the first 3 days (Table 1). After 2 weeks the mean serum glucose concentration had increased markedly but it was still below that of the SZ treated controls. Three DMU pretreated C57BL/KsJ mice and none of the control animals had a serum glucose < 12.5 mmol/l at the time of death. It is worthy of note that the C57BL/KsJ mice had markedly higher serum glucose concentrations than the NMRI mice 2 weeks after the SZ injection and the difference between the control groups at this time was highly significant ($p < 0.001$).

The results of histological examination of the pancreases are summarized in Table 2. Fifteen of the 22 C57BL/KsJ mice given DMU before SZ exhibited pancreatic insulitis (Fig. 1; Table 2, class C), which was observed in only one of the 18 control animals. In some of the C57BL/KsJ mice treated with DMU + SZ, areas of inflammatory reactions were observed in the exocrine pancreas with a pronounced connective tissue proliferation containing a pleomorphic mass of cells (Fig. 2), similar to that which we have observed previously in multi-SZ-treated C57BL/KsJ mice [18].

Ten of the control NMRI mice had only a few small islets containing a majority of pycnotic cells (Table 2, class D), whereas only one of the DMU-treated NMRI mice displayed a similar islet morphology. In separate control experiments both C57BL/KsJ and NMRI mice were given DMU only. No long-term effects on the pancreatic morphology or serum glucose concentrations

Table 2. Pancreatic islet morphology 14 days after a single dose of streptozotocin in C57BL/KsJ and NMRI mice pretreated with either dimethyl urea or saline

Islet morphology	A	B	C	D
C57BL/KsJ mice				
DMU + SZ ($n = 22$)	4	2	15[a]	1
Saline + SZ ($n = 18$)	0	3	1	14
NMRI mice				
DMU + SZ ($n = 15$)	8	5	1	1[a]
Saline + SZ ($n = 15$)	4	1	0	10

The islet morphology of each animal was evaluated according to four arbitrary classes by light microscopy. A: Normal islet morphology; B: Essentially normal islet appearance, but with some mononuclear cell infiltration especially in the peri-insulinar tissue; C: Heavy infiltration of mononuclear cells into a large number of islets (Fig. 1); D: 'Diabetic' appearance with only a few, small islets and islets displaying vast cellular disarray and pycnotic nuclei. The different groups were compared by Fisher's exact test for 2 × 2 tables [17]. [a] $p < 0.001$ compared with the saline treated control group

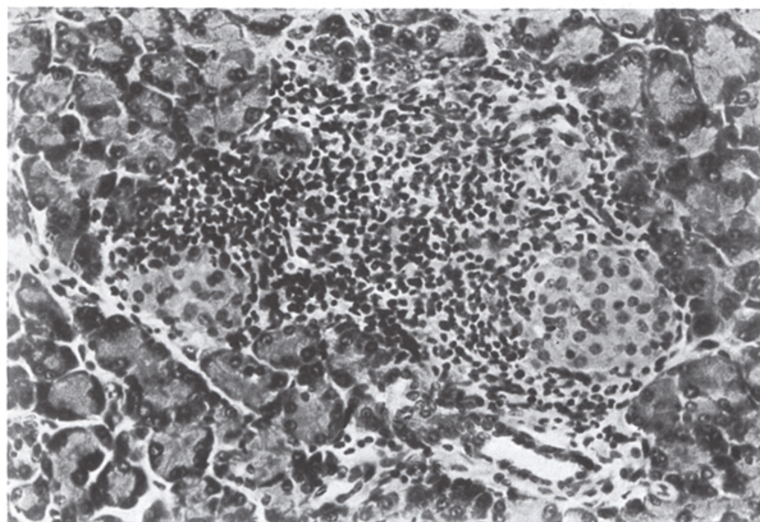

Fig. 1. Pancreatic islets of a C57BL/KsJ mouse treated with dimethyl urea and streptozotocin showing severe round cell infiltration. The serum glucose after 14 days (at death) was 16.5 mmol/l. Hematoxylin and eosin (× 250)

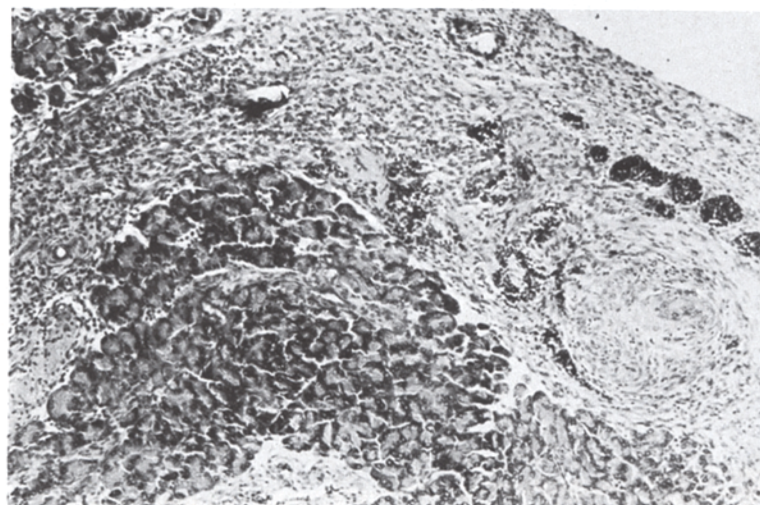

Fig. 2. Pancreas of a C57BL/KsJ mouse treated with dimethyl urea and streptozotocin, showing an area of proliferative connective tissue. Hematoxylin and eosin (× 100)

Table 3. Rates of proinsulin biosynthesis in islets exposed to streptozotocin and dimethyl urea in vitro for 30 min at 5.5 mmol/l glucose

SZ (mmol/l)	DMU (mg/ml)	Proinsulin biosynthesis (% of control)	No of experiments
0.55	0	93.2 ± 5.4	5
1.1	0	74.9 ± 7.4[a]	7
2.2	0	60.2 ± 5.6[b] ┐[c]	17
2.2	6	86.5 ± 4.9[a] ┘	11
4.4	0	34.3 ± 2.7[b] ┐[d]	38
4.4	6	55.5 ± 5.1[b] ┘	13

Results expressed as mean ± SEM. Proinsulin biosynthesis was estimated by measuring the incorporation of L-[4,5-^{3}H] leucine into the proinsulin fraction of islets incubated for 2 h in a medium supplemented with 16.7 mmol/l glucose. The mean c.p.m. values per 20 islets for each experimental group were calculated. The data were expressed as the percentage proinsulin biosynthesis of the experimental group compared with the control group, which was not exposed to SZ. The statistical significance of the difference between SZ-exposed and non-exposed control islets was tested by means of a paired t-test: [a] $p < 0.05$ and [b] $p < 0.001$. An unpaired t-test was used for calculating the statistical significance of the difference obtained by the addition of DMU to islets: [c] $p < 0.01$ and [d] $p < 0.001$

were observed during the 2-week follow-up period (results not shown).

In order to test the hypothesis that the DMU-induced acute elevation of the serum glucose (Table 1) contributed to the protective effect against SZ, NMRI mice were pretreated with glucose 30 min before the SZ injection (160 mg/kg body-weight, IV). Such mice developed a more marked hyperglycaemia 3 days after the SZ injection than the corresponding control group pretreated with saline (21.4 ± 2.4 and 14.2 ± 1.7 mmol/l glucose, respectively; $p < 0.05$; $n = 18$ for both groups). On days 7 and 14 no significant differences were observed in the serum glucose of the two groups of mice. Four of the 18 mice pretreated with glucose died before the end of the 2-week observation period. The islets of all but one of these surviving mice were very small and disintegrated (corresponding to class D, Table 2).

Effects of Dimethyl Urea in Vitro

A dose-dependent inhibition of glucose-stimulated proinsulin biosynthesis was observed in islets exposed to SZ for 30 min in vitro (Table 3). No significant depression of the biosynthetic rate could be demonstrated at 0.55 mmol/l SZ, but it was significant at all higher concentrations. Moreover, the percentage contribution of proinsulin to the total protein biosynthesis showed a similar dose-dependent decrease (data not shown). The SZ inhibited proinsulin biosynthesis was partially normalised by DMU and a significant protection was found both at 2.2 and 4.4 mmol/l SZ.

Discussion

In the present investigation dimethyl urea was found to protect partially NMRI mice against the hyperglycaemic effect of streptozotocin. It seems plausible that the protective effect of DMU in vivo is not due to its short-lasting hyperglycaemic effect, which is well known to protect against alloxan action [19, 20]. We found, in contrast, an enhancing effect of glucose on the SZ action, which is in agreement with the results of Brodsky and Logothetopoulos [21] on C57BL/6J mice. Dulin and Wyse [22] and Ganda et al. [23] likewise were unable to protect rats by administration of glucose prior to SZ. The fact that DMU partially prevented the SZ-induced depression of proinsulin biosynthesis in vitro further supports the view that the protection of DMU in vivo is not due to its hyperglycaemic effect.

When C57BL/KsJ mice were given DMU and a single diabetogenic dose of SZ, hyperglycaemia developed more slowly than in the control animals and was accompanied by insulitis in 15 of the 22 animals. Like and Rossini have shown that C57BL/KsJ mice receiving five sub-diabetogenic doses of SZ developed insulitis and a gradual hyperglycaemia [24]. These authors have suggested that this treatment in susceptible mouse strains, may induce a direct B cell cytotoxicity and in combination with virus induction within the B cells may initiate a cell-mediated auto-immune reaction [25]. It could be speculated that DMU when given to C57BL/KsJ mice before a high dose of SZ, reduces the immediate B cell cytotoxicity and a gradual hyperglycaemia develops, similar to that of the multi-SZ model. The pathogenetic significance of insulitis for hyperglycaemia in these animals may, however, be doubtful as suggested by Leiter [26].

The chemical reaction by which SZ causes B cell cytotoxicity and the nature of hydroxyl radical involvment remains obscure. Recently, Yamamoto and Okamoto [27] demonstrated an increased activity of polyadenosine diphosphoribose (poly (ADP-rib)) synthetase, an enzyme participating in DNA-repair, in nuclei from rat pancreatic islets exposed to SZ in vitro. This enzyme uses NAD as a substrate, which is concordant with the documented observation that SZ depletes the NAD content of pancreatic islets [28–29]. It has been demonstrated that enzymically generated oxygen radicals in vitro can cause DNA single strand breaks [30], which presumably activates the enzyme. Furthermore, Skidmore et al. [31] have observed similar effects on NAD concentrations and poly(ADP-rib) synthetase activity in mouse leukaemia cells after methylnitrosourea (the aglucone moiety of SZ) and γ-radiation treatment. The latter is well known to produce free radicals. In support of such a mechanism, we have found that DMU inhibits the NAD depletion of mouse pancreatic islets exposed to SZ in vitro [32].

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