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Zinc sulphate induces metallothionein in pancreatic islets of mice and protects against diabetes induced by multiple low doses of streptozotocin

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Abstract

Aims/hypothesis. Diabetes is induced by multiple low doses of streptozotocin (MLD-STZ) in male mice of susceptible strains. In this model beta-cell injury and T-cell-mediated inflammatory reactions are induced. Probably, reactive oxygen species (ROS) participate in the destruction of beta cells. The effects of ROS can be counterbalanced by several antioxidant systems. One of these is metallothionein (MT), cytosolic proteins that are induced by zinc ions (Zn^{2+}) and scavenge hydroxyl radicals ($^{\cdot}OH$). The effect of Zn^{2+} on MLD-STZ-diabetes was studied.
Methods. We gave C57BL/6 and (C57BL/6 × SJL)F_1 hybrid mice either MLD-STZ or in addition Zn^{2+}-enriched drinking water. We analysed metallothionein ex vivo in pancreatic islets for protein and mRNA concentration for the isoforms 1 and 2. Pancreatic sections were examined by immunohistochemistry for metallothionein and histologically for insulitis.
Results. In both strains, Zn^{2+}-enriched drinking water significantly up-regulated metallothionein and prevented MLD-STZ-diabetes and loss of beta-cell function. In the F_1 hybrid mice a variant of MLD-STZ-diabetes was observed. These mice developed hyperglycaemia 10 weeks after the first injection of STZ (in contrast to 2 weeks observed in other mouse strains) and pronounced insulitis. The mRNA of the metallothionein isoforms 1 and 2 were constitutively expressed and slightly up-regulated by Zn^{2+}-enriched drinking water. All islets cells stained for metallothionein.
Conclusions/interpretations. Drinking water enriched with Zn^{2+} significantly up-regulated metallothionein production in pancreatic islets of mice and prevented diabetes induced with MLD-STZ. [Diabetologia (2000) 43: 1020–1030]

Keywords Streptozotocin, metallothionein, zinc sulphate, diabetes, beta cells

T cell-mediated inflammatory autoimmune reactions are considered to cause Type I diabetes (insulin-dependent) mellitus [1, 2]. Reactive oxygen species (ROS) and nitrogen monoxide ($NO^{\cdot}$) contribute to inflammatory diseases [3] and are implicated as mediators of beta-cell destruction in animal models of diabetes [4–11]. Reactive oxygen species are continuously produced by respiring mitochondria [12] and reduced nicotinamide adenine dinucleotide phosphate oxidase [13]. Certain antioxidants specifically protect against their damage: superoxide dismutase (SOD) against superoxide radical ($O_2^{\cdot -}$), catalase against hydrogen peroxide (H_2O_2), and glutathione peroxidase against H_2O_2 and lipid peroxides. It is, however, not known if there is a specific defense against hydroxyl radicals ($^{\cdot}OH$) in vivo, the most strongly reactive of ROS [14].

Recently, very low concentrations of antioxidants were found in isolated pancreatic islets of rats and in

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Abbreviations: AEC, 3-Amino-9-ethylcarbazole; B6SJL/F_1, (C57BL/6 × SJL)F_1 male hybrids; Fe^{2+}, iron; HBSS, Hank's balanced salt solution; M, DNA molecular weight marker; MLD-STZ, multiple low doses of streptozotocin; MT, metallothionein; N, negative control; $NO^{\cdot}$, nitrogen monoxide; $O_2^{\cdot -}$, superoxide radical; $^{\cdot}OH$, hydroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase; Zn^{2+}, zinc sulphate

insulin-producing RINm5F cells compared with other rat tissues [15]. The authors concluded that beta cells are vulnerable to ROS because of their poor antioxidant system. This assumption was corroborated by the observation that up-regulation of catalase and glutathione peroxidase in transfected RINm5F cells conferred resistance to ROS [16]. Furthermore, overexpression of copper/zinc (Cu/Zn) SOD in transgenic mice [17] or targeted to beta cells [18] averted attacks on beta cells by ROS generated by streptozotocin (STZ) or alloxan or both.

Metallothionein (MT) are a group of low molecular weight (approximately 7000 M_r), cysteine-rich (23–33 mol%) cytosolic proteins found in all eukaryotic species [19]. Metallothionein are potent ·OH scavengers in vitro [14, 20–22] and 38.5-fold to 50-fold more effective in protecting DNA from ·OH attacks than glutathione [23, 24]. There are four isoforms of MT [25]. The isoforms MT-1 and MT-2 are present in major organs, MT-1 being more abundant than MT-2 [19, 26]. Metallothionein synthesis is stimulated by numerous non-metallic agents and by metal ions [14, 19]. In rats and mice, Zn salt-induced MT were localized in the cytoplasm of pancreatic exocrine cells [26–31]. In rats, islets stained uniformly for MT by immunohistochemistry [26] but in mice the results were discrepant. By immunohistochemistry, an antibody reacting with both MT-1 and MT-2 isoforms failed to stain islets of normal and of transgenic mice overexpressing MT-1 [31]. Our laboratory applying quantitative measurements [32] found, however, constitutive concentrations and induction of MT with Zn sulphate (Zn^{2+}) in islets of mice in vitro [33] and ex vivo [34].

Pretreatment with Zn^{2+} prevents diabetes in animals. In rats, s.c. injection of Zn^{2+} reduced hyperglycaemia induced with one toxic dose of STZ injected i.p. [30]. Our laboratory reported that i.p. pretreatment with Zn^{2+} prevented diabetes induced with multiple low doses of streptozotocin (MLD-STZ) in mice [35]. Both groups of investigators proposed that Zn^{2+}-induced MT scavenged ·OH triggered by STZ. Further support for the protective effect of Zn^{2+} on MLD-STZ-induced diabetes in mice is presented.

Materials and methods

Animals. We obtained C57BL/6 male mice at 5–6 weeks of age from Harlan Winkelmann (Borchen, Germany) and purchased (C57BL/6 × SJL)F_1 male hybrids (B6SJL/F_1) at 5–6 weeks of age from The Jackson Laboratories (Bar Harbor, Me., USA). Mice were 7–8 weeks old at the beginning of the experiments. They were kept under specific pathogen-free conditions, received a rodent diet (Ssniff M, Ssniff, Soest, Germany) and had free access to drinking water. The animal studies were conducted in accordance with the "Principles of laboratory animal care" (NIH publication no. 85–23, revised 1995) as well as the current version of the German Law on the Protection of Animals.

Reagents. Collagenase (0.42 U/mg), HEPES, bovine haemoglobin and TRIS were supplied by Serva (Heidelberg, Germany) and reagents for the Krebs-Ringer buffer, $ZnSO_4$, haematoxylin and Kaiser's glycerol gelatin by Merck (Darmstadt, Germany). Streptozotocin was obtained from Roche Diagnostics (Mannheim, Germany), D-glucose, BSA, and RPMI 1640 culture medium without D-glucose from Sigma (Deisenhofen, Germany), FCS, Hank's balanced salt solution (HBSS), PBS and Penicillin-Streptomycin were supplied by Gibco BRL (Eggenstein, Germany). Lymphocyte separation medium was purchased from Biochrom (Berlin, Germany), carrier-free ^{109}cadmium (Cd) was obtained from Amersham Buchler (Braunschweig, Germany), Bio-Rad dye solution from Bio-Rad (München, Germany), insulin RIA 100 from Pharmacia & Upjohn (Freiburg, Germany) and anti-GAD-RIA from BRAHMS Diagnostica (Berlin, Germany). For staining by immunohistochemistry, guinea pig antiserum to porcine insulin, rabbit anti-guinea pig IgG, goat anti-rabbit IgG, normal serum of guinea pigs and rabbits, substrate 3-amino-9-ethylcarbazole (AEC) and washing solutions were supplied by Dako (Hamburg, Germany). Polyclonal antiserum to rat liver MT-1 and MT-2 was generated in rabbits and kindly provided by Dr. K. H. Summer (Institute of Toxicology, Neuherberg, Germany).

Treatment of mice. To investigate whether Zn^{2+}-enriched drinking water induces MT synthesis in pancreatic islets, groups of five mice each had free access to water enriched with 25 mmol/l Zn^{2+} for 1 week [25]. Mice whose water had no additive served as controls. Islets were isolated from individual mice [34] and prepared for measurement of MT concentrations [33].

To induce diabetes, mice were injected i.p. with 5 × 40 mg STZ/kg body weight on each of 5 consecutive days according to the MLD-STZ protocol [36]. Streptozotocin was dissolved in 0.1 mol/l sodium citrate buffer (pH 4.0) at a concentration of 0.4% and injected within 5 min after preparation. The day of the first STZ injection was designated day 0. For treatment with Zn^{2+}, two protocols were applied. In the first protocol groups of C57BL/6 and B6SJL/F_1 mice were given free access to drinking water enriched with 25 mmol/l Zn^{2+}; this treatment was started 1 week before the first STZ injection and conducted throughout the whole experimental period until the mice were killed. In the second protocol C57BL/6 mice were given free access to Zn^{2+}-enriched drinking water from 1 week before the first STZ injection until 1 day after the last STZ injection. Groups of mice that had only received either Zn^{2+}-enriched drinking water or i.p. injections of MLD-STZ served as controls. The Zn^{2+}-enriched drinking water was freshly prepared and replaced daily.

For the OGTT, D-glucose was dissolved in 0.9% saline at a concentration of 20%. After a fasting period of 16 h, each mouse received an oral load of 2.0 g D-glucose/kg body weight through an intubation tube. Blood glucose concentrations were measured just before (0 min) and at 15 and 30 min after the glucose challenge. The C57BL/6 mice underwent an OGTT at week 4 and B6SJLF_1 at week 6 after the onset of treatment with MLD-STZ. Mice matched with them for age served as controls.

Determination of plasma glucose. Blood samples were collected weekly from non-fasted animals between 0900 and 1100 hours from the retro-orbital venous plexus, using 20 µl-capillary glass tubes. Glucose concentration was measured by the hexokinase method using an autoanalyser (Eppendorf APC 5040, Hamburg, Germany). Hyperglycaemia was defined as a blood glucose concentration of 13.9 mmol/l or more persisting for 3 or more consecutive weeks.

Determination of MT. Metallothionein were measured by the ^{109}Cd-haemoglobin saturation assay as described previously [32, 34]. Briefly, isolated islets from individual mice were washed three times with PBS, covered with 50 µl double-distilled water and stored at –80 °C. For measurement of total protein and MT in cytosolic preparations, the deep-frozen islets were thawed, kept at 4 °C, lyophilised and resuspended in 300 µl TRIS/HCl (10 mmol/l, 85 mmol/l NaCl, pH 7.4). After centrifugation (1 000 *g*, 5 min, 4 °C), 100 µl of the supernatant were used for total protein measurement by Bio-Rad protein assay and 200 µl were kept at –80 °C until measurement of MT. To quantify concentrations of total MT, ^{109}Cd was added in excess to 100 µl of the cytosolic probes. To recover ^{109}Cd bound to MT, excess ^{109}Cd was complexed by addition of bovine haemoglobin (4 %) and the Cd-haemoglobin complex was removed by heat treatment. After centrifugation (4 000 *g*, 5 min, 20 °C) the supernatant containing MT was analysed for ^{109}Cd with a Packard Auto-Gamma 5780 (Packard, Frankfurt/Main, Germany). Addition of the haemolysate and heat treatment were repeated three times. The calculation of MT content was based on a molar ratio of 7 g-atom Cd/mol MT [37] and a molecular weight of 6 600.

Immunohistochemistry. After the mice had been killed by cervical dislocation, the pancreas and liver were removed and specimens were snap-frozen in liquid nitrogen. Cryostat sections (5 µm thick) were placed on slides, air dried, acetone-fixed for 10 min, and stained for either MT or insulin by using an immunoperoxidase method. A polyclonal rabbit anti-rat MT antiserum, cross-reacting with mouse MT was used for staining of MT. Sections were overlayed with 50 µl of the primary antibody to rat MT (diluted 1:100) and incubated for 30 min at room temperature in a humid chamber. After two washing procedures in PBS, the sections were incubated with 50 µl of peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:100) for 45 min at room temperature and washed twice with PBS. The staining reaction was activated by incubation of the sections with the substrate solution AEC for 20 min at 37 °C then stopped by thorough washings in distilled water. Finally, the sections were counterstained with Mayer's haemalaun and mounted in Kaiser's glycerol gelatin. Controls for the immunohistochemical staining were incubated with non-immune serum of the animal species used to produce the primary antibodies and omission of the primary antibody reagents from the procedure. Microscopical examinations were done independently by two of the authors. The degree of staining was scored as follows: 0 = no staining; 1 + = dots of staining loosely scattered throughout the cell cytoplasm; 2 + = 50 % staining of the cytoplasm; 3 + = more than 75 % staining of the cytoplasm.

Histological examination. For light microscopy, groups of 5 B6SJL/F_1 mice each were killed on day 10 as well as 4 weeks after the first injection of MLD-STZ. Sections from untreated mice and mice treated with Zn^{2+}-enriched drinking water served as controls. Preparation of pancreas sections, staining and examination were done as described previously with slight modifications [38]. Briefly, the pancreata were removed and fixed in Bouin's solution. After routine processing, three step-sections 5 µm thick at a distance of 50 µm were prepared from each paraffin-blocked pancreas and sections were stained with haematoxylin-eosin. After the slides were coded, examination of the sections for the presence of infiltrates with mononuclear cells at both islet-pole and intra-islet sites were done independently by two of the authors. The degree of intra-islet infiltrates (insulitis) was scored as follows: 0 = no infiltrate; 1 + = mild infiltrate (≤ 30 % of islet cells are mononuclear cells); 2 + = moderate infiltrate (> 30 to ≤ 75 % of islet cells are mononuclear cells); 3 + = severe infiltrate (> 75 % islet cells are mononuclear cells). Perivascular or periductular sites or both at islet poles were examined for absence or presence of infiltrates.

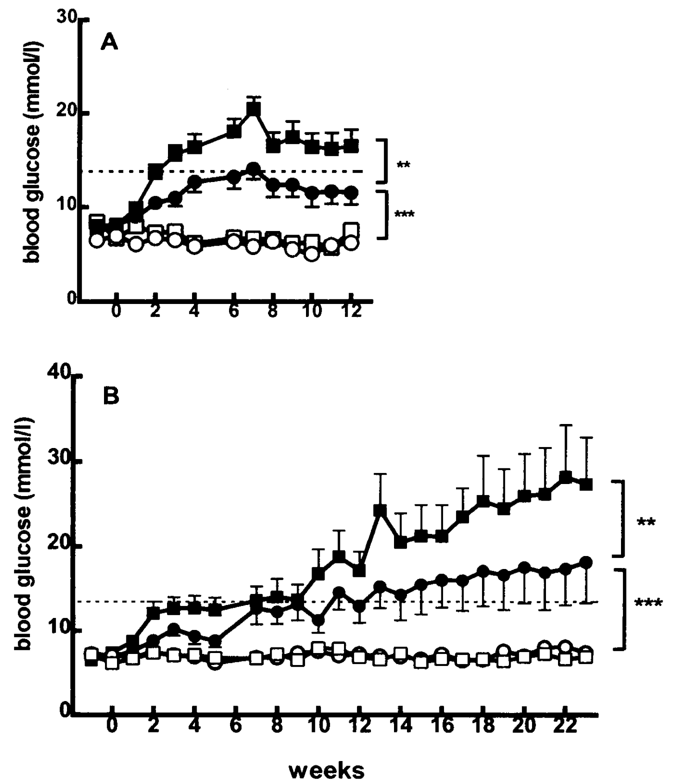

Fig. 1 A, B. Effect of Zn^{2+}-enriched drinking water on diabetes induced by MLD-STZ in C57BL/6 **A** and B6SJL/F_1 **B** mice. Blood glucose concentrations (means ± SEM) over time in weeks are shown. Bars of very small SEM are hidden in the curves symbols. Male mice were treated with either MLD-STZ only (■), MLD-STZ plus Zn^{2+}-enriched (25 mmol/l) drinking water (●), Zn^{2+}-enriched drinking water only (○) or were not treated (□). Each group treated with MLD-STZ consisted of 10–15 mice and for the control groups 5 mice each were used. Zn^{2+}-enriched drinking water prevented hyperglycaemia induced with MLD-STZ. ** $p < 0.01$ comparing the areas under the curves of MLD-STZ-treated vs MLD-STZ-treated plus Zn^{2+}-treated groups; *** $p < 0.001$ comparing the areas under the curves of MLD-STZ- plus Zn^{2+}-treated vs control groups

Determination of GAD_{65}-autoantibodies. Samples of sera from 6 untreated and 20 mice treated with MLD-STZ were analysed for GAD_{65}-autoantibodies using a radioligand assay as described previously [39]. The cut-off was defined at 571 cpm.

RNA preparation and RT-PCR. Total RNA was extracted from pooled islets isolated from groups of ten mice each and from liver tissue using the TRIzol reagent kit (Life Technologies, Gaithersburg, Md., USA) [40]. The RNA preparation was stored at –80 °C until use. By using Moloney murine leukemia virus RT (Life Technologies), 1 µg of total RNA from islets and liver were reversibly transcribed into cDNA, followed by amplification of target genes by PCR [41]. For amplification of mouse MT-1, MT-2 and the housekeeping gene β-actin as internal control, primer pairs were commercially synthesized by

MWG-Biotech (Ebersberg, Germany): MT-1, 5'-TCCTGAGTACCTTCTCCTC-3', 3'-GGTGGTGCACATTTATCAT-5'; MT-2, 5'-TGCGCTGGCGCCTGCAAAT-3', 3'-TCTCCGAAGGCTGTTCACG-5'; proinsulin, 5'-GGCTTCTTCTACACACACCCA-3', 3'-ATGGTCGACCTCTTGATGAC-5'; β-actin, 5'-GTGGGCCGCTCTAGGCACCAA-3', 3'-CTCTTTGATGTCACGCACGATTTC-5'. The RT reaction was amplified using *Taq* polymerase (Roche Diagnostics, Mannheim, Germany). The cycle numbers were chosen to be on the linear, i.e. exponential phase of the amplification of the three genes: 35 for MT-1 and MT-2, 26 for proinsulin and 30 for β-actin. For separation, the amplified PCR products, 7 μl of each, i.e. the target product and β-actin, were loaded on 1.5 % agarose gels containing ethidium bromide (0.1 μg/ml). The resulting bands were photographed with Polaroid Instant Pack Film 665 (Polaroid, Cambridge, Mass., USA). To exclude the possibility of genomic DNA contamination during RNA preparation, negative controls were set up for each PCR amplification, using purified RNA as a template.

Data analysis. Data presented are means ± SEM or SD. For PCR, intensities of bands of PCR products on the film were determined using scanning densitometry. The ratio of the intensity integral of target PCR products to that of β-actin was calculated. Comparisons between groups were done by the unpaired Student's *t* test. We considered *p* less than 0.05 statistically significant.

Results

Prevention of MLD-STZ-induced diabetes by Zn^{2+}. Treatment with Zn^{2+}-enriched drinking water prevented diabetes induced by MLD-STZ in both C57BL/6 and B6SJL/F_1 mice (Fig. 1). The C57BL/6 mice that were treated with MLD-STZ only started to develop hyperglycaemia 2 weeks after the first injection of STZ and hyperglycaemia persisted for the further observation period of 10 weeks. Treatment with Zn^{2+}-enriched drinking water, however, significantly reduced the high blood glucose concentrations induced by MLD-STZ. Although the mean blood glucose concentrations in this group did not exceed the euglycaemic threshold of 13.9 mmol/l they were significantly higher compared with the values measured in the two control groups which were treated with either Zn^{2+}-drinking water or tap water alone (Fig. 1). Treatment with Zn^{2+}-enriched drinking water until 1 day after the last injection of STZ did not suffice to protect against MLD-STZ-induced hyperglycaemia (data not shown).

Notably, B6SJL/F_1 mice started to develop severe hyperglycaemia just 10 weeks after the first injection of STZ. Treatment with Zn^{2+}-enriched drinking water significantly reduced the high blood glucose concentrations induced by MLD-STZ during the observation period of 23 weeks. Yet, in this group, the mean blood glucose concentrations slightly exceeded the euglycaemic threshold of 13.9 mmol/l and they remained significantly higher than those in the two control groups that had either not been treated or had re-

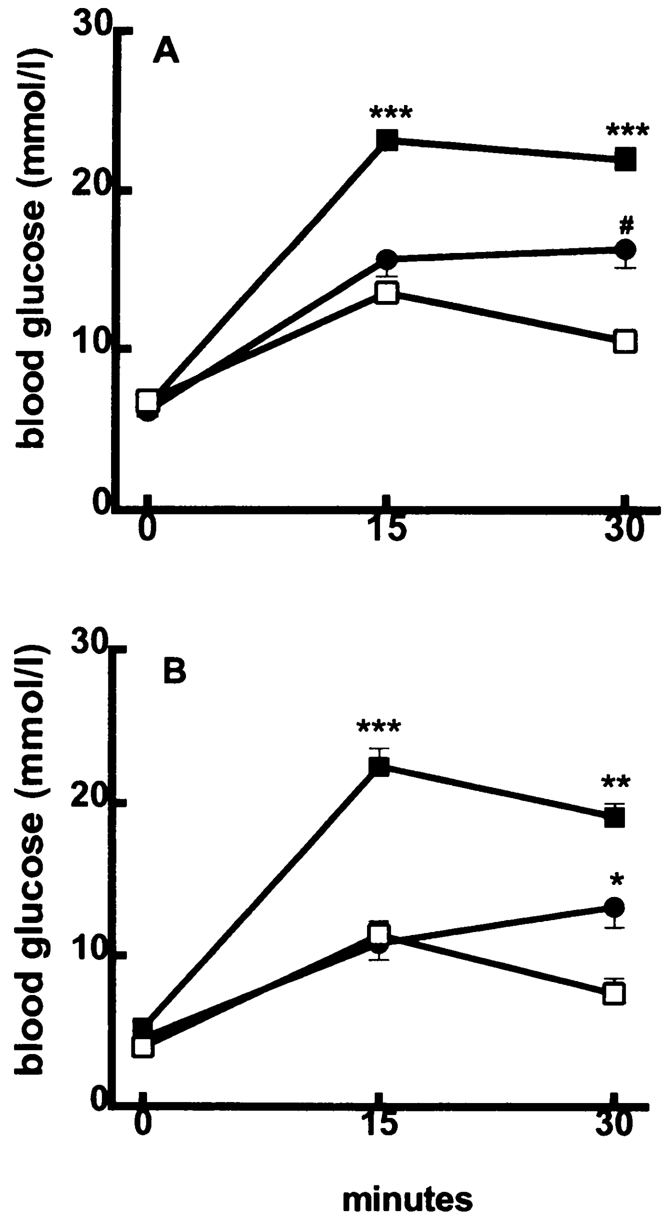

Fig. 2 A, B. Effect of continous treatment with Zn^{2+}-enriched drinking water on oral glucose tolerance in mice treated with MLD-STZ. Blood glucose concentrations (means ± SEM) before and after a glucose load are given. Bars of very small SEM are hidden in the curves symbols. **A** 4 weeks after starting treatment of C57BL/6 mice. **B** 6 weeks after starting treatment of B6SJL/F_1 mice. Mice were treated with MLD-STZ either alone (■) or in addition with Zn^{2+}-enriched (25 mmol/l) drinking water (●) or were not treated (□). Groups of 15 mice each were used for the treatment with MLD-STZ and groups of 5 mice each for controls. Zn^{2+}-enriched drinking water prevented loss of glucose tolerance induced with MLD-STZ. ** $p < 0.01$ and *** $p < 0.001$ comparing MLD-STZ-treated vs MLD-STZ- plus Zn^{2+}-treated groups; * $p < 0.05$ and # $p < 0.001$ comparing MLD-STZ- plus Zn^{2+}-treated vs untreated control groups

Table 1. Effect of Zn^{2+}-enriched drinking water on the variables indicated

Variable	C57BL/6 mice		$B6SJL/F_1$ mice	
	Zn^{2+}	control	Zn^{2+}	control
MT (ng/µg islet protein)	1.34 ± 0.26[a]	0.61 ± 0.11	2.20 ± 0.34[b]	0.35 ± 0.05
Body weight (g)	23.5 ± 0.7	22.2 ± 0.3	25.2 ± 0.3	25.8 ± 0.4
Blood glucose concentration (mmol/l)	7.2 ± 0.3	8.3 ± 0.5	7.5 ± 0.3	6.7 ± 0.2
Islets/mouse *(n)*	124 ± 6	135 ± 10	130 ± 11	142 ± 7
Total protein concentration/Islet (ng)	88.6 ± 4.9	78.6 ± 5.5	153.30 ± 24.11	194.93 ± 31.03

Mice were treated with Zn^{2+}-enriched drinking water or tap water (control) for 1 week. Results were obtained from groups of four to five C57BL/6 and nine to ten $B6SJL/F_1$, hybrid mice each. Data are means ± SEM. [a] $p < 0.05$; [b] $p < 0.001$ compared with untreated controls

ceived Zn^{2+}-enriched drinking water only (Fig. 1). The protective effect induced by Zn^{2+} was not caused by starving because the body weight and the individual daily intake of approximately 7 ml water containing 7.1 mg Zn^{2+} were similar in both control groups (data not shown). Note that it has been proved that Zn^{2+} is essentially non-toxic in human. It is neither carcinogenic, mutagenic nor teratogenic [42]. Because hyperglycaemia determines the intake of water the individual consumption was not calculated. Nevertheless, the protective effect of Zn^{2+} was exerted before diabetes developed.

Zn^{2+}-enriched drinking water prevented essential beta-cell dysfunction in mice injected with MLD-STZ. In vivo, beta-cell function was assessed by using the OGTT. As expected, the groups in mice injected with MLD-STZ had a high glucose tolerance at 15 and 30 min after the glucose load compared with the untreated control groups (Fig. 2). Treatment with Zn^{2+}-enriched drinking water, however, significantly countered MLD-STZ-induced loss of glucose tolerance. At week 4 after the first injection of STZ, C57BL/6 mice showed only slightly reduced glucose tolerance similar to the results obtained at week 6 in $B6SJL/F_1$ mice (Fig. 2).

Zn^{2+}-enriched drinking water induced MT in pancreatic islets. Treatment of C57BL/6 and $B6SJL/F_1$ mice with Zn^{2+}-enriched drinking water for 1 week resulted in significant induction of MT synthesis in pancreatic islets, as measured by the ^{109}Cd-haemoglobin assay (Table 1). Metallothionein were measured in islets isolated from individual mice from groups of five C57BL/6 and ten $B6SJL/F_1$ donors. The index of MT induction, i.e. the ratio of MT concentrations in islets from mice treated with Zn^{2+}-enriched drinking water over that of untreated control mice, was 2.3 ± 0.5 in C57BL/6 mice and 7.5 ± 1.6 in $B6SJL/F_1$ mice.

The Zn^{2+}-enriched drinking water was well tolerated. Mice thus treated failed to show any pathological signs or symptoms as judged by clinical inspection and several laboratory variables, such as body weight, blood glucose concentrations, number of pancreatic islets isolated and total protein concentrations/pancreatic islet (Table 1).

Localization of pancreatic MT by immunohistochemistry. Metallothionein proteins were clearly localized in the cytoplasm of pancreatic islets. The islets in snap-frozen pancreatic sections prepared from untreated C57BL/6 control mice stained uniformly for MT (Fig. 3). Pancreatic sections of 11 untreated C57BL/6 donors were prepared and of the 76 islets examined the intensity of staining was as follows: 1 + = 0 %, 2 + = 52.5 % and 3 + = 47.5 %; staining of the exocrine pancreatic cells was considerably less and was scored as follows: 1 + = 86.0 %, 2 + = 14 %, and 3 + = 0 %. Similar results were obtained in pancreatic sections that were prepared on day 8 after starting treatment with either Zn^{2+}-enriched drinking water alone, on day 6 after starting treatment with MLD-STZ alone or on day 6 after starting treatment with MLD-STZ plus Zn^{2+}-enriched drinking water (data not shown). Thus, the statistically significant ex vivo increment of MT protein in isolated islets of mice treated with Zn^{2+}-enriched drinking water (Table 1) was not indicated by immunohistochemistry, a method which is not unequivocally suitable for quantitative analysis. No staining was detected when sections were incubated with non-immune serum of rabbits (Fig. 3) or when the primary antibody was omitted from the procedure (data not shown). The presence of beta cells in the islets was confirmed by staining with guinea pig anti-porcine insulin antibody as primary reactant (data not shown). The results obtained in parallel or serial sections were indistinguishable.

Modest increase of mRNA expression of MT-1 and MT-2 by Zn^{2+}-enriched drinking water. The mRNA of the MT isoforms MT-1 and MT-2 were constitutively expressed in pancreatic islets and liver of both C57BL/6 and $B6SJL/F_1$ mice (Fig. 4). The density of MT-1 mRNA considerably exceeded that

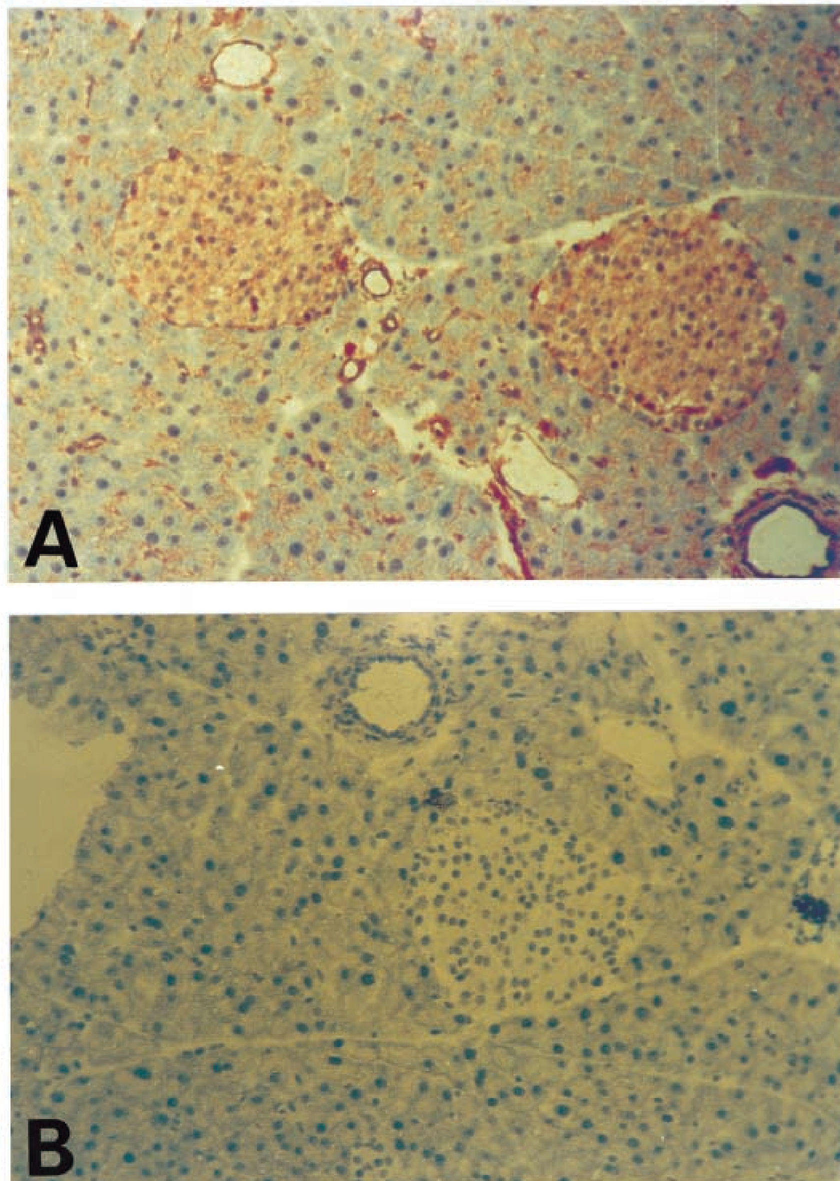

Fig. 3 A, B. Immunohistochemical staining of MT in pancreatic sections of untreated male C57BL/6 mice. **A** uniform staining for MT, scored 3 + , of islet cells and weak staining, scored 1 + , of exocrine cells. **B** no staining of the section with non-immune serum of rabbits. Original magnification × 400

of MT-2 mRNA in both tissues. Treatment of mice with Zn^{2+}-enriched drinking water modestly increased the density of both MT-1 and MT-2 mRNA expression in pancreatic islets as well as in liver tissue (Fig. 5). A statistically significant increment was only observed for MT-1 mRNA in pancreatic islets of B6SJL/F_1 mice.

Zn^{2+}-enriched drinking water failed to affect the mRNA expression of proinsulin. We tested if the protective effect of Zn^{2+}-enriched drinking water against hyperglycaemia induced by MLD-STZ resulted from an increment of proinsulin. Similar density of proinsulin mRNA expression was found in islets prepared from C57BL/6 mice treated with Zn^{2+}-enriched drinking water and in islets isolated from untreated controls (Fig. 6). Similar results were also obtained in islets of B6SJL/F_1 mice (data not shown).

MLD-STZ-induced insulitis in B6SJL/F_1 mice. Histological examination of pancreatic sections were done to test if treatment of B6SJL/F_1 mice with MLD-STZ also stimulates immune reactions with infiltration of islets by mononuclear cells similar to other inbred strains such as CD-1 [36] and C57BL/6 [38]. Inflammatory infiltrates were observed at numerous islet poles and intra-islet sites. As measured against a scoring system from 0 to 3 + , Zn^{2+}-enriched drinking water failed to essentially change the degree of insulitis [38]. Insulitis was absent in untreated mice and

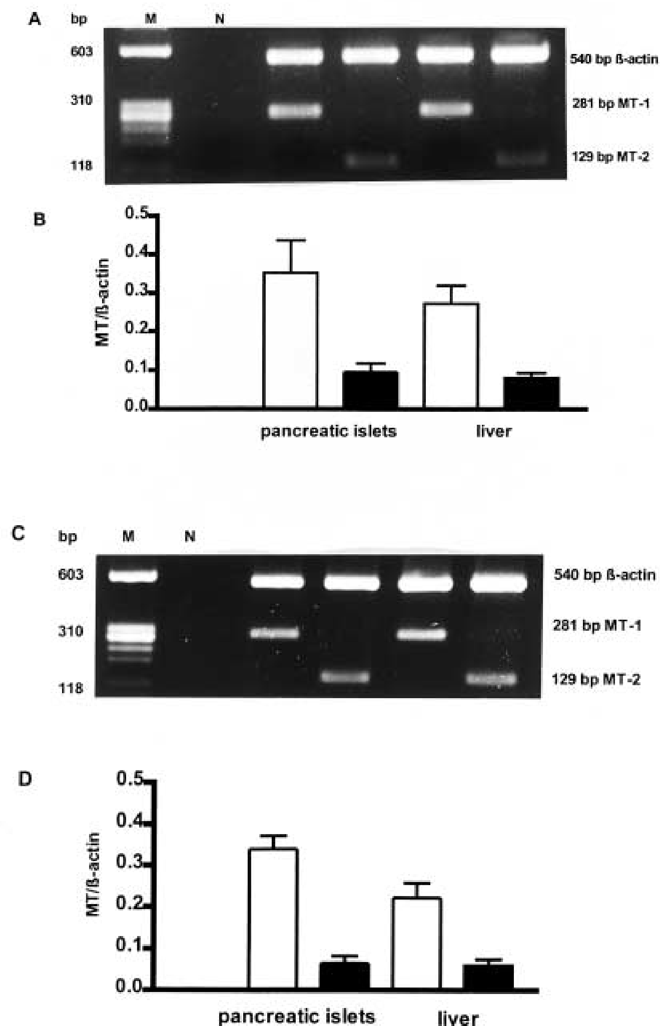

Fig. 4 A–D. RT-PCR determination of MT-1, MT-2 and β-actin mRNA in isolated pancreatic islets and liver of C57BL/6 **A** and B6SJL/F_1 **C** mice. Means ± SEM of the ratios of MT-1 (□) to β-actin and MT-2 (■) to β-actin mRNA of C57BL/6 **B** and B6SJL/F_1 **D**. The values were calculated from three independent experiments. MT-1 and MT-2 mRNA are constitutively expressed in pancreatic islets; MT-1 mRNA expression is dominant over that of MT-2. A similar pattern of MT-1 and MT-2 mRNA expression was detected in the liver tissue

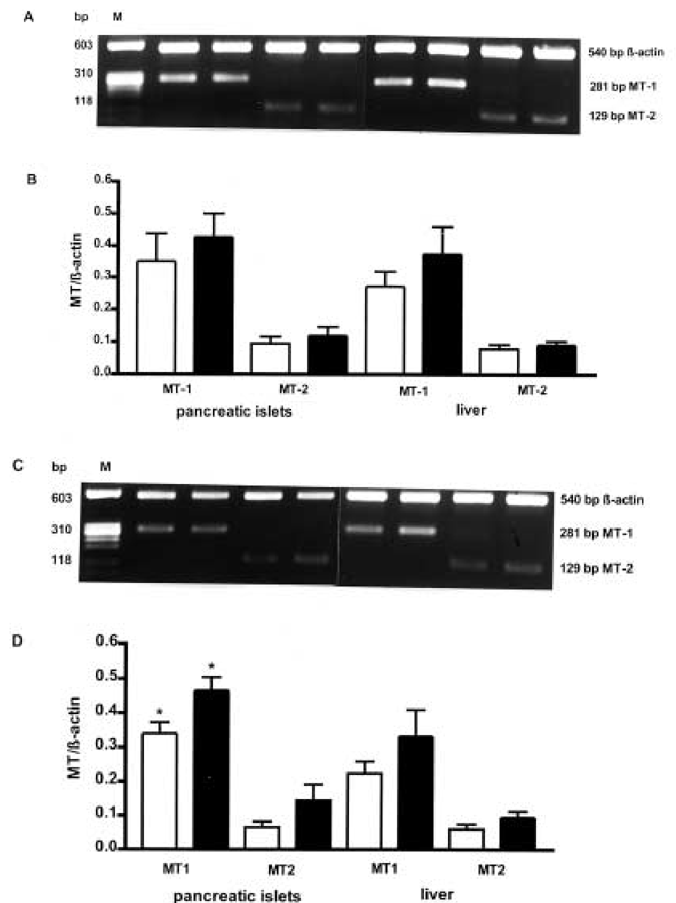

Fig. 5 A–D. RT-PCR determination of MT-1, MT-2 and β-actin mRNA in islets isolated from pancreata and liver of mice that had received Zn^{2+}-enriched drinking water for 7 days or were not treated. RT-PCR products of C57BL/6 **A** and B6SJL/F_1 **C** mice. Means ± SEM of the ratios of MT-1 to β-actin and MT-2 to β-actin mRNA in isolated islets and liver of C57BL/6 **B** and B6SJL/F_1 **D** mice, which were treated with Zn^{2+}-enriched drinking water (■) or were not treated (□). The values were calculated from three independent experiments. In general, treatment of mice with Zn^{2+}-enriched drinking water modestly increased the mRNA expression of MT-1 and MT-2, a significant (* $p < 0.05$ between the marked groups) induction, however, was only observed for MT-1 in pancreatic islets of B6SJL/F_1 mice

rarely visible in mice treated with Zn^{2+}-enriched drinking water (Table 2, Fig. 7).

MLD-STZ failed to induce GAD_{65}-autoantibodies in B6SJL/F_1 mice. As B6SJL/F_1 mice showed pronounced insulitis we wanted to evaluate whether the intense immune reactivities induce production of autoantibodies to diabetes-associated antigens [43]. Therefore, sera of mice were analysed for GAD_{65}-autoantibodies at different time points after treatment. The concentrations of GAD_{65}-autoantibodies (means ± SD) were 355 ± 54 cpm in untreated control mice. The concentrations after 10 days, 4 weeks and 10 weeks of treatment with MLD-STZ were 388 ± 67, 477 ± 103 and 561 ± 233, respectively. Treatment with MLD-STZ induced a continuous increment of the mean autoantibody concentration with time, a significant difference ($p < 0.05$) was, however, observed only 4 weeks after treatment when compared with the control value.

Discussion

In our study, treatment with Zn^{2+}-enriched drinking water prevented diabetes induced with MLD-STZ in C57BL/6 and B6SJL/F_1 mice. This effect was associated with a statistically significant up-regulation of MT protein and increment of mRNA expression of

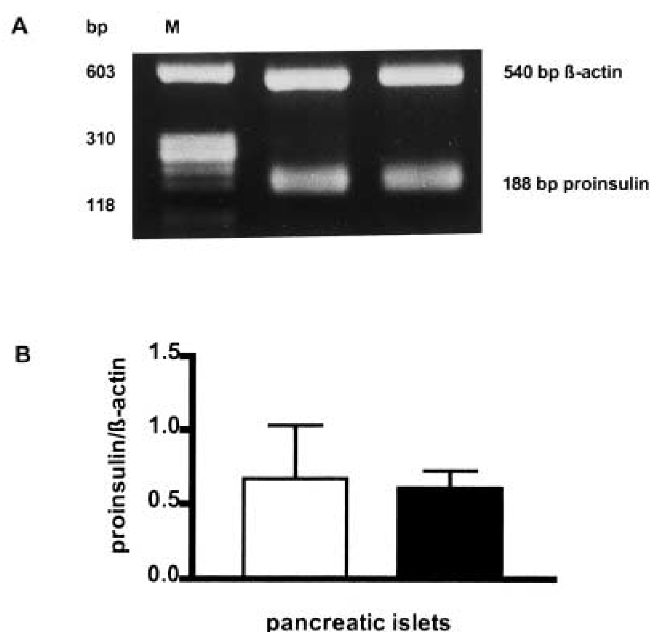

Fig. 6 A, B. RT-PCR determination of proinsulin mRNA in isolated islets of C57BL/6 mice that had received Zn^{2+}-enriched drinking water for 7 days or were not treated. Samples used for proinsulin mRNA were the same preparation as that used for MT-1 and MT-2 mRNA amplification. **A** RT-PCR products. **B** means ± SEM of the ratios of proinsulin to β-actin mRNA in isolated islets of mice, which were treated with Zn^{2+}-enriched drinking water (■) or were not treated (□). Treatment of mice with Zn^{2+}-enriched drinking water failed to affect mRNA expression of proinsulin

the isoforms MT-1 and MT-2 in islets. Possibly, the slight increment of MT mRNA reflects mRNA stabilization facilitating more frequent translation with statistically significant MT protein production. Metallothionein were present in all islet cells including beta cells and prevailed in the cytoplasm whereas the exocrine cells stained only weakly.

These results extend previous findings from our laboratory [34, 35], using different experimental protocols. Injections of Zn^{2+} i.p. dose-dependently upregulated MT synthesis in islets [34] and protected against MLD-STZ diabetes [35]. In vitro, incubation of islets with Zn^{2+} also induced MT [33] and pre-incubation with Zn^{2+} protected against STZ toxicity [35]. In our present study, diabetes was prevented by giving Zn^{2+} orally, a route that is more suitable for long-term treatment than i.p. injections. Note the long prediabetic period of 10 weeks in B6SJL/F_1 mice treated with MLD-STZ, which is five times that generally observed in other mouse strains [44]. These mice developed insulitis histologically similar to other mouse strains [44] that remained unaffected by treatment with Zn^{2+}-enriched drinking water.

Discussing the mechanism underlying protection against MLD-STZ by Zn^{2+}, we assumed that Zn^{2+}-induced MT provides a defense against $^{\cdot}OH$ generated by MLD-STZ. This assumption is based on the following observations: ROS are increased during inflammatory reactions [3] and participate in beta-cell destruction [45]. Because T cell-dependent inflammatory reactions are also involved in diabetes induced by MLD-STZ [36, 46, 47], ROS could be generated and destroy beta cells. Of the group of ROS, $^{\cdot}OH$ are generated by the Fenton reaction from H_2O_2 in the presence of adventitious Fe^{2+}. As MT scavenged $^{\cdot}OH$ in vitro [20, 22] they could also protect against it in vivo.

There is no evidence of $^{\cdot}OH$ liberation from STZ. It is, however, possible that STZ triggers the chain reaction to generate $^{\cdot}OH$ by interacting with respiring mitochondria or other cellular or subcellular fractions or both. Therefore, the production of $^{\cdot}OH$ in beta cells might occur in addition to the STZ-induced inflammatory reaction. In this context, we address the participation of reactive nitrogen species such as

Table 2. Effect of treatment with MLD-STZ and Zn^{2+}-drinking water on infiltrations with mononuclear cells at pancreatic islet sites of B6SJL/F_1 mice

Treatment of mice	Time point of examination	Islet sites examined *(n)*		Islets with mononuclear cell infiltrates, *n* (%)					
		Islet pole	Intra-islet	At islet pole		At intra-islet sites			
				Absent	Present	0	1+	2+	3+
–	12 weeks old	26	87	26 (100)	0 (0)	87 (100)	0 (0)	0 (0)	0 (0)
Zn^{2+}-enriched drinking water	18 weeks old	7	339	5 (71.4)	2 (28.6)	331 (97.6)	5 (1.5)	1 (0.3)	1 (0.3)
MLD-STZ	10 days after the first injection	106	284	63 (59.4)	43 (40.6)	258 (90.8)	10 (3.5)	5 (1.8)	7 (2.5)
MLD-STZ	4 weeks after the first injection	134	462	69 (51.5)	65 (48.5)	380 (82.2)	42 (9.1)	18 (3.9)	17 (3.7)
Zn^{2+}-enriched drinking water + MLD-STZ	10 days after the first injection	43	349	19 (44.2)	24 (55.8)	291 (83.4)	19 (5.4)	4 (1.1)	4 (1.1)
Zn^{2+}-enriched drinking water + MLD-STZ	4 weeks after the first injection	59	357	28 (47.5)	31 (52.5)	314 (88.0)	20 (5.6)	4 (1.1)	5 (1.4)

Data were obtained from groups of five mice each and three step-sections of each pancreas were examined

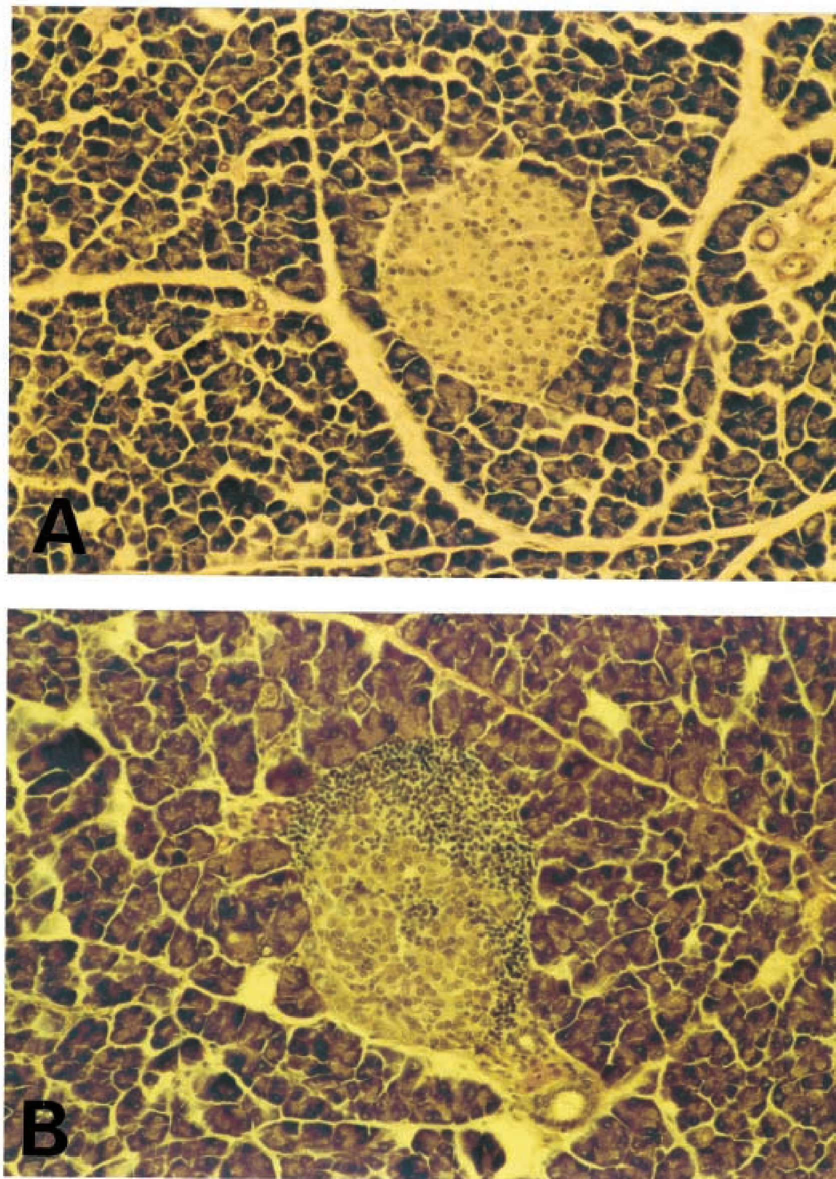

Fig. 7 A, B. Representative histology of pancreatic sections prepared from B6SJL/F_1 mice and stained with haematoxylin and eosin. **A** no infiltrates of mononuclear cells in islets of untreated control mice. **B** intra-islet infiltrates scored as 2 + of MLD-STZ-treated mice 4 weeks after the first injection with MLD-STZ. Original magnification × 200

$NO^{\cdot}$ as mediators of beta-cell toxicity. Results of investigations focussing on the role of $NO^{\cdot}$ in beta-cell destruction are contradictory. In vitro, $NO^{\cdot}$ produced by activated macrophages destroyed beta cells [48, 49]. In vivo, inhibition of $NO^{\cdot}$ generation prevented diabetes induced by MLD-STZ [5, 50]. Other investigators, in contrast, neither observed $NO^{\cdot}$ generation in islets isolated from mice treated with MLD-STZ [51] nor prevented diabetes induced by MLD-STZ with inhibitors of $NO^{\cdot}$ generation [52]. When generated, $NO^{\cdot}$ probably synergizes with ROS for beta-cell destruction. The interaction of $NO^{\cdot}$ with $O_2^{\cdot-}$ forms peroxynitrite, which can decompose to $^{\cdot}OH$ [53, 54]. In cell-free systems, STZ failed to liberate $NO^{\cdot}$ by spontaneous decomposition [55]. The exact mechanism underlying STZ-mediated beta-cell injury is not known because the effect of $NO^{\cdot}$ as a direct mediator of STZ-induced beta-cell toxicity has not been unequivocally proved.

We propose that the underlying mechanisms for the results obtained with Zn^{2+}-enriched drinking water were that either MT up-regulated by Zn^{2+} scavenged $^{\cdot}OH$ generated by STZ in beta cells or by inflammatory reactions induced by MLD-STZ or both or that MT prevented $^{\cdot}OH$ generation by inhibiting the Fenton reaction through binding of Fe^{2+} [56]. It is unlikely that Zn^{2+} was the protecting element. Although Zn^{2+} is required for insulin production and insulin action [57], it failed to affect mRNA expression of proinsulin in mice (present data) and mRNA ex-

pression of insulin in rats [28] or to lower the blood glucose concentrations due to increased insulin release (present data).

Treatment with Zn^{2+}-enriched drinking water statistically significantly up-regulated MT synthesis in pancreatic islets and protected mice from diabetes induced by MLD-STZ. A variant of the classic MLD-STZ diabetes model has been observed in B6SJL/F_1 mice with a prolonged prediabetic period of 10 weeks. This extended prediabetic phase could involve pathogenic pathways similar to those observed in human Type I diabetes. Because the dose of Zn^{2+} used was non-toxic, interventions with Zn salts can also be considered for other models of Type I diabetes and for humans at risk of developing this disease.

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