Tolbutamide reduces the incidence of diabetes mellitus, but not insulitis, in the non-obese-diabetic mouse

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Summary. The functional state of beta cells may influence the rate of their destruction in Type 1 (insulin-dependent) diabetes mellitus. We examined the effect of diazoxide, which inhibits insulin secretion, or tolbutamide, which stimulates insulin secretion, upon the incidence of diabetes in the non-obese-diabetic (NOD) mouse. Female mice were treated from 3–30 weeks of age with diet containing diazoxide 250 mg·kg^{-1} or tolbutamide 125 mg·kg^{-1}. The cumulative incidence of diabetes at 35 weeks was similar in the diazoxide (16 of 24) and control (18 of 24) groups, but reduced in the tolbutamide group (10 of 23, $p < 0.04$ vs control group). In a second experiment, treatment was started from 9 weeks of age, by which time insulitis is already present. The cumulative incidence of diabetes at 35 weeks was 16 of 24 in controls, 15 of 24 on diazoxide and 11 of 24 on tolbutamide ($p = $ NS vs control). A third experiment compared the effect of treatment from 3 weeks with control diet or diet containing tolbutamide 125 mg·kg^{-1} or 500 mg·kg^{-1}. Diabetes was reduced by tolbutamide treatment, with a cumulative incidence of 25 of 31 in controls, 18 of 30 on tolbutamide 125 mg·kg^{-1} ($p < 0.04$) and 14 of 32 on 500 mg·kg^{-1} ($p < 0.002$), although the difference between the two treatment groups failed to reach statistical significance. A fourth experiment showed that treatment from 3–12 weeks with diazoxide 1000 mg·kg^{-1} increased the extent of insulitis compared with controls and animals treated with tolbutamide 500 mg·kg^{-1}. Elucidation of the mechansims by which tolbutamide reduces the incidence of diabetes in the NOD mouse has implications for human intervention trials.

Key words: NOD mouse, tolbutamide, diazoxide, Type 1 (insulin-dependent) diabetes mellitus

Prophylactic insulin therapy has been found to reduce the incidence of diabetes mellitus in two spontaneous animal models of Type 1 (insulin-dependent) diabetes, the Bio-Breeding (BB) Wistar rat [1, 2] and the non-obese-diabetic (NOD) mouse [3]. In order to explain these findings, it was proposed that "a reduction of endogenous insulin secretion might reduce beta-cell antigen expression to such a level that the autoimmune cascade is not initiated or is alleviated" [1]. Furthermore, a trial of intensive insulin therapy in newly-diagnosed Type 1 diabetic patients concluded that suppression of endogenous insulin may improve beta-cell function over the subsequent year [4].

These observations have prompted clinical trials of prophylactic insulin therapy in islet cell antibody-positive non-diabetic subjects, in an attempt to protect beta cells from autoimmune destruction [5]. Since insulin requires repeated injection, oral agents also deserve consideration.

We have examined whether tolbutamide and diazoxide, drugs which modify insulin secretion, affect the incidence of diabetes in the NOD mouse. Both drugs are thought to act via the ATP-sensitive potassium channel of beta cells [6]. Tolbutamide closes this channel causing stimulation of insulin secretion, whereas diazoxide opens the channel leading to inhibition of insulin secretion. By exploiting the opposing effects of these drugs, we set out to clarify whether the functional state of the beta cells influences the rate of their destruction in Type 1 diabetes.

Our initial experiment showed that prophylactic tolbutamide treatment reduced the cumulative incidence of diabetes in the NOD mouse [7]. Diazoxide, on the other hand, did not significantly alter the incidence of diabetes. These findings challenged the belief that suppression of endogenous insulin secretion may protect the beta cells from destruction. We therefore performed a further series of experiments in order to confirm and to extend our original observations.
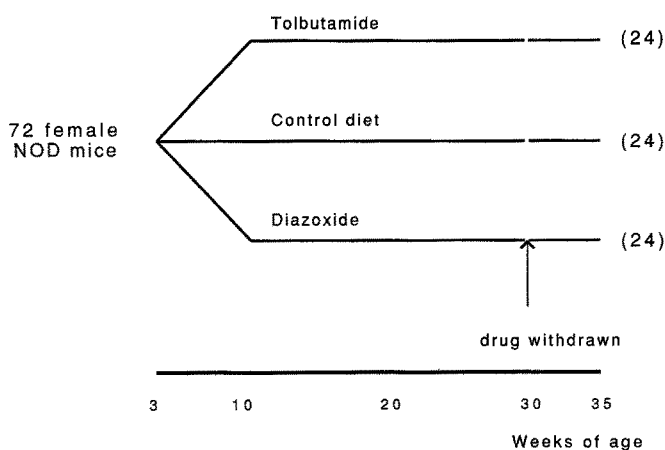
Fig. 1. The protocol for investigating the effect of either 250 mg·kg^{-1} diazoxide or 125 mg·kg^{-1} tolbutamide in the diet, on the incidence of diabetes in the NOD mouse

Materials and methods

Development of diabetes

Experiment 1

The first experiment investigated the effect of prophylactic diazoxide or tolbutamide treatment from 3 weeks of age, on the development of diabetes in the NOD mouse. Seventy-two female NOD mice were split into three litter-matched groups at 3 weeks of age and given either a control diet, diet containing diazoxide (250 mg·kg^{-1}) or diet containing tolbutamide (125 mg·kg^{-1}). Therapy was withdrawn at 30 weeks of age and the animals followed-up for a further 5 weeks (Fig. 1) to ensure that the hypoglycaemic effect of tolbutamide did not mask occult diabetes and to reveal the long-term effects of treatment. The mice were weighed weekly from the start of therapy and tested weekly for glycosuria from 10 weeks of age, with diabetes being diagnosed on finding a urine glucose level greater than 56 mmol/l on more than one occasion (Diabur-Test 5000; Boehringer Mannheim, Mannheim, FRG). The animals were killed at 35 weeks of age or when diabetes was confirmed and the pancreata were then frozen for histological investigation.

Experiment 2

Therapies aimed at preventing the onset of Type 1 diabetes in humans, require the identification of individuals at high risk of developing the disease. Since the presence of islet cell antibodies gives the best prediction of disease, therapeutic intervention may only be attempted when the autoimmune process is already underway [8]. A further 72 female mice were therefore treated according to the same protocol, but with therapy started at 9 weeks of age, to determine whether tolbutamide could reduce the incidence of diabetes, even when insulitis is already present [9].

Experiment 3

A third experiment was performed to determine whether a further reduction in diabetes incidence could be obtained, by increasing the dose of tolbutamide. Ninety-six female NOD mice were split into three litter-matched groups and treated from 3 weeks of age with either control diet, or diet containing either 125 mg·kg^{-1} or 500 mg·kg^{-1} tolbutamide. Animals were monitored for diabetes from 10 weeks of age, the drug was withdrawn at 30 weeks, the mice

were killed at 31 weeks of age and the pancreata frozen for histological investigation.

Insulitis and metabolic studies

Experiments 4 and 5

Two further experiments were performed in order to discover whether the drug treatment altered the progression of insulitis and influenced glucose metabolism. Seventy-two litter-matched female NOD mice were treated from 3 weeks of age according to the same protocol with control diet, 500 mg·kg^{-1} tolbutamide or 1000 mg·kg^{-1} diazoxide. At 12 weeks of age a basal blood glucose sample was collected immediately after food was withdrawn. An intraperitoneal glucose load (2 g/kg body weight) was administered and a further sample obtained, at either 30 or 60 min, for blood glucose and serum insulin measurements. The mice were killed and the pancreata frozen for histological investigation. A further 48 litter-matched female NOD mice were treated according to the same protocol but killed at 8 weeks of age, 30 min after a glucose load, with blood being taken for glucose and insulin measurements.

Pancreatic insulin content

Experiment 6

Pancreatic immunoreactive insulin (IRI) content was measured in order to determine the possible effects of treatment on beta-cell mass or granulation. Twenty-four litter-matched female and 24 litter-matched male NOD mice were treated according to the same protocol with control diet, 250 mg·kg^{-1} diazoxide or 125 mg·kg^{-1} tolbutamide from 3 weeks of age. At 11 weeks of age the mice were killed and the pancreata removed, frozen and stored at −70 °C until extraction.

Mouse colony

The NOD mice used in these experiments were obtained from the breeding colony at St. Bartholomew's Hospital, which was established in 1987 and derived originally from the colony of Dr. E. Leiter (Jackson Laboratory, Bar Harbor, Me., USA). The mice are kept in a conventional unit and the colony maintained by parallel line inbreeding. The colony is characterised by the onset of insulitis at 3–5 weeks of age and the onset of diabetes from approximately 10 weeks of age [10].

Treatment

Diazoxide (gift of Schering Plough, Mildenhall, Suffolk, UK) or tolbutamide (Sigma, Poole, Dorset, UK) were given as an admixture in a standard rodent diet (Quest Nutrition, Wingham, Kent, UK).

Blood glucose and serum insulin measurements

Blood glucose was measured by a glucose oxidase method (Yellow Springs Instruments, Yellow Springs, Ohio, USA). Serum insulin was determined using a double-antibody radioimmunoassay [11] with guinea-pig anti-human insulin first antibody (in house) and sheep anti-guinea pig F$_c$ (International Laboratory Service, London, UK) as the second antibody. ^{125}I-labelled human insulin (Amersham International, Amersham, Bucks, UK) was used as tracer, with human insulin standards (Novo Biolabs, Bagsvaerd, Denmark).
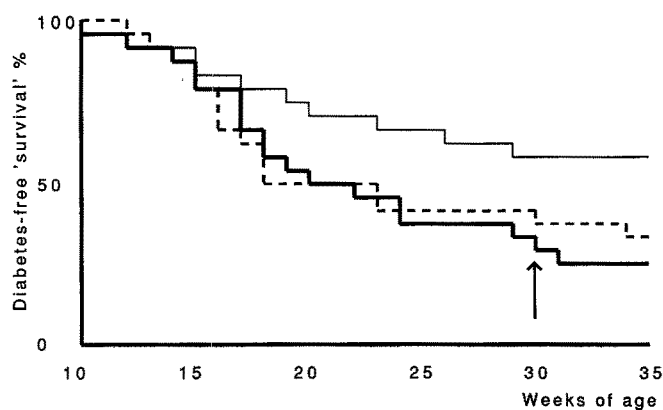
Fig. 2. The cumulative incidence of diabetes in female NOD mice treated from weaning with control diet (bold line) or diet containing either 250 mg·kg^{-1} diazoxide (dotted line) or 125 mg·kg^{-1} tolbutamide (fine line). The incidence of diabetes in tolbutamide-treated animals was significantly less than control ($p < 0.04$). The age at which therapy was withdrawn is denoted by an arrow
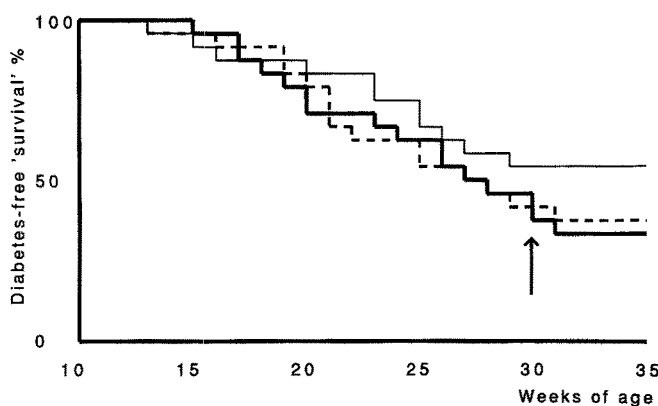

Fig. 3. The cumulative incidence of diabetes in female NOD mice treated from 9 weeks of age with control diet (bold line) or diet containing either 250 mg·kg^{-1} diazoxide (dotted line) or 125 mg·kg^{-1} tolbutamide (fine line). There were no significant differences in the incidence of diabetes between the different treatment groups. The age at which therapy was withdrawn is denoted by an arrow
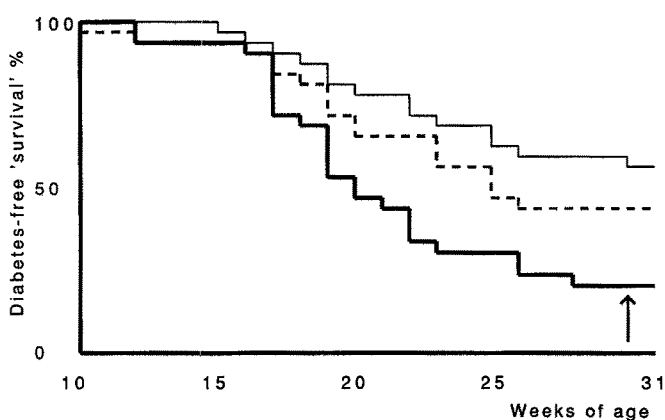

Fig. 4. The cumulative incidence of diabetes in female NOD mice treated from 3 weeks of age with control diet (bold line) or diet containing either 125 mg·kg^{-1} (dotted line) or 500 mg·kg^{-1} tolbutamide (fine line). The incidence of diabetes was significantly less than control in both the 125 mg·kg^{-1} ($p < 0.04$) and 500 mg·kg^{-1} ($p < 0.002$) tolbutamide-treated groups. The age at which therapy was withdrawn is denoted by an arrow

Histology

Ten 6 μm frozen sections were cut at 200 μm intervals from each pancreas, mounted on glass slides and stained with haemotoxylin and eosin. The sections were assessed 'blind' for insulitis, with the islets being classified according to the severity of lymphocytic infiltration (uninfiltrated, peri-insulitis = < 10 % of islet area infiltrated, moderate = < 50 % of islet area infiltrated, severe = > 50 % of islet area infiltrated). The percentage of islets falling within each classification was then calculated for each mouse.

Immunoreactive insulin (IRI) extraction

After weighing, pancreata were hand-homogenized in 4 ml of ice-cold 0.1 mol/l HCl containing enzyme inhibitors (2 mmol/l p-Hydroxymercuribenzoate, 2 mmol/l EDTA, 2 mmol/l N-Ethylmaleimide). A total of 200 μl of phenylmethylsulphonyl fluoride (2 mmol/l) in methanol was added in 50 μl aliquots during the extraction, in order to inactivate serine proteases. The extracts were centrifuged (2500 g at 4°C for 1 h), and the supernatants stored at −70°C until assayed. After dilution in assay buffer, insulin in the supernatants was measured by radioimmunoassay as described previously, but with rat insulin standards (Novo Biolabs). Dilutions of the extracts were shown to be parallel with the standard curve and in three different experiments using immunoprecipitation with insulin antibody, recovery of ^{125}I-labelled insulin added during extraction was 81 %, 69 % and 65 %.

Statistical analysis

Lifetable analysis, with the logrank test, was used to compare the cumulative incidence data [12]. Animals dying from causes unrelated to diabetes were counted as censored observations. Body weights, insulitis, blood glucose, serum insulin levels and pancreatic insulin contents of the different groups were compared using the Kruskal-Wallis test. If a significant result was obtained using the Kruskal-Wallis test, differences between the groups were analysed using the Mann-Whitney U test. Two-tailed tests were used for comparison between groups.

Results

Diabetes outcome

Experiment 1

The effects of treatment from 3 weeks of age on diabetes incidence are shown in Figure 2. At 35 weeks of age, 10 of 23 animals in the tolbutamide-treated group had developed diabetes compared with 18 of 24 in the control ($p < 0.04$) and 16 of 24 in the diazoxide-treated (p = NS) groups. There were no significant differences between the groups in either the extent or severity of insulitis in non-diabetic survivors at 35 weeks of age (data not shown).

Experiment 2

The effects of treatment from 9 weeks of age on diabetes incidence are shown in Figure 3. At 35 weeks, 11 of 24 animals in the tolbutamide-treated group had developed diabetes compared with 16 of 24 in the control (p = NS) and 15 of 24 in the diazoxide-treated (p = NS) groups.
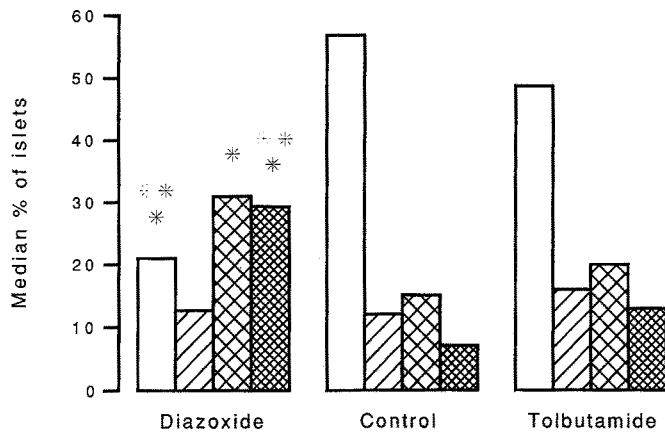
Fig. 5. Insulitis in female NOD mice at 12 weeks of age, following treatment from weaning with diet containing either 1000 mg·kg⁻¹ diazoxide or 500 mg·kg⁻¹ tolbutamide. Islets are classified according to the severity of lymphocytic infiltration (uninfiltrated □, peri-insulitis = < 10 % of islet area infiltrated ▨, moderate = < 50 % islet area infiltrated ▩, severe = > 50 % of islet area infiltrated ▩). Significant differences ($p < 0.05$) are indicated: * diazoxide vs control, ** diazoxide vs tolbutamide

Experiment 3

Treatment from 3 weeks of age with 125 mg·kg⁻¹ tolbutamide in the diet caused a significant reduction in diabetes incidence when compared to control(18 of 30 vs 25 of 31, $p < 0.04$) and a further reduction was observed when the dosage of tolbutamide was increased to 500 mg·kg⁻¹ (14 of 32, $p < 0.002$ vs control) (Fig. 4), although the difference between the two doses was not significant. There were no significant differences in either the extent or severity of insulitis in non-diabetic survivors at 31 weeks of age (data not shown).

Histology and metabolic status

Experiments 4 and 5

The mean number of islets investigated in each mouse at 12 weeks of age was 127 with a minimum of 52. The degree and severity of insulitis in the three groups at this age is illustrated in Figure 5. The diazoxide-treated group had a significantly lower percentage of uninfiltrated islets than the control group (Median 21, range 0–100 % vs 57, 4–98 %, $p < 0.05$) and the tolbutamide-treated group (49, 1–94 %, $p < 0.05$). The diazoxide-treated animals also had a significantly higher percentage of moderately infiltrated islets (31, 0–39 %) than the control group (15, 0–35 %, $p < 0.02$) and a significantly higher percentage of severely infiltrated islets (29, 0–65 %) than either the control (7, 0–54 %, $p < 0.03$) or tolbutamide-treated (13, 1–53 %, $p < 0.04$) groups. There were no significant differences between the control and tolbutamide-treated animals, in either the extent or severity of insulitis.

Basal and stimulated blood glucose levels and stimulated serum insulin levels are shown in Table 1. There were no significant differences between the groups in basal and stimulated blood glucose levels or stimulated insulin levels at either 8 or 12 weeks of age.

Table 1. Blood glucose and serum insulin results of NOD mice treated with diet containing either 1000 mg·kg⁻¹ diazoxide or 500 mg·kg⁻¹ tolbutamide, before and after a 2 g/kg glucose load. Serum insulin was measured using human insulin standards. There were no significant differences between the treatment groups

Treatment	Age (weeks)	Glucose (mmol/l) Basal	30 min	Insulin (pmol/l) 30 min
Diazoxide	8	4.9 (4.1–5.4)	6.1 (4.2–17.8)	141 (66–198)
Control	8	4.7 (3.9–5.3)	6.1 (4.5–8.4)	132 (102–378)
Tolbutamide	8	5.1 (3.8–6.6)	6.6 (5.4–8.8)	186 (96–264)
Diazoxide	12	4.6 (3.7–5.3)	5.7 (4.5–8.7)	90 (42–144)
Control	12	4.9 (4.4–5.4)	6.0 (4.9–14.3)	144 (96–270)
Tolbutamide	12	4.7 (3.8–5.6)	6.4 (4.6–11.0)	90 (66–126)
		Basal	60 min	60 min
Diazoxide	12	4.1 (3.0–5.7)	4.4 (3.5–9.0)	81 (30– 204)
Control	12	4.0 (3.5–4.8)	4.6 (3.1–5.6)	81 (48–1200)
Tolbutamide	12	4.2 (3.3–8.4)	4.8 (3.9–5.9)	99 (42– 438)

Values are medians with ranges in brackets

Pancreatic IRI content

Experiment 6

There were no significant differences in the extractable IRI content of the pancreata of either male (control = median 23, range 19–34 nmol/g, diazoxide = 24, 19–31 nmol/g, tolbutamide = 23, 20–32 nmol/g) or female (control = 29, 25–41 nmol/g, diazoxide = 29, 14–39 nmol/g, tolbutamide = 36, 21–60 nmol/g) mice.

Body weights

There were no significant differences between any of the treatment groups in the incremental area for body weight of animals, from 3–8 weeks of age (data not shown).

Discussion

This study shows that prophylactic tolbutamide treatment from weaning consistently reduces the incidence of diabetes in the NOD mouse. The reduction in diabetes incidence is not accompanied by a reduction in either the extent or severity of insulitis at 12 weeks of age, indicating that the initial stages of the autoimmune process are not affected. When tolbutamide treatment was started at 9 weeks of age however, the reduction in diabetes incidence did not reach statistical significance. The failure to reach significance could be due to an insufficient sample size or may indicate that early intervention is important, if tolbutamide is to be effective in preventing diabetes.

Diazoxide at 1000 mg·kg⁻¹ was found to increase the degree of insulitis in the NOD mouse at 12 weeks of age, but did not significantly alter the incidence of diabetes at a dose of 250 mg·kg⁻¹. This discrepancy may be due to the different dosages of drug used for the investigation of diabetes incidence and insulitis. These findings however, contrast with those of a previous study performed in BB rats, in which diazoxide significantly reduced the in-

cidence of diabetes [13]. The different outcomes of the experiments in mice and rats may be explained by the different effects of diazoxide treatment on insulin secretion, since temporary hyperglycaemia was observed in the rats but not in the mice.

There were no significant differences between the treatment groups in blood glucose or insulin levels at either 8 or 12 weeks of age. No hypoglycaemic effects of tolbutamide were found, nor were significant hyperglycaemic effects of diazoxide treatment observed. Both treatment groups however, tended to have lower 30-min insulin levels at 12 weeks of age than the control group, even though the blood glucose levels were very similar. These findings suggest that peripheral insulin sensitivity may be increased in the treated animals, an effect of sulphonylurea treatment which has previously been observed [14–16]. Since diabetes in NOD mice is characterized by a state of insulinopenia and insulin resistance [17], an increase in insulin sensitivity could lead to a reduction in diabetes incidence.

The success of prophylactic insulin therapy [1, 2, 3, 18] in reducing the incidence of diabetes in BB rats and NOD mice has been attributed to a reduction in beta-cell autoantigen expression. In support of this hypothesis, it has been shown that expression of a beta-cell surface antigen is reduced upon fasting or after insulin treatment [19]. This mechanism is unlikely to explain our findings however, since tolbutamide reduces the incidence of diabetes but does not cause significant alterations in either insulitis or pancreatic IRI content. Unlike insulin [20], tolbutamide is unlikely to act as a foreign antigenic stimulus, although the drug may have a suppressive effect on lymphocyte beta-cell cytotoxicity. Indeed, another sulphonylurea, glipizide, was shown to reduce the incidence of diabetes in BB rats and to have immunosuppressive properties in vitro [21].

There were no significant differences in insulitis between the groups of non-diabetic survivors from either experiment 1 or experiment 3. Although these observations were made after withdrawal of the drugs and few control animals survived, they indicate that the autoimmune process is not reversed by prolonged treatment with tolbutamide. The fact that few tolbutamide-treated animals became diabetic after withdrawal of the drug, could mean that these mice have passed through the phase of high disease susceptibility. In this context, it has been shown in rats that islet-cell mass increases [22] and immunological activity decreases with age [23].

A beta-cell trophic action of tolbutamide both in vivo [24] and in vitro [25, 26], has been described, although this effect of the drug is still disputed [27]. An increase in the number of beta cells could reduce the metabolic stress on individual cells, thereby delaying the onset of diabetes. The similarity in pancreatic insulin contents of the different treatment groups however, make this an unlikely explanation for the reduced incidence of diabetes in tolbutamide-treated animals.

Diet is a factor known to influence diabetes incidence in the NOD mouse [28]. No significant influence of diazoxide or tolbutamide treatment on body weight was observed however, indicating that changes in food intake are unlikely to explain the effects of these drugs on insulitis and diabetes.

In conclusion, the incidence of diabetes in NOD mice is reduced by prophylactic treatment with tolbutamide but not diazoxide. The reduction in diabetes incidence could not however, be related to alterations in the functional state of the beta cells. Elucidation of the precise mechanisms by which tolbutamide affords protection from diabetes in the NOD mouse, may clarify the sequence of events leading to Type 1 diabetes. This may be important when designing new therapeutic strategies for disease prevention during the pre-diabetic period.

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