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*Originals*

# Predisposing effect of anti-Beta cell autoimmune process in NOD mice on the induction of diabetes by environmental insults

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**Summary.** In NOD mice, 50–70% of females and 10–20% of males develop diabetes, although almost all the animals show insulitis. To see if environmental insults could induce diabetes in subjects with pre-clinical anti-Beta cell autoimmunity, non-diabetic NOD mice were selected and injected with a sub-diabetogenic dose of streptozotocin at 6 or 20 weeks of age. The streptozotocin failed to induce diabetes in 16 male and 16 female NOD mice within 4 weeks when they were injected at the age of 6 weeks. In contrast, 6 of 16 male and 10 of 16 female NOD mice developed diabetes within 4 weeks when they were injected at the age of 20 weeks. In untreated age-matched control NOD mice, none of the male and only 2 of 16 female mice became diabetic during the same 4 week period. On histologic examination, the degree of insulitis in streptozotocin-treated NOD mice (at the age of 24 weeks) was not significantly different from that of untreated control NOD mice. However, the streptozotocin-treated animals showed significantly lower pancreatic insulin content than the control mice. These results show that an anti-Beta cell autoimmune process in NOD mice has a predisposing effect on the induction of diabetes by a sub-diabetogenic dose of streptozotocin, and suggest that the precipitation of clinical diabetes by some environmental insults in subjects with pre-existing pre-clinical autoimmune Beta-cell destruction may be one mechanism of disease presentation in human Type 1 (insulin-dependent) diabetes.

**Key words:** Non-obese diabetic (NOD) mice, streptozotocin, autoimmunity, environmental factor, cumulative insults.

The rather low concordance rate in identical twins for Type 1 (insulin-dependent) diabetes (30–50%) suggests that environmental factors influence the development of this disease [1]. Most of the previous searches for environmental factors concentrated on the insults which can cause near-total destruction of pancreatic Beta cells in an acute process. However, current knowledge indicates that in the majority of Type 1 diabetic patients, a rather long immunopathologic process precedes the onset of clinical diabetes [2]. Thus, in addition to their possible action as primary injurious agents to Beta cells, environmental factors (viruses, toxins) may also act either as a trigger of autoimmunity in genetically susceptible individuals or as a final insult that can result in the clinical onset of diabetes in individuals in whom an autoimmune process is causing a decrease in Beta-cell mass.

Diabetes in non-obese diabetic (NOD) mice is characterized by the establishment of insulitis which leads to Beta-cell destruction and culminates in hypoinsulinaemia and hyperglycaemia. Although insulitis develops in essentially all of the animals, overt hyperglycaemia develops in only 50–70% of females and 10–20% of males [3]. Unless the Beta-cell mass is decreased by more than 90%, compensation by remaining intact Beta cells can maintain normoglycaemia [4]. Thus, it is quite possible that the degree of Beta-cell destruction by an autoimmune process in non-diabetic NOD mice is not severe enough to produce hyperglycaemia. Additional Beta-cell damage by some environmental insults might precipitate the onset of clinical diabetes in these instances. Our investigation was initiated to see if some environmental insults, which do not induce diabetes in healthy subjects, can trigger clinical diabetes in these normoglycaemic NOD mice.

## Materials and methods

Our NOD mouse colony was produced from a breeding stock obtained from Clea Japan (Tokyo, Japan). These animals were maintained as previously described [5]. The overall cumulative incidence of diabetes among our colony of NOD mice was about 50% in females and 15% in males at 30 weeks of age. ICR mice, from which

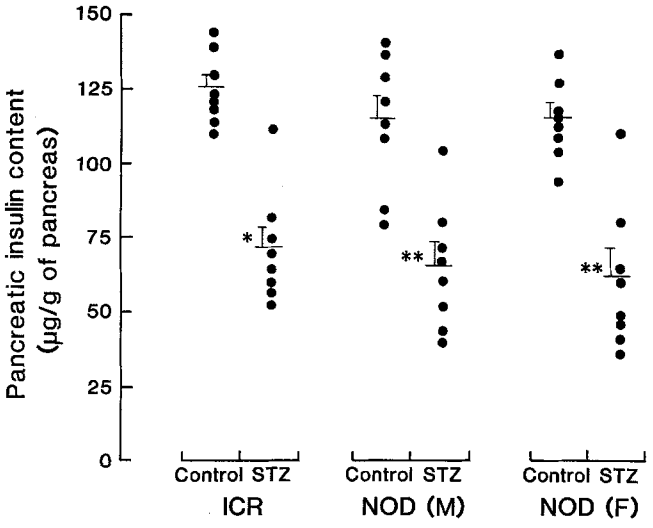


**Fig. 1.** Pancreatic insulin content in ICR and NOD mice with and without streptozotocin (STZ) injection (100 mg/kg). Mice were given STZ at the age of 6 weeks and were killed at 10 weeks. Horizontal bar denotes mean and vertical bar denotes SEM (* $p < 0.001$, ** $p < 0.01$

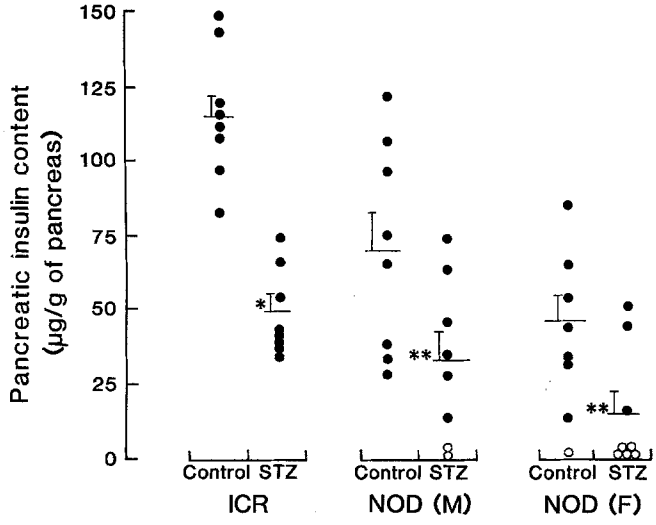


**Fig. 2.** Pancreatic insulin content in ICR and NOD mice with and without streptozotocin (STZ) injection (100 mg/kg). Mice were given STZ at the age of 20 weeks and were killed at 24 weeks. Horizontal bar denotes mean, vertical bar denotes SEM, and open circles denote animals which developed diabetes (* $p < 0.001$, ** $p < 0.05$)

the NOD mouse strain was established, were obtained from the same source.

Streptozotocin (STZ) (Sigma, St. Louis, Mo., USA) was dissolved immediately before use in sodium citrate buffer (pH 4.5) and injected intraperitoneally at a dose of 100 mg/kg into non-diabetic male and female NOD mice at 6 weeks of age in one group ($n = 32$) and at 20 weeks of age in another group ($n = 32$). Our preliminary study showed that this dose was not sufficient to induce diabetes, but was sufficient to damage enough Beta cells. Age-matched ICR male mice were also injected with the same dose of STZ. Groups of age- and sex-matched NOD and ICR mice were not treated and were used as controls.

A total of 32 male and 32 female 6-week-old NOD mice from 12 litters, divided into two groups and 32 male and 32 female non-diabetic 20-week-old NOD mice from 12 litters, divided into two groups (one STZ-treated group and one untreated group) were used for this experiment. All animals were tested twice weekly for glycosuria with Diastix reagent strips (Miles, Ontario, Canada). When the animals were 10- or 24-weeks-old, blood glucose levels were measured as described previously [6], and the pancreata were removed for histologic examination and the measurement of immunoreactive insulin (IRI). Individual mice were classified as diabetic if glycosuria was persistently present and the plasma glucose level was higher than 12 mmol/l (mean + 5 SD of 40 control mice). Part of the pancreas was fixed in formalin and paraffin-embedded sections were stained with haematoxylin and eosin. The severity of insulitis was evaluated according to the grading system described elsewhere [5]. The remainder of the pancreas was weighed and stored at −70 °C, and the extractable insulin content was measured by radioimmunoassay [7].

### *Statistical analysis*

Statistical analysis was performed with the $X^2$-test for the incidence of diabetes and with the Wilcoxon rank sum test and $t$ test for pancreatic insulin content [8].

## Results

A STZ dosage of 100 mg/kg failed to induce diabetes in either group (6-week-old or 20-week-old) of ICR mice. When 6-week-old NOD mice were injected with STZ, none of the animals became diabetic within 4 weeks. When 20-week-old, non-diabetic NOD mice were injected with STZ, 6 of 16 male and 10 of 16 female mice became diabetic at 24 weeks of age. In contrast, none of the untreated male and only 2 of 16 untreated female NOD mice became diabetic during the same 4-week period (Table 1).

On histologic examination, the degree of insulitis at 24 weeks of age in STZ-treated NOD mice was not significantly different from that of control mice (Table 2). However, the STZ-treated animals showed significantly lower pancreatic insulin content than their untreated controls (Fig. 1 and 2, $p < 0.001$–$p < 0.05$). These results indicate that treatment with a sub-diabetogenic dose of STZ results in the reduction of Beta-cell mass in all strains of mice tested but is insufficient to cause diabetes within 4 weeks in ICR mice and 6-week-old NOD mice. In contrast, the same dose of STZ causes clinical diabetes in 20-week-old NOD mice since their Beta-cell mass had already been reduced by an autoimmune insulitis process.

## Discussion

The concordance rate for developing Type 1 diabetes between monozygotic twins approaches 50%, suggesting that genetic factors are necessary but that non-genetic factors such as environmental factors also influence the clinical expression of this disease [1, 9]. An increasing body of evidence indicates that in the major portion of Type 1 diabetes, a rather long immunopathologic process precedes the onset of the disease. Thus, if environmental factors such as viruses or toxins are involved in the pathogenesis of autoimmune Type 1 diabetes, they may act either as a trigger of autoimmunity or as a final insult leading to the clinical onset of diabetes in individuals with an

**Table 1.** Effect of streptozotocin (100 mg/kg) on incidence of diabetes

| Strain | Sex | Age (week) at the time of STZ injection | Incidence of diabetes | |
|---|---|---|---|---|
| | | | Control | STZ-treated |
| NOD | M | 6 | 0/16 | 0/16 |
| NOD | F | 6 | 0/16 | 0/16 |
| NOD | M | 20[a] | 0/16 | 6/16[b] |
| NOD | F | 20[a] | 2/16 | 10/16[b] |
| ICR | M | 6 | 0/8 | 0/8 |
| ICR | M | 20 | 0/8 | 0/8 |

[a] non-diabetic at the time of STZ injection;
[b] $p < 0.05$ compared with control animals

**Table 2.** Effect of STZ (100 mg/kg) on insulitis in NOD mice

| Strain | Sex | Age (week) at the time of STZ injection | Number of animals per group | Insulitis grade[b] at the age of 24 week | |
|---|---|---|---|---|---|
| | | | | Control | STZ-treated |
| NOD | M | 20[a] | 16 | 2.8 ± 0.3 | 2.9 ± 0.3 |
| NOD | F | 20[a] | 16 | 3.4 ± 0.2 | 3.2 ± 0.3 |

[a] non-diabetic at the time of STZ injection;
[b] grade 0 – normal islet; grade 1 – less than 25% of area with mononuclear cell infiltration within an islet; grade 2 – 25–50%; grade 3 – greater than 50%; grade 4 – small retracted islet with few mononuclear infiltrates; At least 20 islets were assessed and the grade was averaged for the number of islets in each pancreas; mean ± SEM

already decreased Beta-cell mass resulting from an autoimmune process. In animal models of the former mechanism, repeated administration of low doses of STZ to susceptible mice resulted in Beta-cell destruction and diabetes which appeared to be mediated by immunologic responses [10]. Recently, we showed that golden Syrian hamsters, infected soon after birth with Beta cell-adapted rubella virus, developed Type 1 diabetes showing insulitis and islet cell antibodies late in life [11]. In man, congenital rubella syndrome provides the best example that viral infection can be associated with the subsequent development of autoimmune Type 1 diabetes [12]. However, there have been no models mimicking the latter mechanism. Therefore, we undertook this study to see if low dose STZ might be a final insult resulting in the clinical onset of diabetes in normoglycaemic NOD mice with a pre-existing autoimmune process.

In NOD mice, mononuclear cells start to invade islets at 4 to 6 weeks of age and almost 100% of the animals show insulitis by 20 weeks of age, but overt hyperglycaemia develops in only 10–20% of males and 50–70% of females [3]. In the present study, we demonstrated that a single sub-diabetogenic dose of STZ administered to non-diabetic 20-week-old NOD mice could precipitate clinical diabetes. In contrast, the same dose of STZ failed to induce diabetes in 6-week-old NOD mice indicating that the effect of low dose STZ on the development of clinical diabetes in non-diabetic NOD mice depends on pre-existing Beta-cell damage. The degree of Beta-cell damage in NOD mice measured by pancreatic insulin content was not uniform in this study, suggesting that the rate of Beta-cell loss by autoimmune mechanisms varies in different subjects. The higher sensitivity to the induction of diabetes by a sub-diabetogenic dose of STZ in female NOD mice seems to reflect the more extensive Beta-cell damage by active autoimmune processes in female than in male NOD mice.

Since STZ is a well-known Beta-cell toxin, it can be easily assumed that direct Beta-cell damage by low dose STZ, which cannot cause diabetes in normal animals, has precipitated clinical diabetes in animals with previously reduced Beta-cell mass. In addition to the direct cytotoxic effect of STZ on the Beta cells, its indirect effect such as the induction of expression of Beta-cell-specific endogenous retroviruses and subsequent acceleration of autoimmune processes cannot be excluded. It was previously shown that multiple STZ treatment of CD-1 mice resulted in the expression of endogenous retroviruses in their Beta cells with the appearance of insulitis and diabetes [13]. Recently, we have found that Beta-cell-specific expression of endogenous retroviruses is highly associated with the development of insulitis and diabetes in NOD mice [14]. In addition, it is possible that STZ might somehow affect not only Beta cells but also immune system cells, particularly islet-infiltrating mononuclear cells. It has been shown that multiple low-dose-STZ induces T-cell stimulation and secretion of lymphokines such as IFN-r [15]. However, the fact that the severity of insulitis at the age of 24 weeks in STZ-treated animals was not significantly different from that of untreated animals suggests that this STZ treatment does not affect the progression of insulitis but significantly reduces the Beta-cell mass. Thus, the mechanism of action of STZ in this study seems to be the direct Beta-cell damage rather than the indirect effects on Beta cells.

Earlier studies in mice showed that previous injection of viruses with little diabetogenicity or of low dose STZ could make normally resistant animals sensitive to the diabetogenic action of EMC-D virus [16, 17]. A more recent study showed that the glucose tolerance and insulin secretion abnormalities appeared to be more pronounced in a genetically susceptible species of monkey (e.g., patas) that received a sub-diabetogenic dose of STZ prior to Coxsackie B4 virus infection [18]. Although all of these observations were unrelated to anti-Beta-cell autoimmunity, these findings support the hypothesis that Type 1 diabetes may result from cumulative Beta-cell damage induced by sequential direct environmental insults. Our findings in this study indicate that an anti-Beta-cell autoimmune process also has a predisposing effect on the induction of diabetes by a single final insult.

On the basis of earlier studies on the cumulative Beta-cell damage induced by sequential environmental insults and the present study on the predisposing effect of anti-Beta-cell autoimmune processes on the induction of diabetes by a single environmental insult, it is concluded that the precipitation of clinical diabetes by some environmental insults in subjects with pre-existing pre-clinical autoimmune Beta-cell destruction may be one mechanism of disease presentation in human Type 1 diabetes.

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