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Nicotinamide prevents the development of diabetes in the cyclophosphamide-induced NOD mouse model by reducing beta-cell apoptosis

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Abstract

The development of diabetes in non-obese diabetic (NOD) mice, which normally takes between 3 and 7 months, can be accelerated by cyclophosphamide (CY) injections, with rapid progression to diabetes within only 2–3 weeks. This insulin-dependent diabetes mellitus (IDDM) can be prevented or delayed in CY-treated NOD mice by nicotinamide (NA). The present study was undertaken to determine the mode of cell death responsible for the development of IDDM in CY-treated male NOD mice and to investigate the effect of NA on beta-cell death. Apoptotic beta cells were present within the islets of Langerhans in haematoxylin and eosin-stained sections of the pancreata harvested from 3- and 12-week-old male NOD mice, from 8 h until 14 days after a single intraperitoneal injection of CY (150 mg/kg body weight). The maximum amount of beta-cell apoptosis in 3-week-old animals occurred 1–2 days after CY treatment (20 apoptotic cells per 100 islets), after which time levels of apoptosis declined steadily throughout the 14-day period studied. The incidence of beta-cell apoptosis in 12-week-old male NOD mice occurred in two peaks; the first was recorded 8–24 h after CY treatment (30 apoptotic cells/100 islets), while the second, at 7 days (36 apoptotic cells per 100 islets), coincided with increased insulitis. Administration of NA 15 min before CY treatment, and thereafter daily, substantially reduced the amount of apoptosis and effectively eliminated (4 apoptotic cells per 100 islets) the second wave of beta-cell apoptosis seen at day 7 in 12-week-old animals given CY alone. These results show that apoptosis is the mode of beta-cell death responsible for the development of CY-induced IDDM and that prevention of IDDM by NA is associated with a reduction in beta-cell apoptosis.

Keywords: non-obese diabetic mouse; insulin-dependent diabetes mellitus; beta cell; apoptosis; nicotinamide; cyclophosphamide

Introduction

IDDM results from the destruction of the insulin-producing beta cells of the pancreatic islets of Langerhans. The events directly responsible for the death of beta cells are unknown. Our findings have shown that in the spontaneous non-obese diabetic (NOD) mouse model of IDDM and in streptozotocin (STZ)-induced IDDM, beta-cell death is by apoptosis [1,2]. Furthermore, in both models, beta-cell apoptosis precedes lymphocytic infiltration into islets.

Studies showing discordance in the incidence of IDDM between monozygotic twins suggest that environmental triggers must play key roles in the disease process. The initial prediabetic phase of the disease, which may span several decades, is clinically silent and a definitive marker to identify predisposed individuals has yet to be elucidated (reviewed in ref. 3). At the time of clinical presentation, most of the beta-cell population has already been eliminated. Identifying the mode of beta-cell death as apoptosis offers the possibility of rendering beta cells resistant to apoptosis, thereby advancing IDDM treatment and prevention.

The NOD mouse [4] is a spontaneous model of diabetes, with clinical and pathological manifestations similar to those seen in human IDDM [5]. In this mouse model, as with humans, the long and the variable time interval between putative triggering factors and the onset of symptoms, representing a functionally significant beta-cell loss, creates difficulties in determining the mechanism of beta-cell destruction. The autoimmune process in NOD mice can be accelerated by cyclophosphamide (CY), with rapid progression to overt diabetes within 2–3 weeks [6]. Nicotinamide (NA) effectively prevents IDDM onset in NOD mice [7]. It has also proven beneficial in delaying the onset of human diabetes [8].

It has been postulated that IDDM development in the NOD mouse occurs in two phases (reviewed in ref. 9). Before 5 weeks of age, no pathological changes are detected within islets. Subsequently, immune cells infiltrate islets and remain harmless until 12 weeks of age, at which time insulitis assumes a destructive role in animals predisposed to diabetes. In the present investigation, CY was used in an attempt to induce IDDM in 3-week-old (before insulitis can be detected)

and 12-week-old (after insulitis has been established) male NOD mice, to determine the mode of beta-cell death responsible for diabetes development and to establish whether the anti-diabetogenic properties of NA are attributable to a reduction in beta-cell apoptosis.

Materials and methods

Animals

Male NOD/Lt mice aged 3 and 12 weeks (Animal Resource Centre, Perth, Australia) were maintained in specific pathogen-free (SPF) conditions on regular mouse chow and water *ad libitum* with 12 h day/night regimes. Test and control groups comprised eight animals for each observation point.

Drugs and reagents

Test groups received either CY alone (group 1; 3- and 12-week-old mice); NA and CY (group 2; 12-week-old mice only); or NA alone (group 3; 12-week-old mice only). Control animals were treated with phosphate buffered saline (PBS, pH 7.4; 3- and 12-week-old mice) only.

CY (Sigma, MO, USA) was diluted in PBS and administered at a dose of 150 mg/kg body weight intraperitoneally to animals in group 1. NA (Sigma, MO, USA) was diluted in PBS and administered at a dose of 500 mg/kg body weight intraperitoneally, 10–15 min before CY treatment to mice in group 2. Animals in group 3 received NA, followed 10–15 min later by an injection of PBS. After the initial NA treatment, mice in groups 2 and 3 received the same dose of NA 3 h later and then each day until the experimental end-point.

Monitoring of diabetes

Blood glucose was measured in fresh capillary whole blood obtained from the tail of mice using an ExacaTech blood glucose sensor (Medisense, MA, USA). The presence of urine glucose and ketones was assessed using Keto-Diastix reagent strips (Bayer Diagnostics, Australia) for urinalysis.

Histological procedures

At 8 h and at 1, 2, 3, 7, and 14 days after CY treatment, the pancreas of each animal was removed and fixed in neutral buffered formalin for 24 h before conventional histological processing and paraffin embedding. It has been reported that all lymphoid organs from NOD mice show greatly diminished cell numbers soon after CY administration [10]. Samples of spleen and small intestine were therefore also taken at 8 and 24 h and the presence of increased numbers of apoptotic cells in these organs was used to confirm drug effectiveness. Serial sections (pancreas, small intestine, and spleen) were cut and every fifth section was stained by haematoxylin and eosin (H&E). Adjacent sections were used for immunohistochemistry.

Immunohistochemical procedures

Indirect biotin/streptavidin immunohistochemistry was performed using diaminobenzidine (DAB) (Sigma, MO, USA) for visualization. Antibodies to detect insulin (Dako, Australia) and CD3 (Dako, Australia) were used to identify beta cells and T-cells respectively. Microwave unmasking (in 0.01 mol/l citrate buffer, pH 6.0) of antigens was necessary prior to immunohistochemistry using the CD3 antibody.

Quantitation of apoptosis

Apoptotic cells were identified in H&E-stained pancreatic sections using established morphological criteria of early (margination of nuclear chromatin to form chromatin caps abutting the nuclear membrane) and late (formation of apoptotic bodies) changes of apoptosis. The number of apoptotic cells was quantified and data were represented as the mean number of apoptotic cells per 100 islets $\pm$ standard error (SE) of the mean for each time point. Sufficient numbers of sections were studied such that for each animal, 500 ± 50 islets were examined for the presence of cells undergoing apoptosis (approximately 4000 islets for each time point studied).

The beta-cell origin of the apoptosis was determined by staining for insulin. Apoptotic T-lymphocytes were identified by immunohistochemistry using an anti-CD3 antibody and were excluded from total cell counts.

Semi-quantitative analysis of insulitis

Each islet was scored as follows: 0 = no detectable mononuclear cells in the islet; 1 = a few peripheral mononuclear cells (peri-insulitis); 2 = up to 50% of the islet area occupied by mononuclear cells; 3 = more than 50% of the islet area occupied by mononuclear cells.

Statistical analysis

Data from control and treated groups concerning apoptosis, insulitis, and blood glucose were analysed using a two-sample *t*-test assuming unequal variances. Variation between treated groups was subjected to multiple comparisons.

Results

Apoptotic cells were identified in H&E-stained pancreatic sections of CY-treated mice throughout the entire experimental period. Some dying cells showed the characteristic morphological changes of early apoptosis; namely, margination of nuclear chromatin to form chromatin caps abutting the nuclear membrane (Figure 1A). More frequently, rounded apoptotic bodies in the later stages of the process were

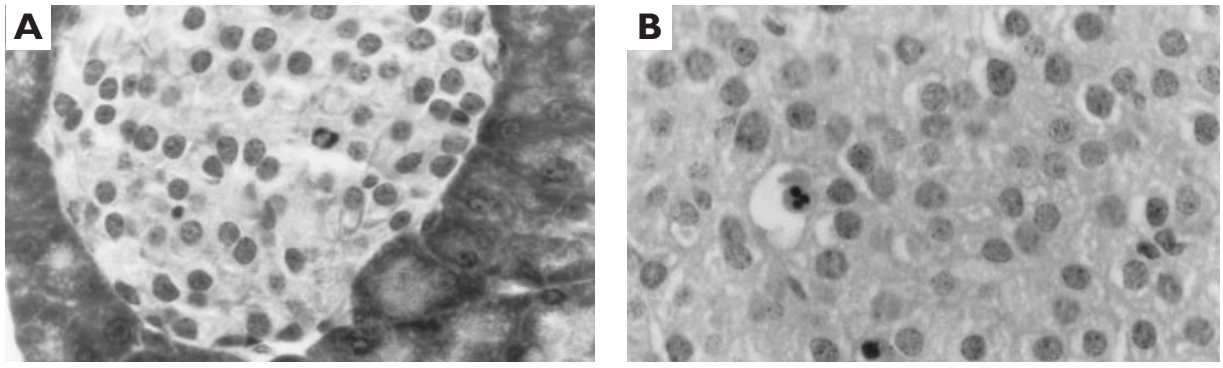

Figure 1. Light micrographs of haematoxylin and eosin-stained sections of pancreatic islets from 12-week-old male NOD mice 8 h (A) and 7 days (B) after treatment with CY. (A) A cell in the early phases of apoptosis showing margination of chromatin against the nuclear membrane ($\times 300$). (B) A condensed rounded apoptotic body containing three dense masses of nuclear chromatin ($\times 300$)

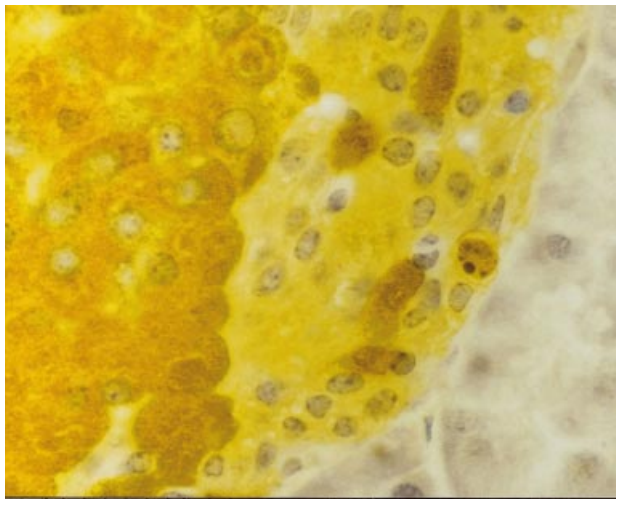

Figure 2. Immunohistochemical staining for insulin ($\times 400$). An insulin-positive apoptotic beta-cell shows characteristic condensation and fragmentation of nuclear chromatin

observed (Figure 1B). Immunohistochemical localization of insulin to the dying cells (Figure 2) confirmed that the apoptosis observed in H&E-stained sections of pancreas from both 3- and 12-week-old NOD mice after a single intraperitoneal injection of CY was beta-cell apoptosis. The amount of beta-cell apoptosis recorded in both 3- and 12-week-old CY-treated NOD mice was significantly higher than that observed in control animals at every time point studied (8 h to 14 days) (Figure 3). The different age groups displayed distinct patterns in the incidence of beta-cell death. The maximum amount of beta-cell apoptosis in 3-week-old animals occurred 1–2 days after CY treatment (20 apoptotic cells per 100 islets at days 1 and 2, $p < 0.01$), after which time levels of apoptosis declined steadily throughout the period studied. In contrast, when 12-week-old male NOD mice were injected with CY, apoptosis occurred in two waves; the first was recorded 8–24 h after treatment (30 apoptotic cells per 100 islets

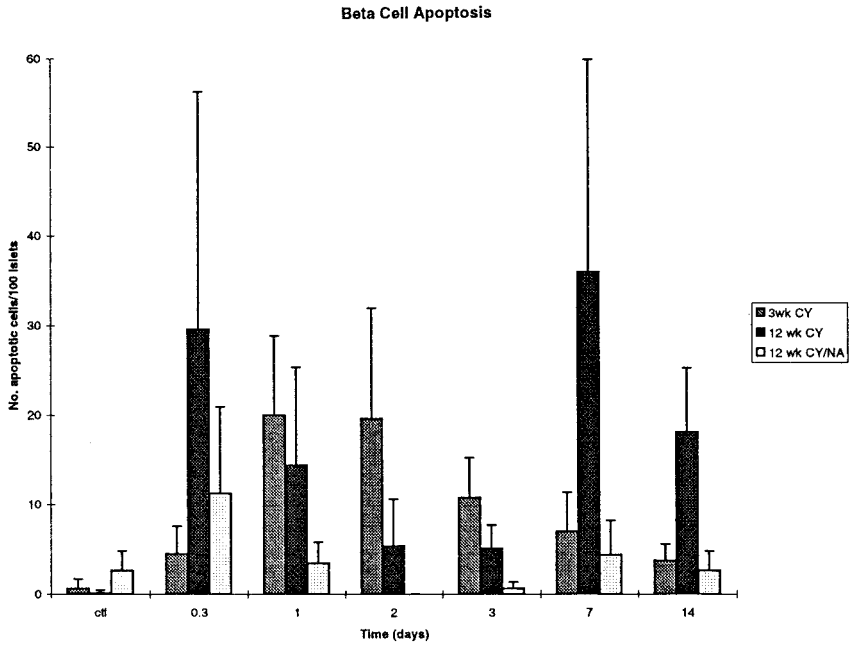

Figure 3. Mean number of apoptotic beta cells per 100 islets from 3- and 12-week-old CY-treated and 12-week-old CY and NA-treated male NOD mice. Data are means +SE. ctl = control; CY = cyclophosphamide treatment only; CY/NA = cyclophosphamide and nicotinamide treatment

at 8 h, $p<0.05$; 14 apoptotic cells per 100 islets at 24 h, $p<0.01$), while the second was at 7 days (36 apoptotic cells per 100 islets, $p<0.01$). There was no difference in the amounts of beta-cell apoptosis in 12-week-old animals recorded at each peak. However, the second peak (at day 7) coincided with significantly increased insulitis compared with controls ($p<0.05$) (Figure 4). Injection of CY induced a greater amount of beta-cell apoptosis in 12-week-old NOD mice than in 3-week-old animals at every time point except day 1, at which time maximum levels were recorded in the younger mice. Apoptotic bodies were markedly increased in number in the intestinal crypts and spleen of all 3- and 12-week-old animals 8 h and 24 h after CY injection.

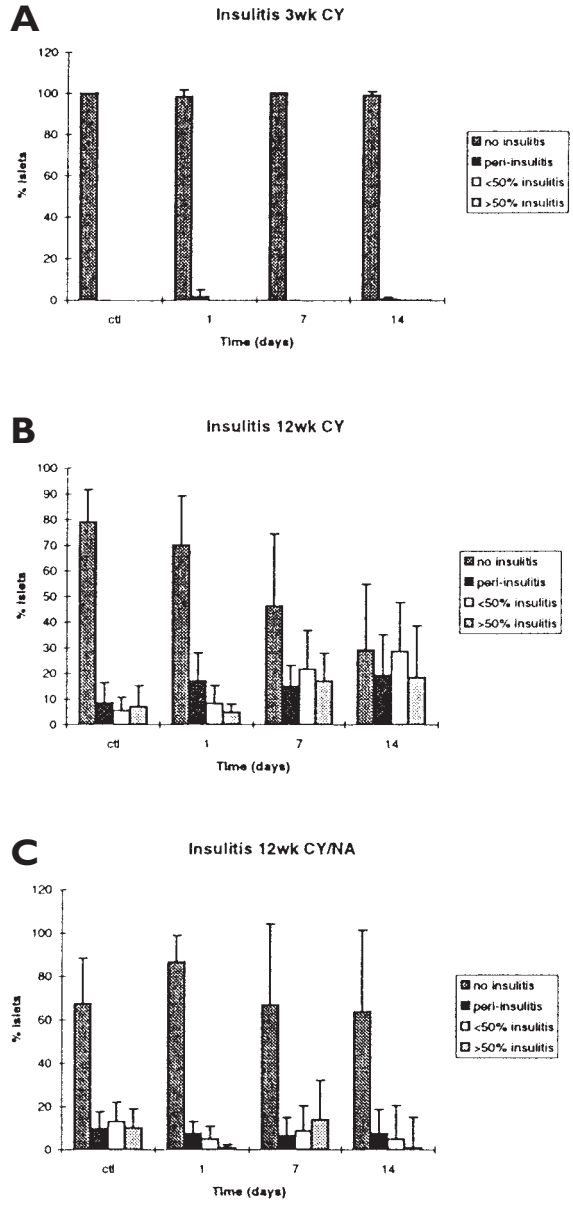

Figure 4. Semi-quantitative analysis of insulitis in male NOD mice aged (A) 3 and (B) 12 weeks treated with CY alone and (C) 12 weeks treated with both CY and NA Islets were graded according to the area occupied by immune infiltrate (no insulitis, peri-insulitis, up to 50% insulitis, and more than 50% insulitis), and the percentage of islets with each grade was calculated at each time point. Only islets from 12-week-old animals showed an increase in the severity of insulitis throughout the experimental period. ctl = control

Monitoring of IDDM development using blood glucose measurements revealed that none of the 3-week-old animals became diabetic throughout the 14-day period studied (Figure 5). On the other hand, 40% of the 12-week-old mice had elevated blood glucose and urinary ketones 14 days after injection with CY. One 12-week-old animal was diabetic on the third day after CY treatment, presumably due to spontaneous development of IDDM. Apart from this animal, none of the 12-week-old NOD mice developed IDDM before day 14 after CY administration.

Treatment of 12-week-old NOD mice with both NA and CY resulted in a reduced peak, at 8 h, in the incidence of beta-cell apoptosis (11 apoptotic cells per 100 islets, $p<0.05$) and the elimination of the second wave of apoptosis which occurred in animals injected with CY alone (Figure 3). NA treatment consistently reduced the amount of beta-cell apoptosis occurring at each time point, compared with CY treatment alone. In contrast to 12-week-old NOD mice that received CY alone, no differences in the severity of insulitis between control and treated groups were recorded throughout the time interval studied when NA and CY were both administered. None of the 12-week-old mice treated with NA and CY developed IDDM by day 14.

Discussion

This study shows that apoptosis is the mode of beta-cell death responsible for the development of CY-induced IDDM in 12-week-old male NOD mice. While CY treatment of 3-week-old male NOD mice did induce an initial wave of beta-cell apoptosis, this was not sustained and progression to IDDM did not occur. Treatment of 12-week-old male NOD mice with NA halted progression to IDDM and this was associated with markedly reduced beta-cell apoptosis. These findings are consistent with the results of our previous investigations, which showed that the mode of beta-cell death in spontaneous (NOD mouse) and induced (multiple low-dose STZ) models of IDDM is apoptosis [1,2]. Furthermore, in both models, beta-cell apoptosis precedes the appearance of immune cells within the islets, suggesting that non-immune mechanisms may play an early role in the pathogenesis of this disease.

Beta cells from both 3- and 12-week-old CY-treated NOD mice were eliminated by apoptosis throughout the entire 14-day period studied. The rapidity with which apoptotic bodies are formed and removed [11] (reviewed in ref. 12) means that the detection of small increases in the number of apoptotic bodies within a tissue represents considerable cumulative cell loss [13]. The absence of sharp peaks in the incidence of beta-cell apoptosis is not surprising. It is well recognized that within a single population of cells, there are variable

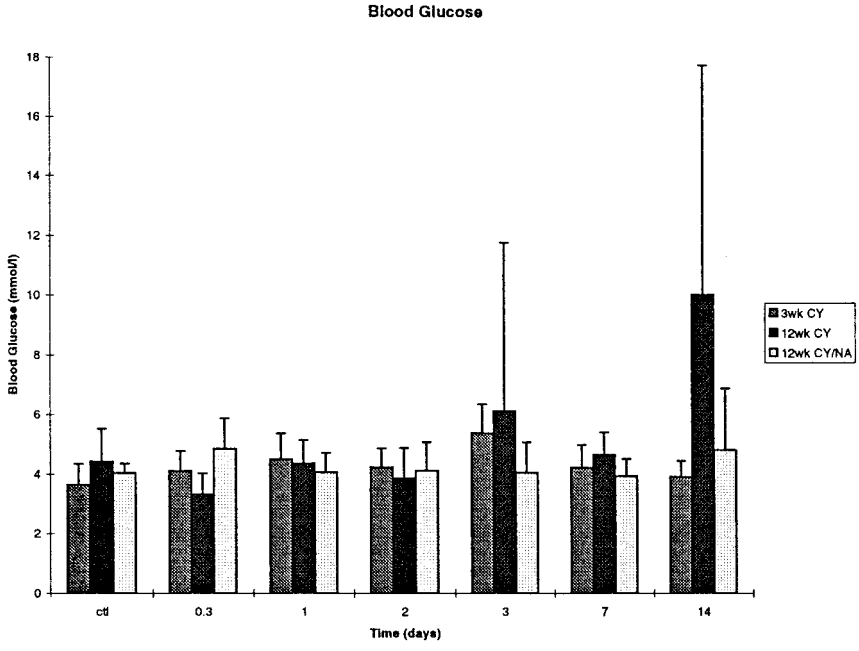

Figure 5. Blood glucose in male NOD mice aged 3 and aged 12 weeks treated with CY and aged 12 weeks treated with CY and NA. Data are means + SE. Twelve-week-old mice administered CY from groups sampled at 3 and 14 days after treatment contained one and three animals, respectively, that had already developed IDDM (elevated blood glucose values; glucose and ketones present in urine). ctl = control; CY = cyclophosphamide treatment only; CY/NA = cyclophosphamide and nicotinamide treatment

response times to apoptotic stimuli (reviewed in ref. 12). Several reports confirm differences in beta-cell sensitivity to cytodestructive agents [14,15].

CY exerts direct toxic effects on cells through the induction of DNA strand breaks [16] and has been reported to induce apoptosis both *in vitro* and *in vivo* [17–19]. Our finding that islets from 3-week-old CY-treated NOD mice contain apoptotic beta cells in the absence of insulitis provides support for the view that CY exerts a direct toxic effect on the beta cells. STZ also induces DNA strand breaks in beta cells [20,21] and stimulates beta-cell apoptosis both *in vivo* [1,2] and *in vitro* [22]. Perhaps both diabetogenic agents (CY and STZ) induce beta-cell apoptosis through a common mechanism.

In NOD mice it has been demonstrated that administration of agents which prevent IDDM does not eliminate insulitis, but appears to divert the disease process to a 'non-destructive' pathway [23]. Immune mechanisms may therefore be necessary, but not sufficient for the development of IDDM. In this study, 3-week-old CY-treated NOD mice exhibited an initial apoptotic peak but failed to show a second, possibly 'immune-mediated', wave of apoptosis and did not develop IDDM. In autoimmune thyroid disease it has been shown that thyroid cells undergo apoptosis in the absence of infiltrating T-lymphocytes, suggesting that immune mechanisms do not initiate the destructive process in this disease [24].

The incidence of beta-cell apoptosis after 12-week-old animals were treated with CY mimics that observed in the multiple low-dose STZ model, where an initial cytotoxic peak is followed by a second wave of apoptosis 7 days after treatment [1]. Consistent with this, 12-week-old animals injected with both CY and NA experienced a single, early peak in the incidence of beta-cell apoptosis, but did not show an increase in the severity of insulitis or a second peak in apoptotic rates at day 7 and did not progress to overt diabetes.

Our results for 12-week-old CY-treated NOD mice are not in conflict with an immune aetiology. Both humoral and cellular immune mechanisms are thought to play a role in IDDM development in the NOD mouse. While the first peak (8 h after CY treatment) in the incidence of apoptosis may be attributed to direct toxicity, the second apoptotic peak (at day 7) coincides with increased insulitis. Immune-mediated killing occurs by apoptosis [25–28]. Transgenic NOD mice which contain a population of monospecific, beta-cell-responsive CD4-positive T-lymphocytes have accelerated progression to IDDM by 2 weeks of age and the beta cells within inflamed islets die by apoptosis [29]. It has been reported that autoantibodies can penetrate cells to mediate apoptosis (reviewed in ref. 30).

IDDM in male mice may exist [31,32]. Exposure of 3-week-old NOD mice to a single toxic insult of CY in the absence of established insulitis may not have been able to destroy adequate numbers of beta cells in the period studied. NA prevented the development of IDDM in CY-treated 12-week-old male NOD mice by reducing beta-cell apoptosis. The generation of DNA strand breaks, which induces poly ADP ribose synthase (PADPRS) activation, and subsequent NAD depletion appear to be common factors in cell death by apoptosis mediated by a variety of factors, including CY (reviewed in ref. 33 and 34). High doses of NA inhibit PADPRS, protect against NAD depletion, and prevent apoptosis [21,35–37]. NA prevents STZ-

induced cleavage of islet DNA and enhances islet survival [15].

The beta-cell mass is the major determinant of the total amount of insulin that can be secreted by the pancreas [38]. The diversity of genetic and environmental factors thought to play key roles in the pathogenesis of human IDDM, coupled with a preclinical period spanning several years, complicates the identification of individuals predisposed to IDDM. In agreement with investigations of other autoimmune diseases [39,40] (reviewed in ref. 41), our previous studies have shown that apoptosis plays a pivotal role in the pathogenesis of IDDM *in vivo* [1,2]. It has been proposed that the multitude of triggers, cellular metabolic events, and multiple signalling pathways of apoptosis converge into a common sequence of events as the morphological features characteristic of apoptosis are highly conserved (reviewed in ref. 12 and 42). With respect to IDDM, it seems that toxin- and immune-mediated beta-cell death involves DNA damage. Furthermore, our studies have provided evidence that apoptosis is the mode of beta-cell death in these situations [1,2] (present study). If the factors which mediate beta-cell apoptosis converge into a single mechanism, the possibility exists to make beta cells resistant to apoptosis, thereby protecting them simultaneously from damage by multiple agents. This may provide a means of limiting or even halting the development of IDDM in susceptible individuals, by maintaining a functional beta-cell mass.

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