

Nicotine Reduces the Incidence of Type I Diabetes in Mice

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ABSTRACT

Nicotine has been previously shown to have immunosuppressive actions. Type I diabetes is an autoimmune disease resulting from the specific destruction of the insulin-producing pancreatic β -cells. Thus, we hypothesized that nicotine may exert protective effects against type I diabetes. The multiple low-dose streptozotocin (MLDS)-induced model and spontaneous nonobese diabetic (NOD) mouse model of type I diabetes were used to assess whether nicotine could prevent this autoimmune disease. Blood glucose levels, diabetes incidence, pancreas insulin content, and cytokine levels were measured in both models of diabetes, both to assess the level of protection exerted by nicotine and to further investigate its mechanism of action. Nicotine treatment reduced the hyperglycemia and in-

cidence of disease in both the MLDS and NOD mouse models of diabetes. Nicotine also protected against the diabetes-induced decrease in pancreatic insulin content observed in both animal models. The pancreatic levels of the Th1 cytokines interleukin (IL)-12, IL-1, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ were increased in both MLDS-induced and spontaneous NOD diabetes, an effect prevented by nicotine treatment. Nicotine treatment increased the pancreatic levels of the Th2 cytokines IL-4 and IL-10. Nicotine treatment reduces the incidence of type I diabetes in two animal models by changing the profile of pancreatic cytokine expression from Th1 to Th2.

Type I diabetes is a disease characterized by the specific destruction of the insulin-producing β -cells of the pancreatic islets of Langerhans by the immune system (Bottazzo and Bonifacio, 1991; Noorchashm et al., 1997). The islet is invaded by immune cells, particularly T-cells that are CD4⁺ and CD8⁺ (Rabinovitch, 1994), forming a pancreatic inflammation termed insulinitis, with the resulting production of immune cell mediators, such as cytokines and free radicals (including nitric oxide and related radicals such as peroxynitrite), inhibiting β -cell function and ultimately inducing β -cell death (Rabinovitch and Suarez-Pinzon, 1998). The induction of nitric oxide synthase (iNOS) and the subsequent formation of nitric oxide and related free radicals have been implicated in the destruction of the β -cells in diabetes.

A variety of cytokines have been found expressed in the insulinitis lesion of the animal models of diabetes and in the pancreata of humans with type I diabetes. It has been proposed that the insulinitis lesion is β -cell destructive when Th1 cytokines (IL-12, IFN- γ , IL-1, and TNF- α), produced by islet-infiltrating T-cells, dominate over Th2 cytokines (IL-4 and IL-10) (Rabinovitch, 1998). Agents that suppress the immune system, including cyclosporine (Baquerizo et al., 1989) and

glucocorticoids (Like et al., 1983), have been shown to reduce the disease incidence in animal models of type I diabetes.

There are two murine models of autoimmune diabetes: the multiple low-dose streptozotocin (MLDS) model and the spontaneous NOD mouse model. The MLDS model of diabetes is characterized by progressive hyperglycemia and insulinitis similar to that observed in recent onset type I diabetics (Like and Rossini, 1976). The NOD mouse model also shares clinical serological and histoimmunological features with human type I diabetes (Bach, 1994). As in humans, the disease is characterized by infiltration of the pancreatic islets by immune cells, insulinitis followed by destruction of the β -cells. Both models have been used extensively to study new therapies to prevent type I diabetes (Mabley et al., 2001; Suarez-Pinzon et al., 2001). The β -cell destructive role of nitric oxide in diabetes has been determined using these animal models; in the MLDS model, a specific iNOS inhibitor protects against development of diabetes (J. G. Mabley and C. Szabó, unpublished observations), and the iNOS-deficient mouse was also found to have reduced sensitivity to MLDS-induced diabetes (Flodstrom et al., 1999). Nitric oxide synthase has been shown to play a role in mediating diabetes in the genetic animal models of type I diabetes both in the BB rat (Kleemann et al., 1993; Wu, 1995) and the NOD mouse (Corbett et al., 1993).

Nicotine has been shown to have immunosuppressive ef-

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ABBREVIATIONS: iNOS, inducible nitric oxide synthase; IL, interleukin; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; MLDS, multiple low-dose streptozotocin; NOD, nonobese diabetic; ELISA, enzyme-linked immunosorbent assay.

fects, having both T-cell-dependent and -independent effects. Nicotine-treated mouse T-cells, which had been stimulated with anti-CD3, produce significantly less Th1 cytokines (IL-2 and IFN- γ) but had increased Th2 cytokine production (IL-4 and IL-10) compared with untreated cells (Zhang and Petro, 1996). Nicotine also reduced immune cell infiltration into the colon in a rat model of colitis (Eliakim et al., 1998). Therefore, the aim of this study was to determine whether nicotine treatment could prevent type I diabetes.

Materials and Methods

Reagents were obtained from the following sources. Streptozotocin, sodium citrate, sodium nitrite, sodium nitrate, nicotine, nitrate reductase, NADPH, sulfanilamide, and naphyl-ethylenediamine were obtained from Sigma Chemical Company (St. Louis, MI). BALB/c mice and NOD mice were purchased from Taconic Farms (Germantown, NY). Insulin ELISA kits were obtained from ALPCO (Windham, NH). Urine glucose Tes-Tape was purchased from Eli Lilly (Toronto, ON, Canada). A One-Touch II hospital blood glucose meter was obtained from Lifescan (a Johnson & Johnson Company, Milpitas, CA). Specific cytokine ELISA kits were from R & D Systems (Minneapolis, MN).

Induction of Diabetes. All animal experiments were carried out in accordance with the guidelines published by the NIH in *Principles of Laboratory Animal Care* (NIH publication 85-23, revised 1985) and with the approval of Inotek's Institutional Animal Care and Use Committee.

Male BALB/c mice were treated with streptozotocin (40 mg/kg dissolved in citrate buffer, pH 4.5) or vehicle (citrate buffer) i.p. for 5 consecutive days. Mice were treated every day starting on day 1 with either nicotine (0.4 mg/kg) or vehicle (saline) s.c. The blood glucose level was monitored over the following 21 days using a one-touch blood glucose meter (Lifescan). Blood glucose was measured on days 1, 7, 14, and 21 from blood obtained from the tail vein. Hyperglycemia was defined as a nonfasting blood glucose level higher than 200 mg/dl. Cumulative incidence of diabetes was calculated as a percentage of hyperglycemic mice per treatment group at each time point. Biopsies of the pancreas were removed on day 21 for further biochemical analysis. Serum was also taken and frozen for the determination of nitrite/nitrate levels.

Female NOD mice were purchased at 4 weeks of age and allowed to acclimatize to Inotek's animal facility for 1 week before daily treatment with nicotine (0.4 mg/kg s.c.) commencing at 5 weeks of age. Two separate studies were set up. In the first, spontaneous diabetes incidence was monitored until 25 weeks of age; these mice had their urine glucose levels checked using Tes-Tape. A mouse was defined as diabetic following 3 days of glucosuria and a blood glucose level on the third day greater than 200 mg/dl. In a second study, NOD mice were treated in the same way, but this experiment was terminated when the mice reached 18 weeks of age, and as for the MLDS experiment, pancreas biopsies were taken for biochemical analysis. The female mice taken at 18 weeks were not diabetic, and the mean blood glucose of both the vehicle and nicotine groups was comparable (data not shown).

Determination of Pancreas Insulin Content and Cytokine Levels. The pancreas biopsy was weighed before being placed into 6 ml of acid ethanol and homogenized (Garcia Soriano et al., 2001). The pancreas was incubated for 72 h at 4°C before being centrifuged and the insulin content of the supernatant determined using a commercially available ELISA kit (ALPCO). Pancreas insulin content was expressed as nanograms of insulin per milligram of protein. A second sample of pancreas was removed and snap frozen in liquid nitrogen; the sample was then homogenized in 700 μ l of a TRIS-HCl buffer containing protease inhibitors. The samples were centrifuged for 30 min, and the supernatant was removed and frozen at -80°C until

assay. Cytokine levels were determined using commercially available kits (R & D Systems).

Determination of Serum Nitrite/Nitrate Levels. Serum nitrate levels were determined by converting the nitrate to nitrite using the enzyme nitrate reductase, followed by addition of Griess reagent to colorimetrically quantify the nitrite concentration (Mabley et al., 2001). The serum was diluted (1:5) in phosphate-buffered saline before a 25- μ l aliquot was added to a mixture of 25 μ l of nitrate reductase (1 U/1.5 ml) and 25 μ l of NADPH (0.134 mg/ml), both dissolved in 40 mM TRIS, pH 7.6, and incubated at room temperature for 3 h. Following this period, 100 μ l of Griess reagent (1:1 mix of 1% sulfanilamide in 5% phosphoric acid and 0.1% naphyl-ethylenediamine) was added and incubated for a further 10 min at room temperature. The absorbency of the samples was read at 540 nm with a 650-nm reference. The concentration of nitrite/nitrate was determined from a standard curve of sodium nitrite and expressed as micromolars nitrite/nitrate.

Statistical Analysis. The data are presented as mean \pm S.E.M.; statistical analysis was performed using either the χ^2 test or Student's *t* test as appropriate, with a *p* value of less than 0.05 considered significant.

Results

Treatment of mice with MLDS resulted in progressive hyperglycemia and an increased incidence of diabetes (Fig. 1). Daily treatment with 0.4 mg/kg nicotine subcutaneously attenuated the hyperglycemia and significantly reduced the incidence of diabetes (Fig. 1); a lower dose of nicotine (0.2 mg/kg/day) had no protective effects (data not shown). On day 21, the pancreas insulin content was dramatically decreased in MLDS-treated mice, an effect reversed by nicotine treatment (Fig. 2A). Serum nitrite/nitrate levels indicative of nitric oxide formation were increased in MLDS-treated mice, an effect again reversed by nicotine treatment (Fig. 2B). Nicotine treatment alone had no effect on blood glucose levels, incidence of diabetes, pancreas insulin content, or serum nitrite/nitrate levels (Figs. 1 and 2). Pancreatic levels of both Th1 and Th2 cytokines were determined following MLDS treatment with or without nicotine. MLDS increased the pancreatic levels of the Th1 cytokines TNF- α , IL-12 IFN- γ , and IL-1 β significantly compared with untreated mice (Fig. 3, A and B). MLDS treatment had no effect on the Th2 cytokines IL-4 and IL-10 (Fig. 3C). MLDS mice treated with nicotine had significantly less Th1 cytokines, with TNF- α , IL-12 IFN- γ , and IL-1 levels being reduced to those observed in the control mice (Fig. 3, A and B). Interestingly, there was a trend for nicotine to increase pancreatic levels of Th2 cytokines, with IL-4 being significantly increased above those seen in control and MLDS-treated mice (Fig. 3C). Although not reaching a level of significance (*p* = 0.09), there was also a trend for nicotine to increase IL-10 levels.

Female NOD mice spontaneously developed diabetes starting at 11 weeks of age. Mice treated daily with nicotine (0.4 mg/kg s.c.) had a significantly delayed onset of the disease to 14 weeks, with a reduced incidence of diabetes being observed up to 25 weeks of age (Fig. 4). However, stopping the nicotine treatment at 25 weeks of age resulted in the remaining mice becoming diabetic over the following 2 weeks (data not shown). The insulin content of 18-week-old female NOD mice was found to be significantly higher in those treated with nicotine (Fig. 5). However serum nitrite/nitrate levels were found to be identical (Fig. 5). Analysis of the pancreas cytokine levels in 18-week old mice showed that nicotine-

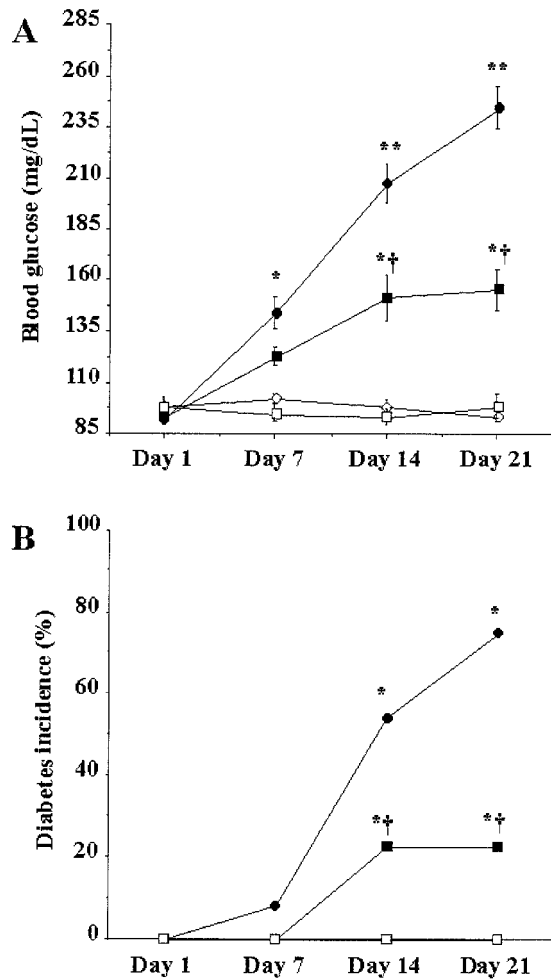


Fig. 1. Daily treatment with nicotine (0.4 mg/kg s.c.) decreases the level of (A) hyperglycemia and (B) incidence of diabetes following MLDS treatment. Blood glucose levels are expressed as milligrams of glucose per deciliter, and the incidence of diabetes is plotted as a cumulative percentage of mice with blood glucose greater than 200 mg/dl. Results are the mean \pm S.E.M. ($n = 24$ to 30 ; three separate experiments; 8–10 mice per experimental group). Statistical analysis was carried out using Student's *t* test or χ^2 test in which $p < 0.05$ was considered significant. *, $p < 0.05$; **, $p < 0.01$ versus control mice; †, $p < 0.05$ versus MLDS-treated mice; ○, vehicle-treated; ●, MLDS-treated; ■, MLDS plus nicotine; □, nicotine alone.

treated mice had significantly lower levels of the Th1 cytokines IL-12 (p40) and TNF- α (Fig. 6A), higher levels of the Th2 cytokine IL-10 (Fig. 6B), and identical levels of IL-4.

Discussion

We have demonstrated here that nicotine is able to reduce the incidence of type I diabetes in both the chemically induced and spontaneous diabetes animal models. Nicotine was able to protect β -cells from destruction by modulating the activity of the immune system, as demonstrated by the attenuation of nitric oxide formation in response to MLDS treatment and the shift from Th1 to Th2 cytokine levels in the pancreas. Nicotine has been shown to protect the β -cell line RIN-5F from combined cytokine treatment (Mabley et al., 2000), but this seems to be a nonspecific protective effect at relatively high concentrations of nicotine and was unrelated to either inhibition of nitric oxide synthase or the en-

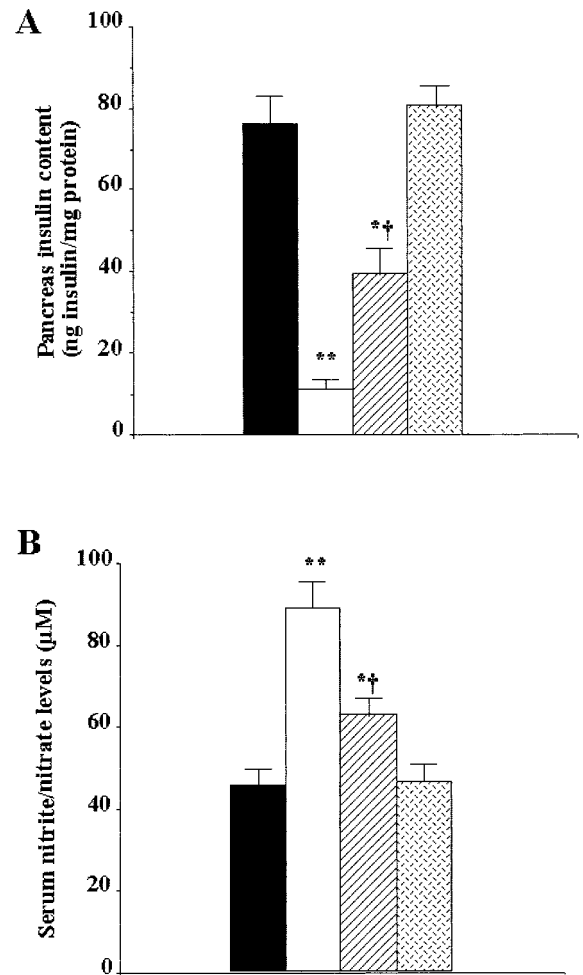


Fig. 2. Nicotine treatment (0.4 mg/kg s.c.) attenuated the MLDS-effects on (A) pancreas insulin content and (B) serum nitrite/nitrate levels. Insulin content of the pancreas is expressed as nanograms of insulin per milligram of protein and nitrite/nitrate levels in micromolar. Results are the mean \pm S.E.M. ($n = 24$; three separate experiments; 8–10 mice per experimental group). Statistical analysis was carried out using Student's *t* test in which $p < 0.05$ was considered significant. *, $p < 0.05$; **, $p < 0.01$ versus control mice; †, $p < 0.05$ versus streptozotocin-treated mice; ■, vehicle-treated; □, MLDS-treated; ▨, MLDS plus nicotine; ▩, nicotine alone.

zyme poly (ADP-ribose) synthetase (data not shown), inhibitors of which have been shown to be potent protective agents in type I diabetes (Mabley et al., 2001; Suarez-Pinzon et al., 2001).

Specific receptors for nicotine have been shown to be present on lymphocytes from human (Hoss et al., 1986), rat (Maslinski et al., 1992), and mouse (Toyabe et al., 1997). Nicotine has been shown to have T-cell-dependent and -independent effects. Nicotine suppresses antibody-dependent cell-mediated cytotoxicity and lymphokine-activated killer cell activities of normal human lymphocytes (Nair et al., 1990) and also prevents production of inflammatory mediators, such as IL-1, IL-8, and prostaglandin E_2 , from human macrophages (Sugano et al., 1998) and IL-2 and TNF- α from human mononuclear cells (Madretsma et al., 1996). An effect that is mediated through modulation of nuclear factor- κ B activation (Sugano et al., 1998). Nicotine also has effects on T-cell responses in the mouse; nicotine exposure of splenic mononuclear cells stimulated with anti-CD3 resulted in a

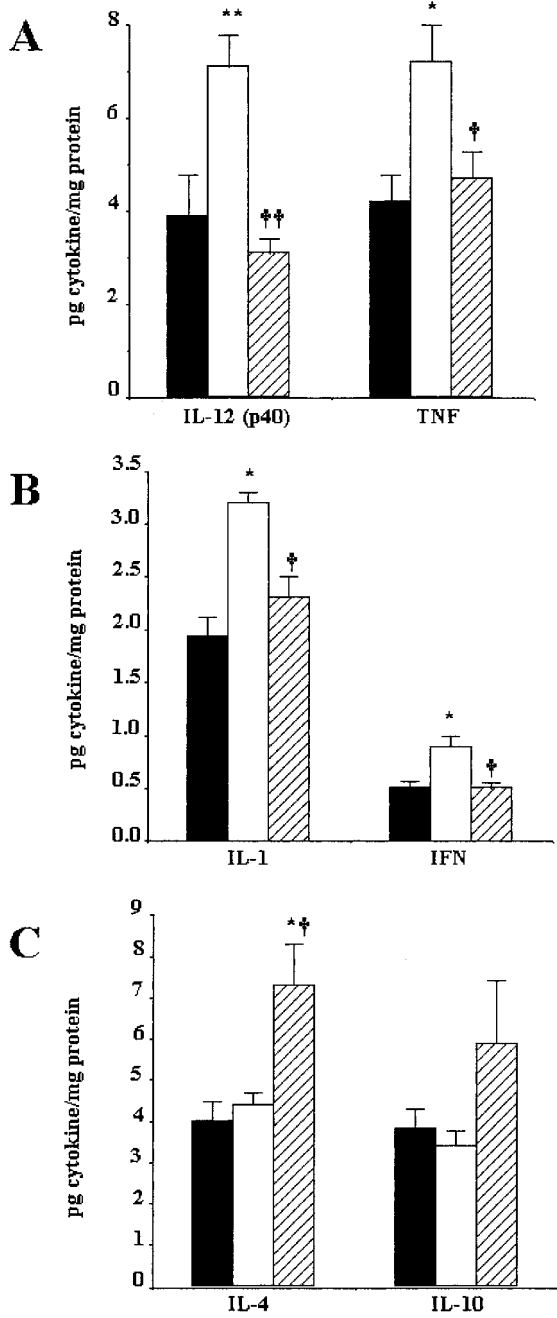


Fig. 3. Pancreatic levels of Th1 and Th2 cytokines. Nicotine (0.4 mg/kg s.c.) prevents the MLDS-induced increase in the Th1 cytokines IL-12, TNF- α , IL-1, and IFN- γ (A and B). MLDS treatment had no effect on the pancreatic the Th2 cytokines IL-4 and IL-10 (C), whereas nicotine treatment significantly increased pancreatic IL-4 levels. Results are the mean \pm S.E.M. ($n = 20$; two separate experiments; 10 mice per experimental group). Statistical analysis was carried out using Student's t test in which $p < 0.05$ was considered significant. *, $p < 0.05$; **, $p < 0.01$ versus control mice; †, $p < 0.05$; ††, $p < 0.01$ versus streptozotocin-treated mice; ■, vehicle-treated; □, MLDS-treated; ▨, MLDS plus nicotine.

decrease in the percentage of CD4⁺ T-cells expressing both CD28 and CTLA-4 and in the intensity of CD28 expression (Zhang and Petro, 1996). There was also a decrease in production of the Th1 cytokines IL-2 and IFN- γ , but significantly more Th2 cytokines, IL-4 and IL-10, were produced (Zhang and Petro, 1996). More recently, vagal nerve stimulation had anti-inflammatory effects against lipopolysaccharide treat-

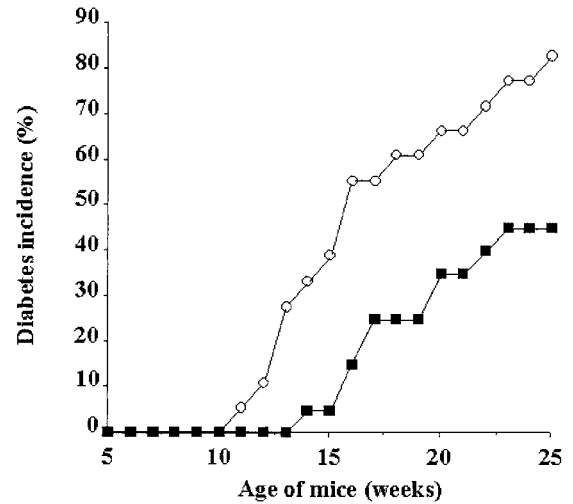


Fig. 4. Nicotine significantly delays the onset and reduces the incidence of spontaneous diabetes in female NOD mice. NOD mice were treated starting at 5 weeks of age with 0.4 mg/kg nicotine s.c. or vehicle, and disease development was monitored over the following 20 weeks. Results are expressed as a cumulative percentage of mice with blood glucose greater than 200 mg/dl. Each experimental group consisted of 18 mice. ○, vehicle-treated; ■, nicotine-treated.

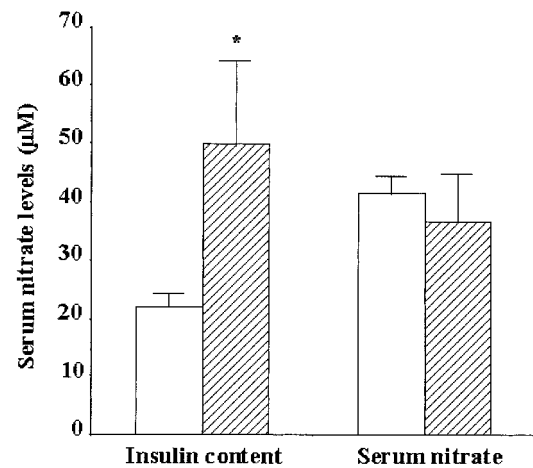


Fig. 5. Biochemical analysis of the pancreas of 18-week-old NOD mice treated with either vehicle or nicotine (0.4 mg/kg s.c.) from the age of 5 weeks. The pancreas of mice treated with nicotine had significantly higher insulin content, however; there was no difference in serum nitrate levels. Insulin content is expressed as nanograms per milligram of pancreatic protein and serum nitrate levels in micromolars. Results are expressed as the mean \pm S.E.M. ($n = 10$). Statistical analysis was carried out using Student's t test in which $p < 0.05$ was considered significant. *, $p < 0.05$ versus vehicle-treated mice; □, vehicle-treated; ▨, nicotine-treated.

ment (Borovikova et al., 2000), effects mediated by the principal vagal neurotransmitter acetylcholine (Borovikova et al., 2000).

The presence of the Th1 and Th2 cytokine subsets in the pancreas and the balance between them has been proposed to be the determining factor in diabetes development (Rabinovitch and Suarez-Pinzon, 1998). Th1 and Th2 cytokines are reciprocally regulated, with Th1 cytokines modulating the actions of Th2 cytokines and vice versa. There have been several reports implicating Th1 cytokine expression involvement in the pathogenesis of type I diabetes (Rabinovitch, 1998). We have presented data here showing that in the pancreas of mice from both the chemically induced and spon-

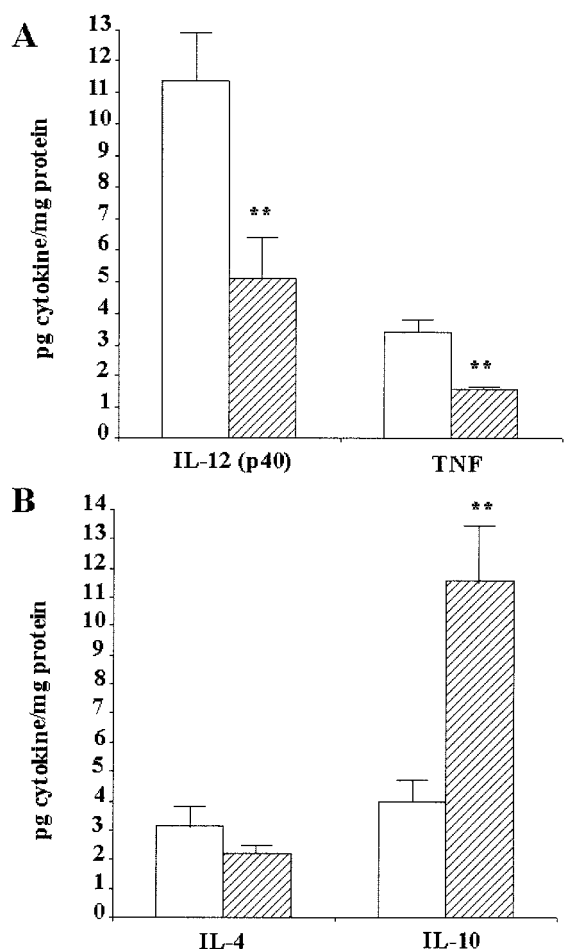


Fig. 6. Analysis of cytokine protein levels in the pancreas of 18-week-old female NOD mice treated with either vehicle or nicotine from 5 weeks of age. Nicotine-treated mice had significantly lower pancreatic levels of the Th1 cytokines IL-12 and TNF (A); however, these mice had significantly higher levels of the Th2 cytokine IL-10 (b). Results are expressed as the mean \pm S.E.M. ($n = 10$). Statistical analysis was carried out using Student's *t* test in which $p < 0.05$ was considered significant. **, $p < 0.01$ versus vehicle-treated mice; □, vehicle-treated; ▨, nicotine-treated.

taneous diabetes models of type I diabetes, there is a significant increase in the levels of the Th1 cytokines IL-12, TNF- α , IL-1, and IFN- γ , all of which have been implicated in the development of diabetes (Rabinovitch, 1998). The expression of IL-1, TNF- α , IL-12, and IFN- γ in the pancreas has been associated with β -cell-destructive insulinitis (Rabinovitch, 1998). In contrast, those mice that had been treated with nicotine had significantly lower levels of Th1 cytokines but, more importantly in the case of the MLDS model, increased levels of IL-4 and in the NOD mouse model increased IL-10 levels, both Th2 cytokines. Expression of IL-4 and IL-10 in the pancreas has been correlated with a benign, nondestructive insulinitis (Rabinovitch, 1998).

Reduction of Th1 levels using antibodies, transcriptional inhibitors, or cytokine secretion inhibitors has proven to be successful in preventing diabetes. Anti-IFN- γ antibodies reduce the incidence of diabetes in both the MLDS-induced model (Herold et al., 1996) and NOD mouse (Campbell et al., 1991); anti-IL-12 antibodies have proved to be effective at suppressing diabetes in the NOD mouse (Fujihira et al., 2000). Antibodies against IL-1 (Cailleau et al., 1997) and TNF- α (Yang et al., 1994) have both been shown to prevent

diabetes in the NOD mouse. Inhibitors of TNF- α transcription, such as MDL 201,449A (Holstad and Sandler, 2001), or secretion, such as troglitazone (Beales et al., 1998), have both prevented diabetes in the MLDS and NOD mouse models of diabetes, respectively.

The Th2 cytokines IL-4 and IL-10 have both been shown to prevent spontaneous diabetes in the NOD mouse (Pennline et al., 1994; Cameron et al., 1997; Nitta et al., 1998), leading to the possibility that it is the skewing of the cytokine profile from Th1 to Th2 in the pancreas that mediates nicotine's protection. The role of the Th2 cytokines in preventing diabetes development has been difficult to definitively prove. Certainly in many in vitro studies application or stimulation of Th2, cytokine release has been shown to antagonize the effects of Th1 cytokines and reduce production of Th1 cytokines (Hasko et al., 1998). The concept that expression of IL-4 or IL-10 in the pancreas protects against β -cell destruction is supported by studies in which increased numbers of IL-4- and IL-10-producing cells were found in the islets of NOD mice protected from diabetes development by oral insulin treatment (Hancock et al., 1995). Administration of IL-4 from 2 weeks of age has been shown to protect NOD female mice from diabetes. The mechanism of action being proposed is the stabilization of a protective Th2-mediated environment in the pancreatic islets, with IL-4 favoring the expansion of regulatory CD4⁺ Th2 cells in vivo preventing the onset of insulinitis and diabetes mediated by autoreactive Th1 cells (Cameron et al., 1997). More recently, King et al. (2001) demonstrated that pancreatic expression of IL-4 prevented virally induced diabetes. The mechanism by which IL-4 exerted protection against diabetes was by the inhibition of the generation of diabetogenic cytotoxic T-lymphocytes via influencing the antigen-presenting dendritic cell (King et al., 2001).

The ability of nicotine to decrease Th1 and increase Th2 cytokine levels seems to play a central role in its mechanism of protection. It is very clear from previous reports that reduction of pancreas levels of IL-12, IL-1, IFN- γ , and TNF- α prevents β -cell destruction (Rabinovitch, 1998). However, what is not certain is the order these effects occur. Does nicotine reduce the levels of Th1 cytokines leading to an increase in the Th2 cytokines or rather does the rise in Th2 cytokines reduce the levels of Th1 cytokines? Unfortunately, there is currently no way to know because the mechanistic effects of nicotine on the immune cells remain to be elucidated; even the nicotinic acetylcholine receptor subtype responsible for these immune-suppressive effects has yet to be determined.

In conclusion, we have presented definitive evidence that nicotine treatment reduces the incidence of type I diabetes by changing the profile of cytokines expressed in the pancreas during diabetes from Th1 to Th2. Nicotine itself as a preventative therapy for diabetes is unlikely due to the many and varied effects on other systems in the body. However, the presence of nicotinic acetylcholine receptors on human lymphocytes and the immunosuppressive action that activation of these receptors provokes may open the door to development of a specific agonist, one that may have no specificity for the other subtypes of nicotinic receptor located on other tissues and would prove an effective therapy to prevent diabetes in humans.

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