

## Evidence from Transgenic Mice That Interferon- $\beta$ May Be Involved in the Onset of Diabetes Mellitus\*

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A number of cytokines have been shown to alter the function of pancreatic  $\beta$ -cells and thus might be involved in the development of type 1 diabetes. Interferon- $\beta$  (IFN- $\beta$ ) expression is induced in epithelial cells by several viruses, and it has been detected in islets of type 1 diabetic patients. Here we show that treatment of isolated mouse islets with this cytokine was able to alter insulin secretion *in vitro*. To study whether IFN- $\beta$  alters  $\beta$ -cell function *in vivo* and leads to diabetes, we have developed transgenic mice (C57BL6/SJL) expressing IFN- $\beta$  in  $\beta$ -cells. These mice showed functional alterations in islets and impaired glucose-stimulated insulin secretion. Transgenic animals presented mild hyperglycemia, hypoinsulinemia, hypertriglyceridemia, and altered glucose tolerance test, all features of a prediabetic state. However, they developed overt diabetes, with lymphocytic infiltration of the islets, when treated with low doses of streptozotocin, which did not induce diabetes in control mice. In addition, about 9% of the transgenic mice obtained from the N3 back-cross to outbred albino CD-1 mice spontaneously developed severe hyperglycemia and hypoinsulinemia and showed mononuclear infiltration of the islets. These results suggest that IFN- $\beta$  may be involved in the onset of type 1 diabetes when combined with either an additional factor or a susceptible genetic background.

Despite an immense research effort, the etiology of type 1 diabetes has not been elucidated. It has been proposed that type 1 diabetes is caused by nongenetic factors, probably environmental, operating in a genetically susceptible host to initiate a  $\beta$ -cell-destructive immune process (1–3). These environmental factors, such as viral infections, may operate over a limited period to induce the immune process (4). Thereafter, there is a long prodrome before the onset of clinical diabetes during which clinical, immunologic, and metabolic changes can be detected in the  $\beta$ -cells (4). During the prediabetic period, a decline in the insulin secretion as well as impaired glucose tolerance can be detected several years before the clinical onset of type 1 diabetes (5, 6). Thus, alterations in  $\beta$ -cell function

might be a previous step to the development of diabetes mellitus.

Cytokines are hormone-like peptides mainly used by immune system cells to control local and systemic events of immune and inflammatory responses (7–9). Studies *in vitro* have demonstrated that certain cytokines, such as IL-1,<sup>1</sup> tumor necrosis factor- $\alpha$ , or IFN- $\gamma$  (10–12), can be cytotoxic to pancreatic  $\beta$ -cells, inhibiting insulin secretion. Furthermore, when combined, these cytokines can destroy  $\beta$ -cells (12). Moreover, these cytokines have been found in the pancreatic insulinitis lesion of NOD mice (13) and BB rats (14), and they may thus be considered mediators of  $\beta$ -cell damage in type 1 diabetes. Furthermore, transgenic mice expressing IFN- $\gamma$  in pancreatic  $\beta$ -cells show lymphocytic infiltration of the islets by mononuclear cells,  $\beta$ -cell destruction, and diabetes (15, 16). However, of all the cytokines that may be involved in the development of the diabetic process, only a few can be produced by normal epithelial cells, such as pancreatic  $\beta$ -cells. Type I interferons (IFNs) are pleiotropic cytokines involved in host defenses against viral infections that can be produced by most cell types in response to a virus (17). There are three families of type I IFNs,  $\alpha$ ,  $\beta$ , and  $\omega$ , that are closely related structurally. These IFNs also bind to a common receptor and have potent antiviral activities (17, 18). Several studies have implicated IFN- $\alpha$  in the development of type 1 diabetes. This cytokine can be detected in the islets of newly diagnosed patients of type 1 diabetes (19, 20). In addition, IFN- $\alpha$  can induce the expression of MHC class I antigens in pancreatic  $\beta$ -cells (21), and it can lead to diabetes when expressed in islets of transgenic mice (22). Similar to IFN- $\alpha$ , IFN- $\beta$  has immunomodulatory properties, it induces MHC class I antigen expression (23, 24), and it has also been detected in newly diagnosed patients of type 1 diabetes (19). However, little is known about the role of IFN- $\beta$  in the development of type 1 diabetes.

Here we studied whether IFN- $\beta$  may lead to type 1 diabetes. To this end, we developed transgenic mice expressing IFN- $\beta$  in  $\beta$ -cells. These mice presented impaired  $\beta$ -cell function, hypoinsulinemia, and altered glucose tolerance test, but they did not develop overt type 1 diabetes. However, transgenic mice treated with multiple low doses of streptozotocin or backcrossed to a CD-1 strain developed overt diabetes, with marked hyperglycemia and lymphocytic infiltration of the islets.

### EXPERIMENTAL PROCEDURES

**Construction of the RIP-I/IFN- $\beta$  Chimeric Gene and Generation of Transgenic Mice**—To obtain the RIP-I/IFN- $\beta$  chimeric gene a *SacI*-*BamHI* fragment (–570 bp to +3 bp) of the rat insulin I promoter

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<sup>1</sup> The abbreviations used are: IL, interleukin; IFN, interferon; MHC, major histocompatibility complex; RIP-I, rat insulin I promoter; kb, kilobase pair(s); bp, base pair(s); RT-PCR, reverse transcriptase-polymerase chain reaction; RIA, radioimmunoassay; MLDS, multiple low doses of streptozotocin.

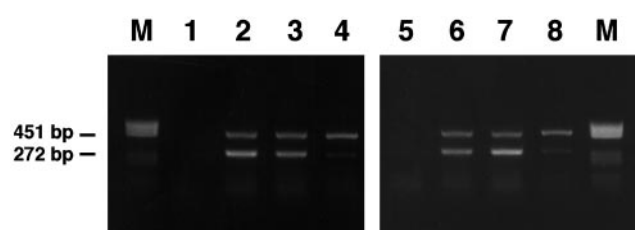
(RIP-I) (25) was linked to a *TaqI*-*TaqI* fragment (+302 bp to +1140 bp) of the human IFN- $\beta$  gene, which contains the entire coding sequence and the polyadenylation signal (26). The construction of the RIP-I/IFN- $\beta$  chimeric gene was initiated by subcloning the *SacI*-*Bam*HI fragment of the RIP-I at the same sites of the Bluescript<sup>R</sup> polylinker. Afterward, the *TaqI*-*TaqI* fragment of the IFN- $\beta$  was introduced at the *ClaI* site of the polylinker. A 1.3-kb *SacI*-*XhoI* fragment, containing the entire RIP-I/IFN- $\beta$  chimeric gene, was microinjected into fertilized mouse eggs from a C57Bl6/SJL background. The general procedures used for microinjection of the RIP-I/IFN- $\beta$  chimeric gene were as described elsewhere (27). At 3 weeks of age, the animals were tested for the presence of the transgene by Southern blot analysis of tail DNA (10  $\mu$ g) digested with *Bgl*II. The RIP-I/IFN- $\beta$  construct contains a unique *Bgl*II site at the end of the IFN- $\beta$  gene. Blots were hybridized with the 1.3-kb *SacI*-*XhoI* fragment containing the entire RIP-I/IFN- $\beta$  chimeric gene, radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech) by random oligopriming (Boehringer Mannheim, Germany). A 1.3-kb fragment was produced by the restriction of *Bgl*II at the site of an RIP-I/IFN- $\beta$  molecule and at the site of a second molecule of the chimeric gene, inserted in the genome in a head-to-tail manner (data not shown). In the experiments described below, heterozygous male mice aged 2 to 3 months were used. However, similar results were obtained in animals more than 6 months old. Transgenic mice expressing the chimeric gene RIP/IFN- $\beta$  with a C57Bl6/SJL genetic background were also back-crossed to CD-1 mice (Charles River), and N1 to N3 generations were analyzed.

Mice were fed *ad libitum* with a standard diet (Panlab, Barcelona, Spain) and kept under a light-dark cycle of 12 h (lights on at 8 a.m.). When stated, mice were fed high carbohydrate diet (ICN Biomedicals Inc., Cleveland, OH) and water *ad libitum* for 1 week. This diet contained 80.5% sucrose, 10.2% casein, 0.3% DL-methionine, 4% cottonseed oil, 2% brewers' yeast, and 2% AIN mineral mix plus 1% AIN vitamin mix. Animals were killed and samples were taken between 9 and 10 a.m. When stated, transgenic mice of both C57Bl6/SJL and CD-1 genetic background were given, on 5 consecutive days, an intraperitoneal injection of streptozotocin (40 mg/kg), dissolved in 0.1 M citrate buffer (pH 4.5) immediately before administration. Control mice were injected only with the citrate buffer. Diabetes was assessed by measuring blood glucose levels.

**Isolation of Pancreatic Islets**—Islets were isolated from the pancreas of control or transgenic mice fed a standard or a high carbohydrate diet. Islets were released from pancreatic acinar tissues by digestion with collagenase P (Boehringer Mannheim) (28). Islets were collected by handpicking under a dissection microscope. For *in vitro* studies, islets from control mice were cultured free-floating in groups of 150–200 in plastic Petri dishes containing RPMI 1640 medium with 5% fetal calf serum, maintained at 37 °C in an atmosphere of 5% CO<sub>2</sub> in air. Experiments were initiated 24 h after culture, by placing islets in RPMI 1640 with 5% fetal calf serum in the absence or in the presence of either murine (10<sup>3</sup> units/ml) or human (10<sup>6</sup> units/ml) IFN- $\beta$ . After 3 days of treatment with the cytokines, insulin secretion by these islets was determined.

**RNA Analysis**—To study the expression of the transgene, total RNA was obtained from pancreas by the guanidine isothiocyanate method (29), and RNA samples (30  $\mu$ g) were electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. Northern blot was hybridized to a <sup>32</sup>P-labeled 0.8-kb *EcoRI*-*XhoI* fragment of the IFN- $\beta$  gene. The  $\beta$ -actin probe corresponded to a 1.3-kb *EcoRI*-*EcoRI* fragment of the rabbit cDNA. To analyze the expression of the  $\beta_2$ -microglobulin, RNA was obtained from islets cultured in the presence of IFN- $\beta$  or from islets of transgenic mice using the acid guanidinium thiocyanate/phenol/chloroform procedure (30). RNA samples were treated with DNase for 30 min at 37 °C, extracted with a phenol/chloroform mixture, and precipitated with ethanol. cDNA synthesis was carried out using 50 ng of total RNA at 42 °C for 60 min in a reaction volume of 20  $\mu$ l. The cDNA was then used for PCR co-amplification of  $\beta_2$ -microglobulin and  $\beta$ -actin with the following temperature program: 30 s at 94 °C, 60 s at 60 °C, and 30 s at 72 °C for 40 cycles. The primer pair used for  $\beta_2$ -microglobulin (sense, 5'-GTATACTCACGGATCCACCGGAGA-3', and antisense 5'-CATGTCTCGATCCAGTAGACGG-3') yielded a 272-bp PCR product. The primer pair used for  $\beta$ -actin (sense 5'-CATCGTGGGCGCCCTAGGCAC-3', and antisense 5'-CCGGCCAGCCAGGTCCAGACGC-3') yielded a 451-bp PCR product. The co-amplified products were analyzed on a 2% agarose gel and visualized following staining with ethidium bromide.

**Protein Analysis**—Western blot analysis was performed by standard procedures (31) from total cellular lysates of islets. Islets were disrupted in 5% sodium dodecyl sulfate (SDS), 80 mM Tris-HCl (pH 6.8), 5



**FIG. 1. Determination of the expression of  $\beta_2$ -microglobulin in islets.** The expression levels of  $\beta_2$ -microglobulin (272 bp) and  $\beta$ -actin (451 bp) were determined by RT-PCR, as indicated under "Experimental Procedures." M, Marker V (Boehringer Mannheim); lanes 1 and 5, negative control of the PCR reaction; lanes 2 and 3, RT-PCR products from islets cultured in the presence of murine or human IFN- $\beta$ , respectively; lane 4, RT-PCR product from islets cultured in the absence of IFN- $\beta$ ; lanes 6 and 7, RT-PCR products from islets of transgenic mice, lines IFN- $\beta$ 28 (Tg1) and IFN- $\beta$ 56 (Tg2), respectively; lane 8, RT-PCR product from islets of control mice.

mm EDTA, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride by sonication. Twenty  $\mu$ g of protein was electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. To detect IFN- $\beta$  a rabbit antiserum to human IFN- $\beta$  (Biogenesis, Bournemouth, UK), diluted at 1:1000, was used.

**Histologic Analysis**—For immunohistochemical detection of IFN- $\beta$  and insulin, sections of pancreas from control and transgenic mice were fixed for 12 to 24 h in Carnoy's reagent, embedded in paraffin, and sectioned. Sections were then incubated with a rabbit anti-human IFN- $\beta$  antibody (Biogenesis), diluted at 1:100, or with a guinea pig anti-porcine insulin antibody (Dako, Carpinteria, CA), at 1:100 dilution, overnight at 4 °C. As secondary antibodies goat anti-rabbit or rabbit anti-guinea pig immunoglobulin G coupled to peroxidase (Boehringer Mannheim) was used. The 3',3'-diaminobenzidine was used as the substrate chromogen. Sections were counterstained in Mayer's hematoxylin. For histologic analysis, sections of pancreas were stained with hematoxylin/eosin.

**Insulin Secretion from Islets**—Batches of six islets were incubated in a shaking water bath for 90 min at 37 °C in 1 ml of bicarbonate-buffered salt solution containing bovine serum albumin (5 mg/ml, fraction V, Sigma) supplemented with 2.8 or 16.7 mM glucose, with 2.8 mM glucose together with either 10 mM leucine plus 10 mM glutamine or 10 mM arginine, or with 16.7 mM glucose plus 5  $\mu$ M forskolin. At the beginning of the treatments, vials were gassed with O<sub>2</sub>-CO<sub>2</sub> (95:5%) for 10 min. At the end of the incubation period supernatants were stored at -20 °C until the insulin was assayed by RIA (CIS, Biointernational, Gif-Sur-Yvette, France). The method allows the determination of 2.5 milliunits of insulin/ml, with a coefficient of variation within and between assays of 6 and 8%, respectively.

**cAMP Measurements**—For cAMP measurements, islets were incubated in groups of 30 for 15 min at 37 °C in Hanks' medium (136 mM NaCl, 1.67 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 5.4 mM KCl, 0.35 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.45 mM KH<sub>2</sub>PO<sub>4</sub>, and 4.2 mM NaHCO<sub>3</sub>) containing 2.8 and 16.7 mM glucose or 16.7 mM glucose plus 5  $\mu$ M forskolin. The incubation was stopped by adding 6% trichloroacetic acid, and islets were frozen and stored at -80 °C, pending analysis. The samples were thawed by sonication on ice and centrifuged at 2000  $\times$  g (4 °C) for 15 min. The pellet was re-sonicated in 200  $\mu$ l of water and used to measure islet cell DNA content. The supernatant was washed 4 times in 5 volumes of water-saturated diethyl ether. The aqueous extract was freeze-dried, and the cAMP content was measured by RIA (using <sup>125</sup>I-cAMP) as described by the manufacturer of the assay kit (Amersham Pharmacia Biotech). In order to increase the sensitivity of the method, samples were acetylated.

**Hormone and Metabolite Assays**—Insulin levels in serum samples were determined by RIA (CIS, Biointernational, Gif-Sur-Yvette, France). Serum glucose concentration was measured enzymatically (Glucosequant<sup>R</sup>, Boehringer Mannheim). Glucose levels were also determined in blood by using a Glucometer<sup>R</sup> analyzer (Bayer, Germany). Serum triglycerides were determined enzymatically (GPO-PAP, Boehringer Mannheim). The intraperitoneal glucose tolerance test was performed between 10 and 11 a.m. in fed control and transgenic mice. After anesthetizing the mice with avertin, a blood sample was obtained from the tail vein to measure the basal level of glucose. Mice were subsequently given an intraperitoneal injection of 1 mg of glucose per g of body weight. Blood samples (5  $\mu$ l) were obtained at different times from the same animals, and the levels of glucose were determined.



**Statistical Analysis**—All values are expressed as the mean  $\pm$  1 S.E. Statistical analysis was carried out using the Student-Newmann-Keuls test. Differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

**Alteration of Glucose-stimulated Insulin Secretion by Islets Cultured in the Presence of IFN- $\beta$** —In this study we examined the role of IFN- $\beta$  in the diabetic process. Since alteration in  $\beta$ -cell function might precede the development of type 1 diabetes, we first analyzed the effect of this cytokine *in vitro*. Mouse islets were cultured in the presence of either mouse ( $10^3$  units/ml) or human ( $10^6$  units/ml) IFN- $\beta$  to determine the effect of these cytokines on insulin secretion. The concentration of the human IFN- $\beta$  used was 1000 times that of the mouse to overcome the species specificity (32). Neither mouse nor human IFN- $\beta$  caused morphologic changes in the islets at the concentrations used (data not shown). To determine the biologic activity of IFN- $\beta$  in the pancreatic islets, the expression of  $\beta_2$ -microglobulin was analyzed by RT-PCR in islets treated with either mouse or human IFN- $\beta$ . The  $\beta_2$ -microglobulin and MHC class I heavy chains complex at the cell surface to form the intact MHC class I antigen (33), and their expression is often coordinately regulated (34). Both mouse and human IFN- $\beta$  induced the expression of  $\beta_2$ -microglobulin after 3 days of culture, while islets cultured in the absence of IFNs showed very

low levels of  $\beta_2$ -microglobulin mRNA (Fig. 1), suggesting an up-regulation of MHC class I antigen by IFN- $\beta$ . The effect of the islet exposure to IFN- $\beta$  on glucose-induced insulin secretion was next studied. Islets cultured for 3 days in the presence of either mouse or human cytokine were incubated for 90 min in the presence of 2.8 or 16.7 mM glucose. At low glucose concentration, insulin levels in the incubation medium of islets treated with IFN- $\beta$  were lower than those of non-treated islets (Table I). When control islets were incubated in the presence of 16.7 mM glucose, a 3-fold increase in insulin secretion was noted. Although islets treated with either mouse or human IFN- $\beta$  showed an increase in insulin secretion at high glucose, a marked reduction (about 50 and 40%, respectively) of the insulin concentration in the incubation medium of these islets compared with non-treated islets was detected (Table I). When

TABLE I  
Effect of IFN- $\beta$  in pancreatic islets insulin secretion

Islets were cultured for 3 days with either  $10^3$  units/ml murine (m) or  $10^6$  units/ml human (h) IFN  $\beta$  (mIFN- $\beta$  or hIFN- $\beta$ ). Insulin secretion at 2.8 and 16.7 mM glucose and 16.7 mM glucose plus 5  $\mu$ M forskolin was determined, as indicated under "Experimental Procedures." Results are the mean  $\pm$  S.E. of four independent experiments.

Culture treatment	Insulin released (ng/islet/90 min)		
	2.8 mM glucose	16.7 mM glucose	16.7 mM glucose + 5 $\mu$ M forskolin
No treatment	1.4 $\pm$ 0.1	4.1 $\pm$ 0.5	17.4 $\pm$ 1.4
mIFN- $\beta$	0.9 $\pm$ 0.1 <sup>a</sup>	2.1 $\pm$ 0.4 <sup>a</sup>	7.1 $\pm$ 1.0 <sup>b</sup>
hIFN- $\beta$	1.0 $\pm$ 0.1 <sup>a</sup>	2.7 $\pm$ 0.4 <sup>a</sup>	7.1 $\pm$ 0.8 <sup>b</sup>

<sup>a</sup>  $p < 0.05$  versus nontreated islets.

<sup>b</sup>  $p < 0.01$  versus nontreated islets.

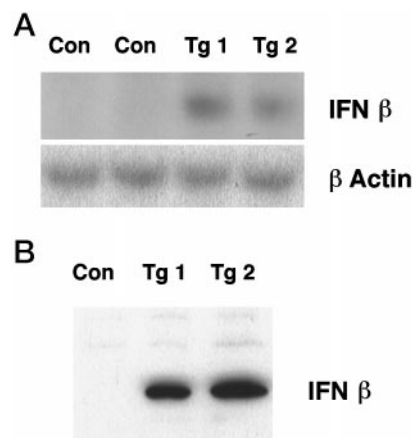


FIG. 2. **Expression of IFN- $\beta$  in  $\beta$ -cells of transgenic mice.** A, total cellular RNA was obtained from the pancreas of control (Con) and transgenic mice from the lines IFN- $\beta$ 28 (Tg1) and IFN- $\beta$ 56 (Tg2) and analyzed by Northern blot as indicated under "Experimental Procedures." B, Western blot analysis was performed from total cellular lysates of islets. Twenty  $\mu$ g of protein was electrophoresed on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. To detect IFN- $\beta$  a rabbit antiserum to human IFN- $\beta$  (Biogenesis) diluted 1:1000 was used.

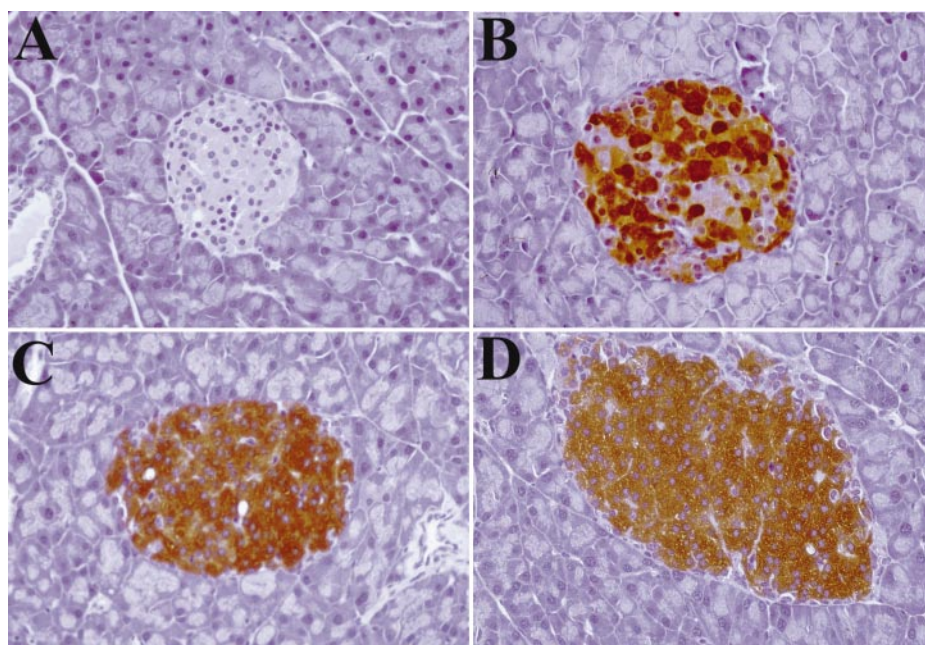


FIG. 3. **Immunohistochemical analysis of IFN- $\beta$  and insulin expression in islets from control and transgenic mice.** A and C, pancreas from normal mice (magnification, 400  $\times$ ); B and D, pancreas from transgenic mice (magnification, 400  $\times$ ) stained for IFN- $\beta$  (A and B) and for insulin (C and D).

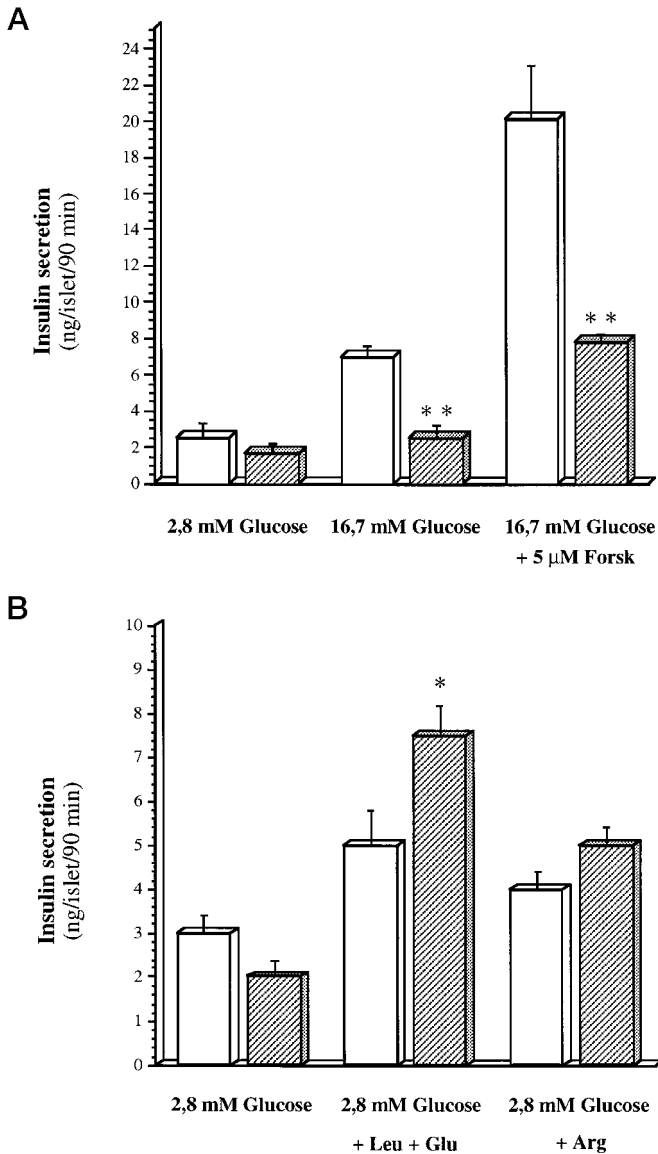


FIG. 4. A, glucose- and forskolin-induced insulin secretion. Insulin secretion at 2.8 and 16.7 mM glucose or at 16.7 mM glucose plus 5  $\mu$ M forskolin was determined, as indicated under "Experimental Procedures," in islets isolated from control (□) and transgenic (▨) mice. B, amino acid-induced insulin release. Insulin secretion at 2.8 mM glucose and 10 mM leucine plus 10 mM glutamine or at 2.8 mM glucose plus 10 mM arginine was determined in islets isolated from control and transgenic mice. Results are the mean  $\pm$  S.E. of six animals in each group. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (versus islets from control mice).

islets were incubated in the presence of 16.7 mM glucose plus 5  $\mu$ M forskolin, an adenylate cyclase activator, non-cytokine-treated islets showed a 4-fold increase in the insulin release over control islets incubated with glucose alone (Table I). In contrast, when incubated with glucose plus forskolin the level of insulin in the incubation medium of IFN- $\beta$ -treated islets was 60% lower than that of control islets (Table I). Thus, these results indicated that IFN- $\beta$  was able to alter  $\beta$ -cell function *in vitro*.

**Expression of IFN- $\beta$  in Pancreatic  $\beta$ -Cells of Transgenic Mice**—To study the potential role of IFN- $\beta$  in the pathogenesis of type 1 diabetes, we developed a transgenic animal model that expresses IFN- $\beta$  specifically in pancreatic  $\beta$ -cells. IFNs are species-specific. However, to avoid the male sterility observed in mice expressing high levels of mouse IFN- $\beta$  (35), the use of the human IFN- $\beta$  gene was considered to be more ap-

TABLE II  
cAMP content in the islets (fmol cAMP/ $\mu$ g DNA)

The concentration of cAMP at 2.8 and 16.7 mM glucose or at 16.7 mM glucose plus 5  $\mu$ M forskolin was determined in islets isolated from control and transgenic mice, as described under "Experimental Procedures." Results are the mean  $\pm$  S.E. of two independent experiments performed in triplicate.

	2.8 mM glucose	16.7 mM glucose	16.7 mM glucose + 5 $\mu$ M forskolin
Control	163 $\pm$ 15	277 $\pm$ 22	1993 $\pm$ 257
RIP/IFN- $\beta$	124 $\pm$ 11 <sup>b</sup>	153 $\pm$ 17 <sup>a</sup>	1152 $\pm$ 127 <sup>b</sup>

<sup>a</sup>  $p < 0.05$  versus islets from control mice.

<sup>b</sup>  $p < 0.01$  versus islets from control mice.

TABLE III  
Serum parameters in transgenic mice expressing the RIP/IFN- $\beta$  chimeric gene

The levels of glucose, insulin, and triglycerides were determined as indicated under "Experimental Procedures." Results are mean  $\pm$  S.E. of 15 animals in each group. \* $p < 0.05$ ; \*\* $p < 0.01$  (versus control mice).

	Control	Transgenic
Glucose (mg/dl)	155 $\pm$ 10	205 $\pm$ 12 <sup>a</sup>
Insulin (ng/dl)	1.3 $\pm$ 0.1	0.8 $\pm$ 0.2 <sup>a</sup>
Triglycerides (mg/dl)	120 $\pm$ 13	210 $\pm$ 16 <sup>b</sup>

<sup>a</sup>  $p < 0.05$  versus control mice.

<sup>b</sup>  $p < 0.01$  versus control mice.

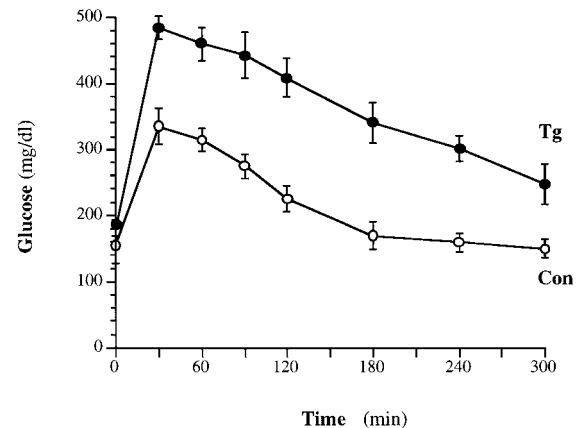


FIG. 5. **Intraperitoneal glucose tolerance test.** Transgenic (Tg) and control (Con) mice were given an intraperitoneal injection of 1 mg of glucose per g of body weight. Blood samples were taken, at the times indicated, from the tail vein of the same animals. Glucose was determined as indicated under "Experimental Procedures." Results are means  $\pm$  S.E. of eight transgenic and eight control mice.

propriate in transgenic mice. Human IFN- $\beta$  is active in mouse cells (36–39), although its efficacy was about 1000 times lower than that of mouse IFN- $\beta$ . Furthermore, here we showed that human IFN- $\beta$  exerted a clear biologic effect in mouse islets, suggesting that it might be used instead of mouse cytokine. Thus, transgenic animals that expressed human IFN- $\beta$  in  $\beta$ -cells, under control of the rat insulin-I promoter (RIP-I/IFN- $\beta$ ), were developed to examine the effect of IFN- $\beta$  *in vivo*. The RIP-I/IFN- $\beta$  chimeric gene was microinjected into fertilized eggs, and four transgenic mice were obtained. In this study, we used F1 and F2 heterozygote mice from the transgenic lines RIP-I/IFN- $\beta$ 28 (TgIFN- $\beta$ -28) and RIP-I/IFN- $\beta$ 56 (TgIFN- $\beta$ -56). TgIFN- $\beta$ -28 and TgIFN- $\beta$ -56 carried about 5 and 20 intact copies, respectively, of the RIP-I/IFN- $\beta$  chimeric gene when analyzed by Southern blot (data not shown). We used littermates as controls for the transgenic animals. A transcript that hybridized with the IFN- $\beta$  probe was detected when total RNA obtained from the pancreas of transgenic mice was analyzed by Northern blot (Fig. 2A). Although these lines of transgenic mice



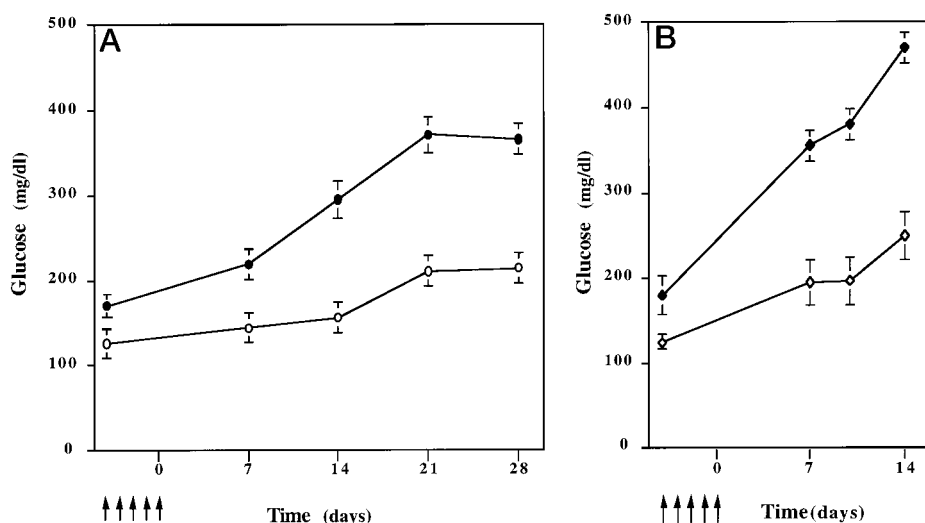


FIG. 6. **Blood glucose levels following treatment with multiple low doses of streptozotocin.** *A*, control (open circle) or transgenic (closed circle) mice, in a C57Bl6/SJL background. *B*, control (open diamonds) or transgenic (closed diamonds) mice, N1 generation obtained from the first back-cross of C57Bl6/SJL mice to CD-1 mice. Arrows indicate the streptozotocin injections (40 mg/kg) for 5 consecutive days. Blood samples were taken, at the times indicated, from the tail vein of the animals. Glucose was determined as indicated under "Experimental Procedures." Results are mean  $\pm$  S.E. of eight transgenic and eight control mice of both strains.

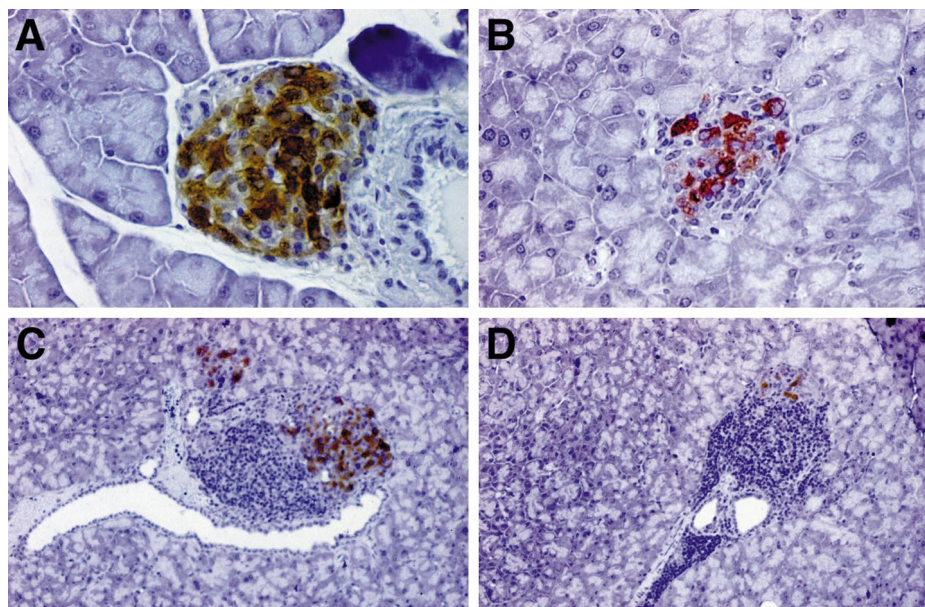
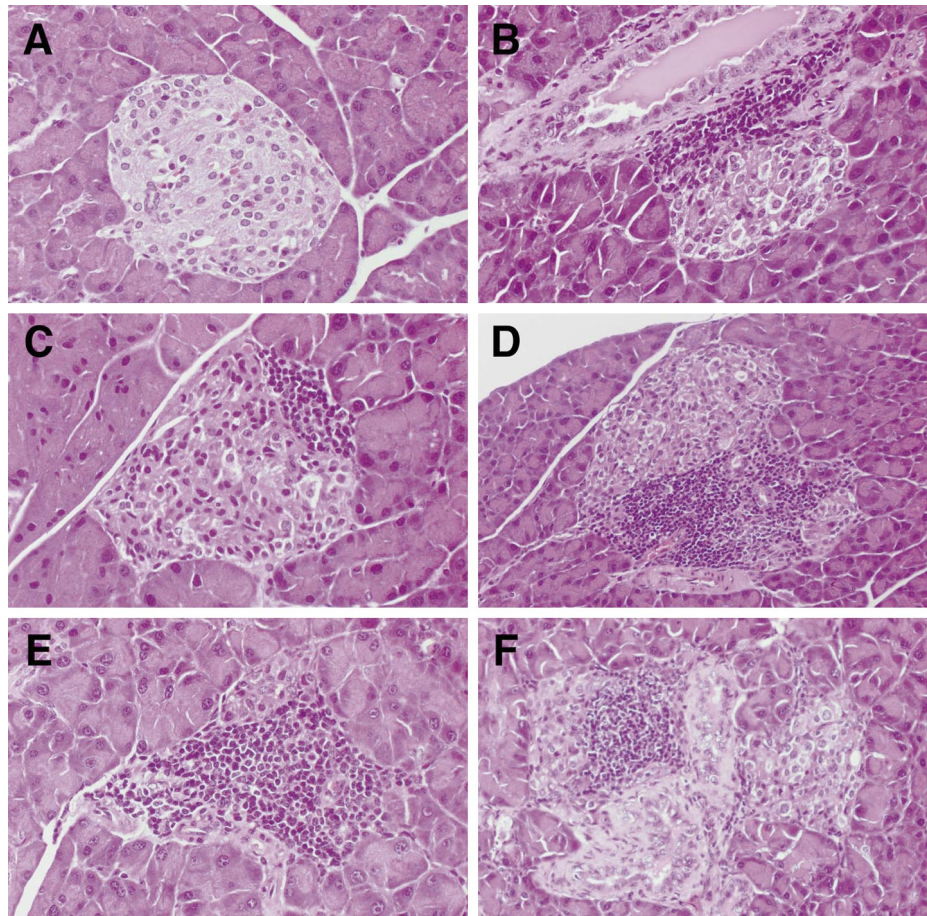


FIG. 7. **Immunohistochemical analysis of insulin expression in islets from C57Bl6/SJL mice 28 days after MLDS treatment.** *A*, islet from control mice (magnification, 400  $\times$ ); *B*, islet from transgenic mice, which showed a marked decrease in insulin-producing cells (magnification, 400  $\times$ ); *C* and *D*, islets from transgenic mice showing mononuclear infiltration (magnification, 400  $\times$ ).

had integrated different number of copies of the transgene, similar levels of IFN- $\beta$  mRNA were noted in the pancreas of both TgIFN- $\beta$ -28 and TgIFN- $\beta$ -56, probably as a result of the site of integration in the genome. Nevertheless, no IFN- $\beta$  mRNA was detected in the pancreas from control animals (Fig. 2A). No expression of the transgene was detected in other tissues examined, like liver and kidney (data not shown). Mice were fed a high carbohydrate diet for 1 week, to induce the expression of the transgene, and afterward islets were obtained and IFN- $\beta$  protein was analyzed by Western blot. The expression of IFN- $\beta$  was parallel to the presence of IFN- $\beta$  protein in islets from transgenic mice, whereas no immunodetectable IFN- $\beta$  was noted in control islets (Fig. 2B). Similarly, after immunohistochemical analysis human IFN- $\beta$  was also detected in insulin-producing cells of islets from transgenic mice fed a standard diet (Fig. 3B). However, the expression of IFN- $\beta$  protein was variegated, probably resulting from differences in the

level of expression of the transgene in the various  $\beta$ -cells within the islet. Similar results were obtained in both TgIFN- $\beta$ -28 and TgIFN- $\beta$ -56 mice. No IFN- $\beta$  immunostaining was observed in islets from control mice (Fig. 3A). Islets from both control and transgenic mice showed similar insulin immunoreactivity (Fig. 3, C and D). All these results indicate that high levels of IFN- $\beta$  were produced in the  $\beta$ -cells of transgenic mice. The expression of the transgene did not cause islet lesions, even in older mice (data not shown).

**Expression of IFN- $\beta$  in Islets Led to an Increase in  $\beta_2$ -Microglobulin**—To examine whether human IFN- $\beta$  was biologically active in the islets of the transgenic mice, the expression of  $\beta_2$ -microglobulin was determined in islets from control and transgenic mice by RT-PCR. The levels of  $\beta_2$ -microglobulin mRNA in islets from control mice were very low. However, islets from both TgIFN- $\beta$ -28 and TgIFN- $\beta$ -56 showed a high induction (about 5-fold) in the expression of  $\beta_2$ -microglobulin



**FIG. 8. Pancreatic sections from 12-week-old N3 generation back-crossed to CD-1.** *A*, islet from control mice (magnification, 400  $\times$ ); *B–F*, islets from transgenic mice. *B*, disorganization of the islet with peri-insulitis can be observed (magnification, 400  $\times$ ); *C*, magnification, 400  $\times$ ; *D*, islets show different degree of mononuclear infiltration (magnification, 200  $\times$ ); *E*, magnification, 400  $\times$ ; *F*, insulitis with total destruction of the islets can be observed (magnification, 200  $\times$ ).

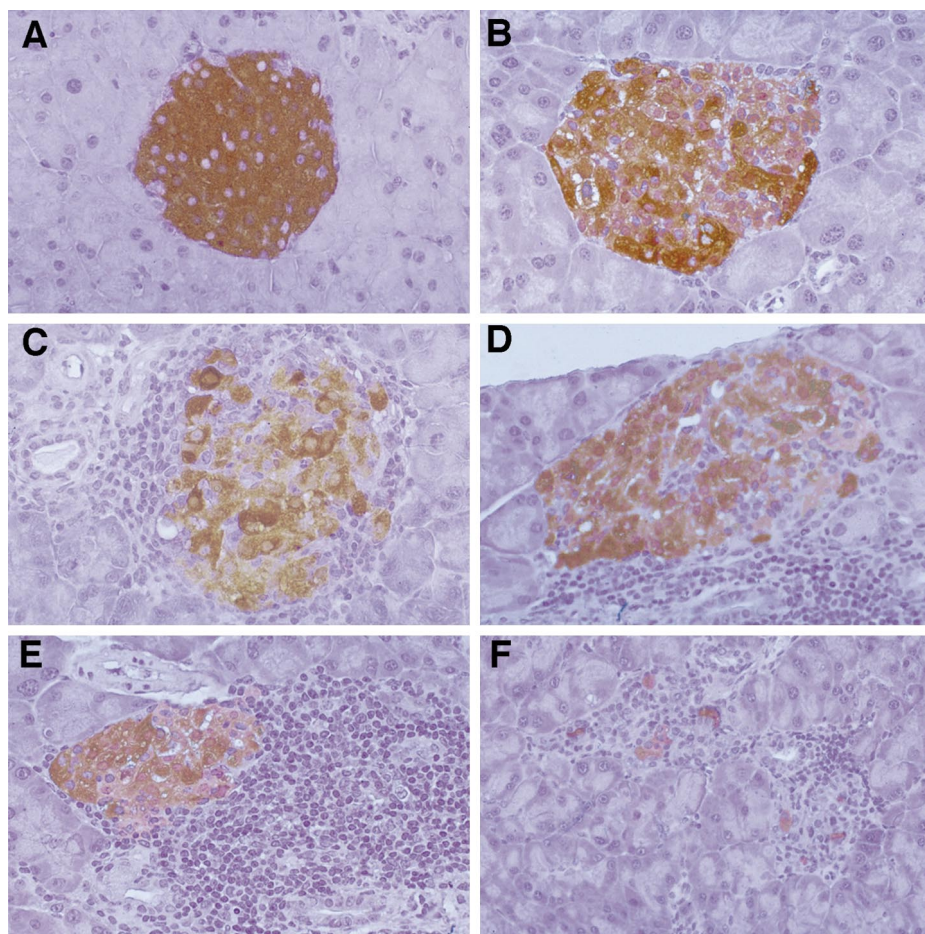
mRNA (Fig. 1). These results suggested that human IFN- $\beta$  produced by the  $\beta$ -cells might induce MHC class I antigen in islets of transgenic mice. The results described below were obtained from the TgIFN- $\beta$ -28 line. However, a similar phenotype was observed in the TgIFN- $\beta$ -56 animals.

**IFN- $\beta$  Expression in Islets Altered  $\beta$ -Cell Function**—To discern whether the glucose-stimulated insulin secretion in  $\beta$ -cells from the transgenic mice was altered, islets from control and transgenic mice were isolated, and the insulin release in response to 2.8 and 16.7 mM glucose was measured (Fig. 4A). Although at both glucose concentrations islets from transgenic mice secreted less insulin than islets from non-transgenic animals, the reduction was higher (about 60%) at 16.7 mM glucose. The concentration of insulin in the medium of islets from transgenic animals incubated with 16.7 mM glucose was similar to that released by islets from control animals incubated with 2.8 mM glucose (Fig. 4A). When amino acid-stimulated insulin secretion was studied no decrease was detected in islets from transgenic mice compared with control mice. Isolated islets cultured in the presence of 2.8 mM glucose together with 10 mM leucine plus 10 mM glutamine or with 10 mM arginine, released more insulin (about 50 and 25% increase, respectively) than islets from control animals (Fig. 4B). Thus, stimulation with leucine plus glutamine and with arginine counteracted the IFN- $\beta$ -induced reduction of glucose-stimulated insulin secretion. When insulin secretion was studied in islets incubated in the presence of 16.7 mM glucose plus 5  $\mu$ M forskolin, islets from control mice showed a 3-fold increase in the insulin release compared with control islets incubated only in the presence of

glucose (Fig. 4A). A similar induction in insulin secretion (about 3-fold) was also observed when transgenic islets were incubated with forskolin and 16.7 mM glucose. However, the level of insulin in the incubation medium of islets from transgenic mice expressing IFN- $\beta$  was 60% lower than that of islets from control mice (Fig. 4A). Furthermore, islets from transgenic mice expressing IFN- $\beta$  showed a decrease in the cAMP content when incubated with low or high glucose concentration (Table II). This suggests that IFN- $\beta$  might either exert a direct negative effect on cAMP production or interfere with the effects of glucose on cAMP production in the  $\beta$ -cells. Incubation of islets from control mice with glucose plus forskolin led to an increase (about 7-fold) in cAMP content. Although a similar increase was noted in islets from transgenic mice, the levels of cAMP reached were 40% lower than those detected in control islets (Table II).

**Transgenic Mice Expressing IFN- $\beta$  Developed a Prediabetic State**—Transgenic mice expressing the RIP/IFN- $\beta$  chimeric gene were mildly hyperglycemic and showed a lower serum concentration of insulin than control mice (about 40% reduction) and a 2-fold increase in serum triglycerides (Table III). In addition, high levels of blood glucose were detected in transgenic mice when intraperitoneal glucose tolerance tests were performed. In contrast to control mice, the glucose concentration reached in transgenic animals had not returned to basal values 180 min after glucose administration (Fig. 5). The alterations in the serum parameters and the impaired response to an intraperitoneal glucose tolerance test suggested that trans-





**FIG. 9. Immunohistochemical analysis of insulin expression of pancreatic sections from 12-week-old N3 generation back-crossed to CD-1.** A, islet from control mice (magnification, 400  $\times$ ); B–F, islets from transgenic mice. B, islet shows a decrease in the number of insulin-producing cells without insulinitis (magnification, 400  $\times$ ); C–E, magnification, 400  $\times$ ; F, different degree of mononuclear infiltration of islets and islet destruction can be observed (magnification, 200  $\times$ ).

genic mice expressing the RIP/IFN- $\beta$  chimeric gene had developed a prediabetic state.

**Treatment of Transgenic Mice with Multiple Low Doses of Streptozotocin Led to Type 1 Diabetes**—Streptozotocin is a toxin that has direct cytotoxic effects on  $\beta$ -cells when injected at high doses. In contrast, multiple low doses of streptozotocin (MLDS) lead to inflammatory autoimmune-mediated destruction of  $\beta$ -cells (40). Certain strains of mice are highly susceptible to MLDS, such as outbred CD-1 mice, whereas others, such as C57Bl6/SJL mice, are resistant and only exhibit a mild increase in blood glucose levels without insulinitis. Transgenic mice were treated with MLDS, to maximize the autoimmune response and minimize the direct toxicity of this drug on the  $\beta$ -cells. Daily injections (intraperitoneal) of 40 mg/kg streptozotocin for 5 consecutive days were performed, and blood samples were taken from the tail vein at 7, 14, 21, and 28 days after MLDS treatment. Transgenic mice expressing the RIP-I/IFN- $\beta$  chimeric gene reached blood glucose levels characteristic of diabetes 14 days after treatment, whereas control mice presented normal levels of serum glucose (Fig. 6A). Immunohistochemical analysis of the pancreas of control mice, following 28 days of treatment, revealed that islets had a slightly lower number of insulin-containing cells, but they did not show any inflammatory infiltration (Fig. 7A). In contrast, not only did islets from transgenic mice show clearly reduced numbers of insulin-containing cells (Fig. 7B) but also 50% of the animals presented insulinitis leading to the destruction of the islets (Fig. 7, C and D).

**Influence of the Genetic Background in IFN- $\beta$ -mediated Di-**

**abetic Process**—To assess the extent to which genetic factors could be involved in the development of the disease, C57Bl6/SJL transgenic mice expressing the RIP/IFN- $\beta$  chimeric gene were back-crossed to CD-1 mice. The aim of this back-cross was to obtain transgenic mice with the genetic background of CD-1 mice, which are more susceptible to develop insulinitis. N1, N2, and N3 generations were obtained. N1 transgenic mice showed a similar phenotype to C57Bl6/SJL mice, *i.e.* hypoinsulinemia, mild hyperglycemia, and an altered glucose tolerance test (data not shown), without progression to overt diabetes. In contrast, when treated with MLDS, N1 transgenic mice presented higher glycemia than MLDS-treated C57Bl6/SJL transgenic mice (Fig. 6B), reaching values characteristic of diabetes 7 days after MLDS injection (Fig. 6B). In contrast, control N1 mice maintained normal values of glycemia at that time, while a slight increase was noted around day 14 after treatment, although glucose levels were always significantly lower than those observed in N1 transgenic mice (Fig. 6B). Histologic analysis of the pancreas showed that 100% of the MLDS-treated N1 transgenic mice developed insulinitis, whereas none of the control mice had histologic alterations.

In contrast to N2 control mice, N2 transgenic mice also showed hypoinsulinemia, mild hyperglycemia, and an altered glucose tolerance test (data not shown), and about 3% of N2 transgenic mice presented increased blood glucose levels (about 300 mg/dl) and showed peri-insular inflammatory infiltration of the islets (data not shown). Furthermore, although N3 control mice were healthy, about 9% of N3 transgenic mice (5 out of 54 animals analyzed) spontaneously developed a diabetic

process at 12 weeks of age, showing polydipsia and polyuria characteristic of type 1 diabetes as well as marked glucosuria. In addition, they showed blood glucose levels above 400 mg/dl and strong hypoinsulinemia (serum insulin levels under 0.2 ng/ml). None of the control mice developed type 1 diabetes. Histologic analysis of the pancreas of N3 diabetic transgenic mice revealed inflammatory infiltration of the islets (Fig. 8, A–F). Fewer insulin-producing cells were detected as the islet lymphocytic infiltration progressed (Fig. 9, A–F).

#### DISCUSSION

The early stages of type 1 diabetes are characterized by a selective inability to secrete insulin in response to glucose, coupled to a better response to other secretagogues (10). The deficient glucose response may be a result of the autoimmune process directed toward the  $\beta$ -cells. Several cytokines may be mediators of immunologic damage of the  $\beta$ -cells, and culture of pancreatic islets in the presence of these cytokines results in alterations in their function (11, 12, 41). In this study we showed that IFN- $\beta$  may alter pancreatic  $\beta$ -cell function both *in vitro* and *in vivo*. Results obtained in islets cultured in the presence of IFN- $\beta$  or islets from transgenic mice expressing the RIP/IFN- $\beta$  chimeric gene suggest that IFN- $\beta$  might alter the signals that lead to an increase in insulin secretion in response to glucose. Similarly, a decrease in the insulin release was noted after incubation of both rat insulinoma cells and rat pancreatic islets with rat IFN- $\alpha$  (42). This decrease was correlated with the concentration and the time of incubation with the cytokine (42). However, while isolated mouse pancreatic islets incubated with either IFN- $\alpha$  or IFN- $\beta$  show 50% inhibition of glucose-stimulated (pro)insulin biosynthesis (43), no change in insulin secretion was detected, probably as a result of the time of incubation and the concentration of the cytokines used. Similarly, IL-1 $\beta$  markedly suppresses glucose-stimulated insulin secretion (10, 41). In contrast, we found that incubation of islets from transgenic mice with leucine plus glutamine or with arginine counteracted the IFN- $\beta$  reduction of glucose-stimulated insulin secretion. It is conceivable that a shift in islet metabolism, favoring amino acid oxidation and decreasing glucose metabolism, might be part of the metabolic responses of the  $\beta$ -cells to an increase in IFN- $\beta$  expression. Similar results have been reported in islets cultured in the presence of IL-1 $\beta$  (10). Exposure to IL-1 $\beta$  led to a marked reduction of glucose-stimulated insulin secretion, but treatment with arginine plus different glucose concentrations and leucine plus glutamine counteracted the IL-1 $\beta$ -induced reduction of insulin release (10).

Glucose-stimulated insulin secretion is also subject to stimulatory and inhibitory modulation by hormones and neurotransmitters (44). Glucagon or glucagon-like peptide-1 (GLP-1) activates adenylate cyclase, leading to an increase in the cellular cAMP concentration. However, the role of cAMP in insulin secretion is not a primary one. Protein kinase A activation potentiates, but does not initiate, insulin secretion (45). After glucose plus forskolin incubation, a marked reduction in insulin release and cAMP concentration was detected in islets cultured in the presence of IFN- $\beta$  and islets from transgenic mice expressing IFN- $\beta$  compared with islets from control mice not treated with the cytokine. Similar inhibitory effects have been described in islets incubated with IL-1 $\beta$  (41, 46). These findings suggest that both glucose- and cAMP-mediated steps (or glucose potentiation of cAMP-mediated steps) in insulin secretion are targets for the action of IFN- $\beta$  or IL-1 $\beta$ .

The alterations in pancreatic  $\beta$ -cell function resulting from the local expression of IFN- $\beta$  led to changes in serum parameters in the transgenic mice. They showed mild hyperglycemia and hypoinsulinemia, and also altered glucose tolerance test.

Moreover, IFN- $\alpha$  injected into healthy subjects led to an impairment of glucose tolerance (47). These findings suggest that transgenic mice expressing IFN- $\beta$  developed a prediabetic state, without progression to type 1 diabetes. Similarly, no incidence of diabetes was detected in transgenic mice expressing IFN- $\alpha$  in  $\beta$ -cells with a C57Bl/6 background (22). However, IFN- $\alpha$  transgenic mice back-crossed to CD1 mice became diabetic with a cumulative incidence of more than 50% (22). An additional factor or a more susceptible genetic background might be required to induce type 1 diabetes in transgenic mice expressing IFN- $\beta$ . In this regard, although in a small percentage (9%), IFN- $\beta$  transgenic mice with a CD-1 genetic background spontaneously developed overt diabetes. This low percentage might result from the fact that the  $\beta$ -cells from these transgenic mice produce human instead of mouse IFN- $\beta$ , and the human cytokine might be less effective in inducing the immune response. However, results obtained in MLDS-treated transgenic mice indicated that IFN- $\beta$  expression exacerbated the toxic effect of streptozotocin and led to type 1 diabetes. Expression of IFN- $\beta$  in islets of transgenic mice might have a synergistic action with other cytokines, since islet expression of IFN- $\alpha$ , IL-6, and tumor necrosis factor- $\alpha$  has been observed after MLDS treatment (48). Similarly, IL-1 administration promotes MLDS-induced insulinitis in strains of mice resistant to the effects of this treatment (49). Furthermore, it has been reported that one line of transgenic mice expressing IFN- $\alpha$  in the pancreas at levels sufficient to induce mild insulinitis, but insufficient to induce type 1 diabetes, show hyperglycemia when treated with MLDS (48), indicating that IFN- $\alpha$  potentiates the effects of streptozotocin in islets. Moreover, simultaneous treatment with MLDS and the type I IFN inducer poly(I/C) exacerbates MLDS-induced insulinitis in mice (48). In addition, poly(I/C) treatment has a diabetogenic effect in BB rats, either by inducing the development of diabetes in the diabetes-resistant BB strain or by accelerating the onset of diabetes in the diabetes-prone strain (48).

All the findings reported here indicate that IFN- $\beta$  may be directly involved in the pathogenesis of type 1 diabetes. Thus, undesirable effects of long term treatment with IFN- $\beta$  to counteract other diseases (like chronic hepatitis or multiple sclerosis) cannot be ruled out. In this regard, it has been reported that long term therapeutic use of type I IFNs induces autoimmunity (50–52) as well as the appearance of type 1 diabetes after treatment with type I IFNs (53–55), supporting the hypothesis that both IFN- $\alpha$  and IFN- $\beta$  might lead to diabetes mellitus.

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