β-Cell Destruction in NOD Mice Correlates With Fas (CD95) Expression on β-Cells and Proinflammatory Cytokine Expression in Islets

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A mechanism of autoimmune destruction of islet β-cells in type 1 diabetes has been proposed to be the binding of Fas ligand (FasL) on T-cells to Fas receptors on β-cells. We investigated this proposal by examining the expression of FasL and Fas on islet-infiltrating T-cells and β-cells in relation to β-cell destruction in a syngeneic islet transplant model in NOD mice. Diabetic NOD mice were transplanted with syngeneic islets and injected with complete Freund's adjuvant, which prevented diabetes recurrence (nondestructive insulitis), and with phosphate-buffered saline, which did not (β-cell destructive insulitis). Two-color immunohistochemical assays revealed that FasL was expressed on $CD4^+$ T-cells, $CD8^+$ T-cells, and β-cells in islet grafts from both diabetic and normoglycemic mice, and the percentage of each type of cell that expressed FasL was greater in islet grafts from normoglycemic compared with diabetic mice. In contrast, Fas was expressed on $CD4^+$ T-cells, $CD8^+$ T-cells, and β-cells in islet grafts from diabetic mice, but it was nearly or totally absent on these cells in islet grafts from normoglycemic mice. Similarly, polymerase chain reaction analysis of islet grafts revealed that Fas mRNA expression was significantly lower in islet grafts from normoglycemic compared with diabetic mice. Also, mRNA levels of interleukin (IL)-1α, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ were significantly lower in islet grafts from normoglycemic mice. Finally, Fas was induced on NOD islet cells by incubation with IL-1β, IFN-γ, and the combination of IL-1β, TNF-α, and IFN-γ. These findings support the concept that cytokine-induced Fas receptor expression on islet β-cells is a mechanism for their destruction by FasL-expressing $CD4^+$ and $CD8^+$ T-cells and, possibly, by FasL-expressing β-cells themselves. *Diabetes* 48:21–28, 1999

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AEC, 3-amino 9-ethylcarbazole; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; FACS, fluorescence-activated cell sorter; FasL, Fas ligand; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IFN, interferon; Ig, immunoglobulin; IL, interleukin; mAb, monoclonal antibody; MRC, Medical Research Council; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TNF, tumor necrosis factor.

A variety of immune effector cells, including $CD4^+$ T-cells, $CD8^+$ T-cells, and macrophages, as well as cytokines and other inflammatory products of these cells, have been implicated as mediators of pancreatic islet β-cell destruction in type 1 diabetes. Two major mechanisms have been described for T-cell–mediated cytotoxicity: *1*) a secretory and membranolytic mechanism involving release of perforin and serine proteases from T-cells and *2*) a receptor-mediated mechanism in which Fas ligand (FasL) (CD95L) molecules on the surface or released from T-cells bind to Fas (CD95) receptor molecules on target cells (1). FasL-Fas interactions between cells usually trigger death of the cells expressing Fas, and cell death occurs by apoptosis, a process of programmed cell death that involves protease activation and DNA fragmentation (2–4). Interestingly, apoptosis has recently been reported to be the mode of β-cell death in NOD mice that develop autoimmune diabetes (5).

Recent studies have suggested a role for FasL-Fas interactions in autoimmune β-cell death. Indeed, Fas expression by β-cells appears to be required for diabetes development, since diabetes did not develop in NOD mice lacking Fas expression (NOD-*lpr/lpr* mice created by crossing NOD mice with MRL-*lpr/lpr* mice that have an incapacitating mutation in the *fas* gene) (6,7). In addition, NOD-*lpr/lpr* mice were resistant to adoptive transfer of diabetes by a NOD-derived islet-reactive $CD8^+$ T-cell clone that otherwise led to Fas induction on β-cells and diabetes after adoptive transfer into young irradiated NOD mice (6). In other studies, cytokines have been demonstrated to induce Fas expression on islet β-cells in vitro. Thus, interleukin (IL)-1β was reported to induce Fas on mouse (8) and human (9,10) β-cells in vitro, and IL-1–sensitized Fas-expressing islet cells were killed by addition of anti-Fas monoclonal antibody (8,10). In a recent study, Fas expression was detected selectively on β-cells in pancreatic sections from two children with recent-onset type 1 diabetes; also, apoptosis was detected in the Fas^+ β-cells located close to $FasL^+$ T-cells infiltrating the islets (11). Collectively, these findings suggest that cytokine-induced Fas expression on β-cells may be an important mechanism for β-cell destruction by FasL-expressing T-cells that infiltrate islets.

In contrast, other studies have suggested that FasL expression by testicular Sertoli cells (12) or myoblasts (13) in the vicinity of islet grafts protects β-cells from immunological destruction. More recent studies, however, have reported that expression of FasL on mouse islet β-cells, either transgenically (14,15) or by gene transduction (15,16),

failed to protect islet allografts (or even isografts) from rejection, but rather FasL was proinflammatory and induced a neutrophilic infiltration into the islet graft. Another perplexing finding was that transgenic expression of FasL by β-cells in NOD mice accelerated diabetes development in some strains of the mice, but it protected against diabetes development in other strains of the mice (6).

Therefore, the aim of the present study was to investigate the expression of FasL and Fas on islet-infiltrating T-cells and islet β-cells in relation to β-cell destruction and autoimmune diabetes development. We used immunohistochemical methods to identify and quantitate FasL- and Fas-expressing $CD4^+$ T-cells, $CD8^+$ T-cells, and β-cells in islets in relation to diabetes recurrence in a syngeneic islet transplant model in NOD mice. Also, we used a semiquantitative polymerase chain reaction (PCR) assay to measure levels of FasL and Fas mRNA expressed in the islet grafts in relation to cytokine mRNA levels in the grafts. We found that destruction of β-cells in islet grafts correlated with Fas expression on the β-cells and expression of proinflammatory cytokines (IL-1, TNF-α, and IFN-γ) in the grafts. Also, these cytokines induced Fas expression on NOD islet cells in vitro.

RESEARCH DESIGN AND METHODS

Animals. NOD mice were purchased from Taconic Farms (Germantown, NY) and were maintained in a pathogen-free facility. Diabetes occurred in female NOD mice beginning at 12 weeks of age and reached 80% by 28 weeks of age. Diabetic mice were treated with daily subcutaneous injections of ultralente beef and pork insulin (1.0 U/100 g body wt) until the day before receiving islet transplants. Female diabetic NOD mice, 17–22 weeks of age and with a diabetes duration of 2–3 weeks, served as islet graft recipients. Islet donors were male NOD mice, 4–5 weeks of age. All mice were cared for according to the guidelines of the Canadian Council on Animal Care.

Islet transplantations. Islets were isolated by stationary collagenase digestion of the pancreas and Ficoll density gradient centrifugation, and then they were hand-picked (17). A total of 500 freshly isolated islets (pooled from approximately four donor mice) were transplanted under the left renal capsule in each diabetic NOD mouse, according to a previously described procedure (18). Transplantation was considered successful if the nonfasting blood glucose returned to normal (<7.0 mmol/l) within 2–3 days. Tail vein blood glucose was monitored daily posttransplantation by using an Accu-Chek IIm glucose monitor (Boehringer Mannheim, Laval, Quebec, Canada). Islet graft rejection was diagnosed by return of hyperglycemia (blood glucose >11.1 mmol/l) accompanied by glucosuria and ketonuria on 2 consecutive days.

Experimental design. Diabetic NOD mice were divided into two groups. Mice in the first group were injected with 50 µl complete Freund's adjuvant (CFA) into each hind footpad at the time of islet transplantation to prevent islet graft destruction and diabetes recurrence (18). Mice in the second group were similarly injected with phosphate-buffered saline (PBS). Diabetes recurred in the PBS-injected mice beginning at 6 days after islet transplantation, and islet grafts were removed for studies within 24 h of diabetes recurrence on days 6–12 posttransplantation (diabetic group). Islet grafts were also removed from the CFA-injected mice on days 6–12 after islet transplantation (normoglycemic group). In the first study, the cellular composition of islet grafts from eight diabetic and eight normoglycemic NOD mice was determined by staining the cells with monoclonal antibodies (mAbs) to β-cells and leukocyte subsets, followed by fluorescence-activated cell sorter (FACS) analysis. In the second study, Fas ($CD95^+$)– and FasL ($CD95L^+$)–expressing cells in islet grafts from 10 diabetic and 10 normoglycemic NOD mice were identified and quantitated by two-color immunohistochemical assays in which the cells were stained with antibodies to Fas and FasL and with mAbs to $CD4^+$ T-cells, $CD8^+$ T-cells, and β-cells. In the third study, islet grafts and spleens from seven diabetic and six normoglycemic NOD mice were extracted for determinations of mRNA levels of Fas, FasL, IL-1α, TNF-α, and IFN-γ by PCR assay. In the fourth study, the effects of cytokines (IL-1β, TNF-α, and IFN-γ) on Fas expression in vitro were examined in isolated islets from NOD mice.

Islet graft cell preparations. Islet grafts were removed from the renal subcapsular transplant site of each mouse and processed separately. Islet grafts were first placed in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Life Technologies, Burlington, Ontario, Canada) and kept on ice, then they were transferred into Eppendorf tubes containing 500 µl Ca^{2+}/Mg^{2+}-free PBS with 0.2 mg/ml EDTA (cell dissociation buffer) (Life Technologies). While on ice, the islet grafts were cut into small pieces with fine scissors and disrupted mechanically by syringe injection through progressively narrower gauge needles. The resulting tissue fragments of the islet grafts were dissociated into single cells by incubation in the cell dissociation buffer at 37°C for 20 min.

FACS analyses. Islet graft cells were washed and resuspended in PBS containing 1 mg/ml bovine serum albumin (BSA) and 0.1 mg/ml sodium azide (FACS buffer). Cell staining was performed in V-bottom microtiter plates at 4°C. Cells ($2–10 \times 10^5$) were incubated in 50 µl FACS buffer containing 20 µg/ml of the following rat mAbs to different mouse leukocyte subsets: Ly-5/T200 (CD45), L3T4 (CD4), Lyt-2 (CD8), M1/70 (Mac-1), and Ly-5/B220 (B-cell) (Cedarlane, Hornby, Ontario, Canada). The buffer also contained a mouse mAb specific to islet β-cells (R2D6, provided by Dr. R. Alejandro, Miami, FL) (19). Cells were washed in FACS buffer and centrifuged at 800*g* for 5 min at 4°C. Supernatants were aspirated, and the cells were resuspended in 50 µl FACS buffer with a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rat mouse-adsorbed immunoglobulin (Ig)G or FITC-conjugated goat anti-mouse IgG (Cedarlane). After incubation for 45 min at 4°C, cells were washed in FACS buffer, fixed in 300 µl of 10% buffered formalin, and analyzed. Analysis of cell fluorescence was performed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) interfaced to a Hewlett-Packard 310 computer. Data analysis was performed using Lysis II software. Logarithmically amplified fluorescence data were collected on 5,000–10,000 cells.

Two-color immunostaining of Fas^+ cells. Islet graft cells were incubated in 4% paraformaldehyde in PBS for 15 min at room temperature and washed twice in PBS by centrifugation at 400*g* for 5 min at 4°C. The fixed cells (20×10^3 in 10 µl) were placed on glass slides coated with Aptex (Sigma, St. Louis, MO), and the slides were stored at –70°C until processed. Slides were thawed, and the cells attached to the slides were treated with 1% paraformaldehyde in PBS at 4°C for 10 min, then washed twice in cold PBS and incubated in 0.3% H_2O_2 in PBS for 10 min. Cells were incubated in PBS containing 10% normal goat serum and 3% BSA for 3 h at room temperature to block nonspecific binding before antibody staining. Cells were incubated overnight at 4°C in a humidity chamber with 1 µg/ml of a hamster anti-mouse Fas (CD95) mAb (Jo2; Pharmingen, San Diego, CA) or 1 µg/ml of a hamster IgG control antibody. Cells were washed in PBS for 30 min and incubated for 20 min at room temperature with a second antibody, biotinylated goat anti-hamster IgG (Pharmingen), diluted 1:40 in PBS. Cells were then washed in PBS for 30 min and incubated for 15 min at room temperature in PBS diluted 1:1 with streptavidin-peroxidase conjugate (Histostain-SP Kit; Zymed Laboratories, South San Francisco, CA). After brief washing with distilled water, cells were treated with the substrate-chromogen 3-amino 9-ethylcarbazole (AEC), staining Fas^+ cells. Slides were left in PBS overnight at 4°C to remove nonspecific binding before proceeding to the following steps intended to identify the phenotypes of the Fas antigen-expressing cells. Cells were treated with 10% normal goat serum and 3% BSA for 1 h at room temperature and incubated for 2 h at 4°C in a humidity chamber with 20 µg/ml of each of the following antibodies, separately: rat mAbs to $CD4^+$ T-cells (L3T4) and $CD8^+$ T-cells (Lyt-2), a rat IgG control antibody, a mouse mAb to islet β-cells (R2D6), and a mouse IgM control antibody. Cells were washed in PBS for 30 min and incubated for 15 min at room temperature with a second antibody, biotinylated goat anti-rat mouse-absorbed IgG or biotinylated goat anti-mouse IgM, diluted 1:30 in PBS. Cells were then washed in PBS, incubated with streptavidin-alkaline phosphatase conjugate (Zymed) for 15 min at room temperature, washed in PBS, and incubated with a 1:2 dilution of alkaline phosphatase Fast-blue (Vector Laboratories, Burlingame, CA) for 3–5 min until staining (blue) for cell surface antigen (CD4, CD8, or R2D6) reached a maximum intensity as observed under the microscope. This was followed by mounting the slides with Crystal Mount (Biomeda, Foster City, CA) at 60°C for 40 min. $CD4^+$ Fas^+ cells,$CD8^+$ Fas^+ cells, and $R2D6^+$ Fas^+ cells were stained blue diffusely over the cell surface ($CD4^+$, $CD8^+$, and $R2D6^+$ cells) and stained red focally at and near the cell surface (Fas^+ cells).

Two-color immunostaining of $FasL^+$ cells. Islet graft cells were fixed in paraformaldehyde and treated with H_2O_2, then nonspecific binding was blocked with normal goat serum and BSA as described above. Cells were incubated with mAbs to $CD4^+$ T-cells, $CD8^+$ T-cells, and β-cells, followed by biotinylated second antibodies, streptavidin-alkaline phosphatase conjugate, and an enzyme-substrate complex (as described above) to identify blue-stained $CD4^+$ T-cells, $CD8^+$ T-cells, and β-cells. Cells were washed in PBS for 30 min, and staining for FasL was carried out by incubating the cells at 34°C for 2 h with 3 µg/ml of a rabbit anti-mouse FasL (CD95L) antibody (Q-20; Santa Cruz Biotechnology, Santa Cruz, CA) or 3 µg/ml of a rabbit IgG control antibody. Cells were then washed in PBS for 30 min and incubated for 30 min at room temperature with a second antibody, biotinylated goat anti-rabbit IgG (Zymed), diluted 1:1 in PBS. Cells were then washed in PBS for 30 min and incubated for 15 min at room temperature in PBS diluted 1:1 with streptavidin-peroxidase conjugate. After brief washing

with distilled water, cells were treated with the substrate-chromogen AEC, staining FasL$^+$ cells red. CD4$^+$ FasL$^+$ cells, CD8$^+$ FasL$^+$ cells, and R2D6$^+$ FasL$^+$ cells were stained blue diffusely over the cell surface (CD4$^+$, CD8$^+$, and R2D6$^+$ cells) and stained red focally at the cell surface and intracellularly (FasL$^+$ cells).

Transfected Fas- and FasL-expressing cells. The following Fas- and FasL-expressing cells were used as positive controls to validate the immunohistochemical staining methods used to identify Fas$^+$ and FasL$^+$ cells in islet grafts. COS-7 cells were transfected by DEAE-dextran with cDNAs for mouse Fas or mouse FasL, both cloned in eukaryotic expression vectors. After 4 h of transfection with mouse Fas cDNA, 30 ± 7% of COS-7 cells stained positively for Fas (Fig. 1*A*), and after 10 h, this increased to 81 ± 4% (mean ± SE, n = 8). After 6 h of transfection with mouse FasL cDNA, 21 ± 3% of COS-7 cells stained positively for FasL (Fig. 1*B*), and after 14 h, this increased to 56 ± 9% (mean ± SE, n = 8). Neither of these antibodies showed any staining of untransfected COS-7 cells or of COS-7 cells transfected with the empty expression vector.

Quantification of Fas- and FasL-expressing cells. Cell preparations were stained in duplicate with each test or control antibody, and 6,000 cells were scored blindly by two independent observers using light microscopy (oil immersion, 100×). COS-7 cells transfected with Fas or FasL cDNAs served as positive controls for cells from islet grafts in each staining run.

PCR analyses. RNA was extracted from islet grafts and spleens by a modification of the guanidinium thiocyanate method of Chirgwin et al. (20), as previously described (21). cDNA synthesis was carried out on 5 µg of total RNA with a Superscript reverse-transcriptase kit (Life Technologies) by using oligo $(deoxythymidine)_{12\text{-}18}$ and Moloney murine leukemia virus reverse transcriptase (20 U) in a 25-µl vol at 37°C for 1.5 h. The reverse transcription product was diluted 1:50, 1:100, and 1:500 in sterile H_2O. PCR amplification of cDNA was performed on 5 µl of each dilution in a 20-µl vol containing 80 ng of each primer, 0.25 mmol/l of each deoxy-NTP, 2.5 µCi of [α-^{32}P]deoxy-CTP (3,000 Ci/mmol; Du Pont, Mississauga, Ontario, Canada), 2 U of AmpliTaq (Perkin-Elmer/Cetus, Norwalk, CT), and 3 mmol/l Mg^{2+}. Samples were amplified through 40 cycles at 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s in a Gene Amp PCR System 9600 (Perkin-Elmer/Cetus). The sequences of the sense and antisense oligonucleotide primers, respectively, were as follows: IL-1α, CGTCAGGCAGAAGT TTGTCA and TGATGAGTTTTGGTGTTTCTGG; TNF-α, CTTAGACTTTGCG GAGTCCG and ACAGTCCAGGTCACTGTCCC; IFN-γ, CGCTACACACTG CATCTTGG and GGCTGGATTCCGGCAACA; Fas, CTCAAGGTACTAATAG CATCTCCGA and CAGTAGAAGTCTGGTTTGCACTTG; FasL, CCCTCTGAAA AAAAAGAGCCGAG and AAGATTGAATACTGCCCCCAGG; and cyclophilin, GACAGCAGAAAACTTTCGTGC and TCCAGCCACTCAGTCTTGG. The PCR reaction products were electrophoresed on 1.5% agarose gels and transferred to nylon membranes. Incorporation of ^{32}P in the PCR product bands was measured by phosphor imager analysis (Fujix BAS 1000; Fuji Photo Film, Tokyo) and expressed as photon-stimulated luminescence per minute. Values obtained for each PCR product were normalized as a percentage of ^{32}P incorporated in the cyclophilin PCR product amplified from the same cDNA preparation. Under the conditions used, the PCR product signal was proportional to the amount of cDNA reverse transcribed from mRNA in the tissue sample (21). Samples of cDNA to be compared were amplified in the same PCR run to avoid interassay variations, and the final results are averages of two separate PCR runs.

Islet incubations. Islets were isolated from NOD mice (males, 4–5 weeks of age) and dissociated into single cells by incubation at 37°C for 10 min in Ca^{2+}/Mg^{2+}-free PBS containing 0.2 mg/ml EDTA, followed by syringe injection through progressively narrower gauge needles. Before experimental incubations, islet cells were incubated (5% CO_2; 37°C) overnight in RPMI 1640 medium containing 11 mmol/l glucose, 2 mmol/l L-glutamine, 0.1 mmol/l sodium pyruvate, 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 12 mmol/l HEPES (control medium). Islet cells (5×10^5/well) were then incubated (5% CO_2; 37°C) for 72 h in control medium without and with IL-1β (50 U/ml), TNF-α (1,000 U/ml), IFN-γ (1,000 U/ml), and a combination of all three cytokines. Recombinant human IL-1β ($2–4 \times 10^7$ U/mg) was provided by Upjohn (Kalamazoo, MI), and recombinant mouse TNF-α (1.2×10^7 U/mg) and recombinant mouse IFN-γ (8×10^6 U/mg) were provided by Genentech (South San Francisco, CA). After the incubations with cytokines, islet cells were stained with a hamster anti-mouse Fas mAb (Jo2; Pharmingen) and an FITC-conjugated goat anti-hamster IgG (Cedarlane), then they were fixed in formalin and analyzed by FACScan as described above (see FACS analyses).

Statistical analysis. Data are presented as means ± SE, and statistical comparisons were made by Welch's unpaired t test, with a two-tailed P value <0.05 considered significant.

RESULTS

Cellular compositions of diabetic and normoglycemic islet grafts. Injection of CFA at the time of syngeneic islet transplantation into diabetic NOD mice protected the islet grafts from β-cell destructive insulitis and autoimmune diabetes recurrence, as previously reported (18). In this study, we show that the β-cell mass (numbers of β-cells) was significantly greater in islet grafts from CFA-protected NOD mice (normoglycemic) compared with islet grafts from control PBS-injected NOD mice (diabetic) (Table 1). Also, Table 1 shows that islet grafts from normoglycemic mice contained significantly fewer leukocytes (CD45$^+$ cells) than did islet grafts from diabetic mice. In addition, the subset compositions of the total leukocyte populations were different in islet grafts from diabetic and normoglycemic mice. Thus, expressed as percentages of total leukocytes in islet grafts, both CD4$^+$ and CD8$^+$ T-cells were significantly decreased, macrophages were not changed, and B-cells were significantly increased in the normoglycemic group (Table 1).

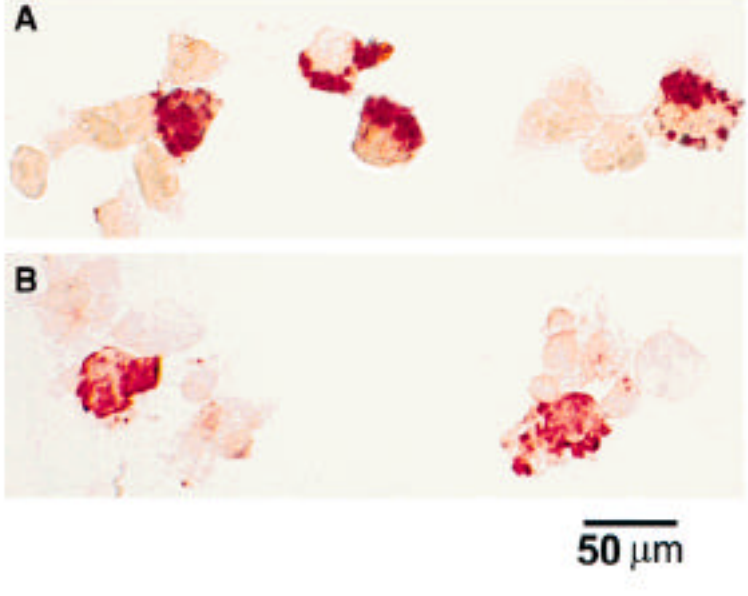

FIG. 1. Photomicrographs of COS-7 cells transfected with cDNAs for mouse Fas or FasL, then immunostained for these proteins. COS-7 cells were transfected with mouse Fas cDNA and stained with a hamster anti-mouse Fas mAb that revealed red-stained (Fas$^+$) cells and unstained (Fas$^-$) cells (*A*). COS-7 cells were transfected with mouse FasL cDNA and stained with a rabbit anti-mouse FasL antibody that revealed red-stained (FasL$^+$) cells and unstained (FasL$^-$) cells (*B*).

TABLE 1
Cellular composition of syngeneic islet grafts from diabetic and normoglycemic NOD mice

	Percent of total cells		Percent of CD45$^+$ cells			
	β-Cells	Leukocytes	T-cells		Macrophages	B-cells
	(R2D6$^+$)	(CD45$^+$)	(CD4$^+$)	(CD8$^+$)	(Mac-I$^+$)	(B220$^+$)
Diabetic NOD mice	13.1 ± 1.1	65.4 ± 4.0	32.0 ± 2.2	12.5 ± 1.4	21.4 ± 1.1	29.5 ± 3.4
Normoglycemic NOD mice	35.1 ± 2.2*	51.6 ± 1.6†	19.0 ± 1.2*	5.4 ± 0.2*	21.7 ± 1.3	51.9 ± 4.2*

Data are means ± SE for eight mice in each group. Diabetic NOD mice were transplanted with syngeneic islets and injected either with CFA to prevent diabetes recurrence or with PBS. Islet grafts were removed from PBS-injected mice within 24 h of diabetes recurrence on days 6–12 posttransplantation (diabetic) and from CFA-injected mice on the same days posttransplantation (normoglycemic). Total numbers of cells in islet grafts were similar in diabetic mice ($42 \pm 4 \times 10^4$) and normoglycemic mice ($38 \pm 2 \times 10^4$). β-Cells, leukocytes, and leukocyte subsets (T-cells, macrophages, and B-cells) in islet grafts were identified by staining with mAbs to cell surface antigens (indicated in parentheses) and quantitated by FACS analysis. *$P < 0.01$, †$P < 0.05$ vs. diabetic group.

Identification of Fas- and FasL-expressing cells in islet grafts. A two-color immunohistochemical method was used to identify the phenotypes of Fas- and FasL-expressing cells in islet grafts. This technique revealed that Fas$^+$ and FasL$^+$ cells were detected among CD4$^+$ T-cells, CD8$^+$ T-cells, and β-cells in islet grafts (Fig. 2). The frequencies of Fas$^+$ and FasL$^+$ CD4$^+$ T-cells, CD8$^+$ T-cells, and β-cells in islet grafts from diabetic and normoglycemic NOD mice are shown in Table 2. The percentage of Fas$^+$ cells among total cells in islet grafts was significantly greater in islet grafts from diabetic mice (8.7 ± 0.6%) compared with normoglycemic mice (0.2 ± 0.1%). Fas$^+$ cells in islet grafts from diabetic mice were approximately equally divided among Fas$^+$ CD4$^+$ cells (2.5 ± 0.3%), Fas$^+$ CD8$^+$ cells (3.0 ±

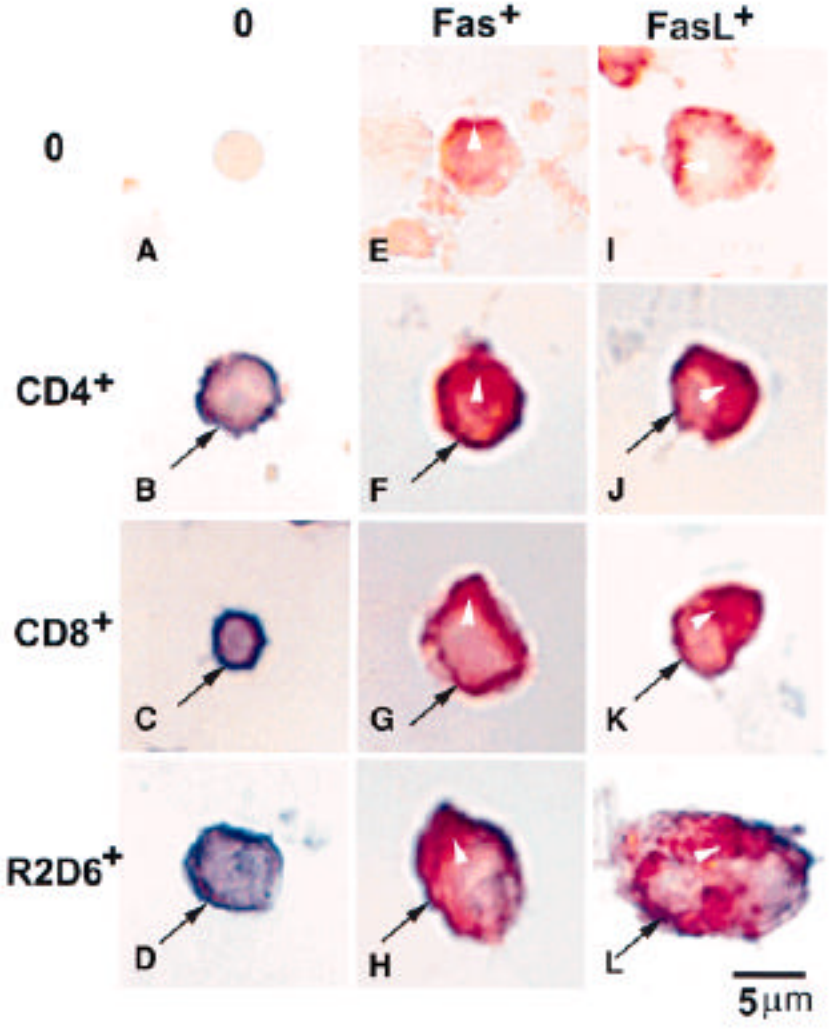

FIG. 2. Identification of cells in islet grafts from NOD mice by two-color immunohistochemical staining. An unstained cell (*A*). CD4$^+$ cells (*B*), CD8$^+$ cells (*C*), and β-cells (R2D6$^+$) (*D*) are stained blue diffusely over the whole cell surface (black arrows). Fas$^+$ cells (*E*) are stained red focally at and near the cell surface (white arrowhead). CD4$^+$ Fas$^+$ cells (*F*), CD8$^+$ Fas$^+$ cells (*G*), and R2D6$^+$ Fas$^+$ cells (*H*) are stained blue diffusely over the whole cell surface (black arrows) and stained red focally at and near the cell surface (white arrowheads). FasL$^+$ cells (*I*) are stained red focally at the cell surface and intracellularly (white arrowhead). CD4$^+$ FasL$^+$ cells (*J*), CD8$^+$ FasL$^+$ cells (*K*), and R2D6$^+$ FasL$^+$ cells (*L*) are stained blue diffusely over the whole cell surface (black arrows) and stained red focally at the cell surface and intracellularly (white arrowheads).

TABLE 2
Identification of Fas- and FasL-expressing cells in syngeneic islet grafts from diabetic and normoglycemic NOD mice

	Percent of total cells		Percent of $CD4^+$ cells		Percent of $CD8^+$ cells		Percent of $R2D6^+(\beta)$ cells	
Cell subset	Diabetic	Normoglycemic	Diabetic	Normoglycemic	Diabetic	Normoglycemic	Diabetic	Normoglycemic
Fas^+	8.7 ± 0.6	0.2 ± 0.1*	—	—	—	—	—	—
Fas^+ $CD4^+$	2.5 ± 0.3	0*	18 ± 4	0*	—	—	—	—
Fas^+ $CD8^+$	3.0 ± 0.3	0.2 ± 0.1*	—	—	53 ± 6	6 ± 2*	—	—
Fas^+ $R2D6^+$	3.2 ± 0.4	0*	—	—	—	—	33 ± 4	0*
$FasL^+$	6.8 ± 0.6	8.7 ± 0.6	—	—	—	—	—	—
$FasL^+$ $CD4^+$	2.0 ± 0.2	2.5 ± 0.4	10 ± 2	21 ± 4†	—	—	—	—
$FasL^+$ $CD8^+$	2.3 ± 0.2	2.5 ± 0.3	—	—	37 ± 6	54 ± 8	—	—
$FasL^+$ $R2D6^+$	1.7 ± 0.4	5.1 ± 0 5†	—	—	—	—	9 ± 1	16 ± 2†

Data are means ± SE for 10 mice in each group. Diabetic NOD mice were transplanted with syngeneic islets and injected either with CFA to prevent diabetes recurrence or with PBS. Islet grafts were removed from PBS-injected mice within 24 h of diabetes recurrence on days 6–12 posttransplantation (diabetic) and from CFA-injected mice on the same days posttransplantation (normoglycemic). Percentages of total cells, $CD4^+$ T-cells, $CD8^+$ T-cells, and $R2D6^+$ β-cells in islet grafts that expressed Fas and FasL were determined by two-color staining with specific antibodies and immunohistochemical assays (see METHODS). *P < 0.01, †P < 0.05 vs. diabetic group.

0.3%), and Fas^+ β-cells (3.2 ± 0.4%). Also, Fas was expressed on 18 ± 4% of $CD4^+$ cells, 53 ± 6% of $CD8^+$ cells, and 33 ± 4% of β-cells in islet grafts from diabetic mice. In contrast, Fas was detected on none of the $CD4^+$ cells, 6 ± 2% of the $CD8^+$ cells, and none of the β-cells in islet grafts from normoglycemic mice. Therefore, Fas was expressed on $CD4^+$ cells, $CD8^+$ cells, and β-cells in islet grafts from diabetic mice, but it was nearly or totally absent on these cells in islet grafts from normoglycemic mice. In contrast, the percentage of $FasL^+$ cells among total cells in islet grafts was similar in islet grafts from diabetic mice (6.8 ± 0.6%) and normoglycemic mice (8.7 ± 0.6%). $FasL^+$ cells in islet grafts from diabetic and normoglycemic mice were distributed among $CD4^+$ cells, $CD8^+$ cells, and β-cells. Interestingly, $FasL^+$ β-cells were significantly more numerous in islet grafts from normoglycemic mice (5.1 ± 0.5%) compared with diabetic mice (1.7 ± 0.4%). Furthermore, the percentages of $CD4^+$ cells, $CD8^+$ cells, and β-cells that expressed FasL were greater in islet grafts from normoglycemic compared with diabetic mice (Table 2).

Fas and FasL mRNA levels in islet grafts. Expression of Fas mRNA was significantly lower in islet grafts of normoglycemic mice than in those of diabetic NOD mice, whereas FasL mRNA expression was somewhat higher in islet grafts from normoglycemic mice than in those from diabetic mice (Fig. 3). Thus, expression of both Fas mRNA (Fig. 3) and protein (Table 2) was significantly lower in islet grafts from normoglycemic compared with diabetic NOD mice, whereas expression of both FasL mRNA (Fig. 3) and protein (Table 2) was the same or greater in islet grafts from normoglycemic compared with diabetic NOD mice. Splenic expression of Fas mRNA was also somewhat lower in normoglycemic than in diabetic mice, and splenic FasL mRNA levels were similar in normoglycemic and diabetic mice (Fig. 3).

Cytokine mRNA levels in islet grafts. IL-1α, TNF-α, and IFN-γ mRNA levels were significantly lower in islet grafts from normoglycemic compared with diabetic NOD mice, whereas mRNA levels of these three cytokines were similar in spleens from normoglycemic and diabetic NOD mice (Fig. 4).

Effects of cytokines on islet cell Fas expression. Fas was expressed on 2% of NOD mouse islet cells in vitro, and incubation with cytokines significantly increased the frequencies of Fas-expressing islet cells to 5 ± 1% with IL-1β, 12 ± 1% with IFN-γ, and 18 ± 1% with the combination of IL-1β, TNF-α, and IFN-γ (Fig. 5).

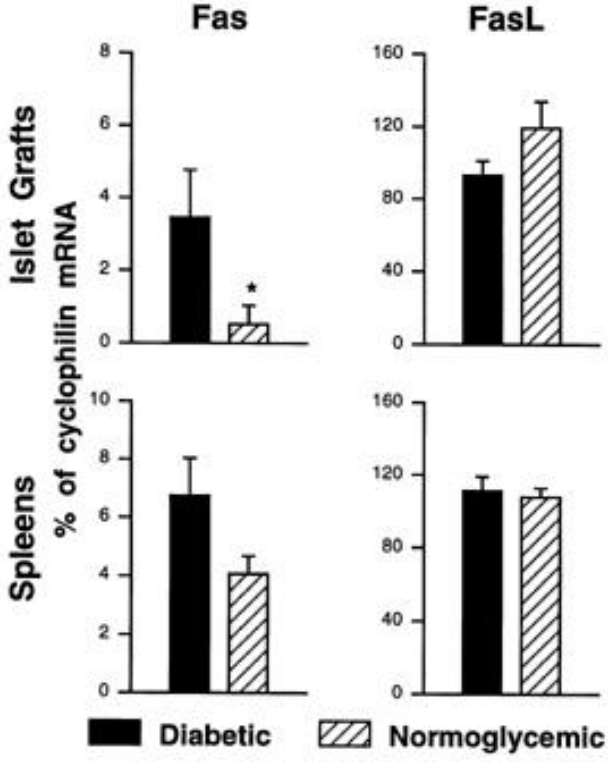

FIG. 3. Fas and FasL mRNA levels (means ± SE) in islet grafts and spleens from two groups of NOD mice at 6–12 days after transplantation with syngeneic islets: PBS-injected, with diabetes recurrence (diabetic, n = 7); and CFA-injected, protected from diabetes recurrence (normoglycemic, n = 6). Fas and FasL mRNA levels were determined by reverse transcriptase PCR assay, and the Fas and FasL PCR assay products (mRNA levels) are expressed as percentages of the cyclophilin PCR product amplified from the same cDNA preparation. *P < 0.05 vs. diabetic mice.

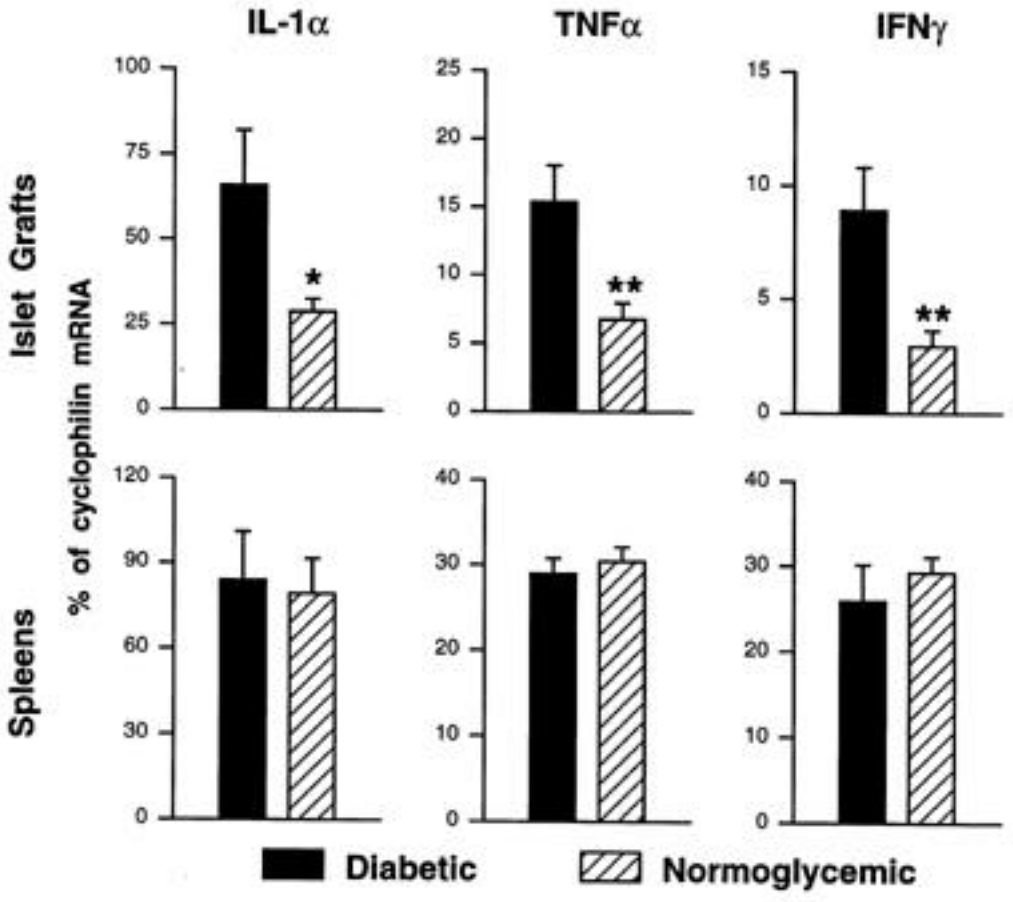

FIG. 4. IL-Iα, TNF-α, and IFN-γ mRNA levels (means ± SE) in islet grafts and spleens from two groups of NOD mice at 6–12 days after transplantation with syngeneic islets: PBS-injected, with diabetes recurrence (diabetic, n = 10); and CFA-injected, protected from diabetes recurrence (normoglycemic, n = 10). Cytokine mRNA levels were determined by reverse transcriptase PCR assay, and the cytokine PCR assay products (mRNA levels) are expressed as percentages of the cyclophilin PCR product amplified from the same cDNA preparation. *P < 0.05, **P < 0.02 vs. diabetic mice.

DISCUSSION

Pancreatic islet infiltration by macrophages and T-cells (insulitis) is a well-recognized antecedent of β-cell destruction in autoimmune diabetes; however, the mechanisms by which β-cells die remain undefined. Recently, attention has been directed at the interaction of FasL (CD95L) on T-cells with a Fas (CD95) receptor on β-cells as a possible mechanism for β-cell killing in type 1 diabetes. The observation that NOD mice lacking Fas expression (NOD-*lpr/lpr*) failed to develop diabetes (6,7) and were resistant to adoptive transfer of diabetes (6) suggested that Fas expression by islet β-cells was essential for diabetes development. Diabetes resistance in NOD-*lpr/lpr* mice, however, might result from a confounding effect of other nondiabetogenic *lpr* genes bred onto the NOD background or from a defective immune system in *lpr* mice. Therefore, it remained to be determined whether Fas expression on β-cells plays a role in the development of spontaneous autoimmune diabetes. In one recent study, Fas expression was detected selectively on β-cells in pancreatic sections from two children with recent-onset type 1 diabetes and not on β-cells in normal human pancreas; also, apoptosis was detected in the Fas^+ β-cells located close to the $FasL^+$ T-cells infiltrating the islets (11). In another recent study, however, Fas was not detected on β-cells (or any other cells) in islets of NOD mice (without or with destructive insulitis), whereas FasL was present, however, only on islet α-cells (22).

In the present study, we used two-color immunohistochemical assays to identify and quantitate FasL- and Fas-expressing cells in islets in relation to β-cell destruction and recurrence of autoimmune diabetes in a syngeneic islet transplant model in NOD mice. We found that FasL was expressed on $CD4^+$ T-cells, $CD8^+$ T-cells, and β-cells in islet grafts from both diabetic and normoglycemic mice, and the percentage of each type of cell that expressed FasL was greater in islet grafts from normoglycemic compared with diabetic mice. Similarly, FasL mRNA expression was greater in islet grafts from normoglycemic compared with diabetic NOD mice; however, the difference was not significant. In contrast, Fas receptor was expressed on $CD4^+$ T-cells, $CD8^+$ T-cells, and β-cells in islet grafts from diabetic mice, but this receptor was nearly or totally absent on cells in islet grafts from normoglycemic mice. Similarly, Fas mRNA expression was significantly lower in islet grafts from normoglycemic compared with diabetic mice. These findings suggest that induction of Fas receptor expression on β-cells may be the mechanism that permits β-cell destruction by Fas ligand-expressing cells in the islets.

Cytokines induce Fas expression on cells (2), and we found that Fas mRNA expression correlated with expression of IL-1α, TNF-α, and IFN-γ in the islet grafts. In addition, we found that IL-1β, IFN-γ, and the combination of IL-1β, TNF-α, and IFN-γ induced Fas expression on NOD islet cells in vitro. Similarly, others have reported that IL-1β induces Fas on normal mouse (8) and human (9–11) β-cells in vitro, and IL-1–sensitized Fas-expressing islet cells are killed by addition of anti-Fas antibody (8,10). These results, taken together with the findings in the present study, suggest that cytokines

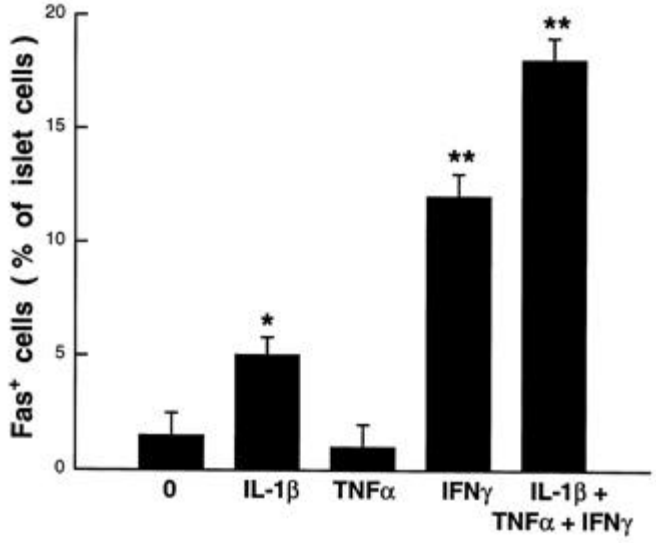

FIG. 5. Cytokines induce Fas expression on NOD mouse islet cells in vitro. Islets were isolated from NOD mice (males, 4–5 weeks of age), and islet cells (5×10^5/well) were incubated for 72 h without (0) and with cytokines as indicated: IL-1β (50 U/ml), TNF-α (1,000 U/ml), IFN-γ (1,000 U/ml), and a combination of all three cytokines. Islet cells were then stained with an mAb to Fas, and Fas$^+$ cells were quantitated by FACS analysis. Values are means ± SE for four separate experiments. *$P < 0.05$, **$P < 0.01$ vs. without (0) cytokines.

(IL-1, TNF-α, IFN-γ) produced by islet-infiltrating macrophages and T-cells induce Fas on β-cells, thereby permitting FasL$^+$ CD4$^+$ and CD8$^+$ T-cells to kill β-cells via FasL-Fas interactions. In addition, because FasL was expressed on β-cells (as well as on CD4$^+$ and CD8$^+$ T-cells) in islet grafts from diabetic mice, FasL$^+$ β-cells might have contributed to the killing of self or other Fas$^+$ β-cells. Although FasL was also expressed on CD4$^+$ and CD8$^+$ T-cells and β-cells in islet grafts from normoglycemic mice, this would not be expected to lead to killing of β-cells, because β-cells did not express Fas receptor in normoglycemic mice.

Our finding that FasL is expressed on islet β-cells, as well as on CD4$^+$ and CD8$^+$ T-cells, is concordant with reports that FasL is constitutively expressed not only in lymphoid cells but also in a wide array of nonlymphoid tissues in mice (23). Also, FasL is constitutively expressed in normal human thyroid follicular cells, and FasL-expressing thyrocytes may be the major effectors of destruction of self or other thyrocytes that have been induced by IL-1β to express Fas in chronic active thyroiditis (24). In contrast, islet cells were reported to be FasL$^-$ in pancreas of both normal subjects and patients with recent-onset type 1 diabetes, while activated T-cells infiltrating the islets of the diabetic subjects were strongly FasL$^+$, and apoptosis was detected in Fas$^+$ β-cells located close to FasL$^+$ T-cells (11). In a more recent report, however, normal human pancreatic β-cells were demonstrated by Western immunoblotting (islets) and immunocytochemistry (pancreatic sections) to express FasL, i.e., FasL did not require cytokines for expression but was expressed constitutively (25). Similarly, we report here that FasL was expressed on β-cells, as well as on CD4$^+$ and CD8$^+$ T-cells, in islet grafts from both diabetic and normoglycemic mice. Interestingly, FasL was expressed more frequently on T-cells and β-cells in islet grafts from normoglycemic mice than in those from diabetic mice; however, Fas receptor was not expressed on these cells in normoglycemic mice. This suggests that cytokine-induced Fas receptor expression rather than expression of FasL may represent a limiting factor for the initiation of β-cell destruction by FasL-expressing cells in the insulitis lesion.

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Author Queries (please see Q in margin and underlined text)