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Genes encoding tumor necrosis factor α and granzyme A are expressed during development of autoimmune diabetes

(insulin-dependent diabetes mellitus/autoimmune disease/nonobese diabetic mouse/*in situ* hybridization)

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ABSTRACT **Progressive destruction of the insulin-producing beta cells in nonobese diabetic mice is observed after infiltration of the pancreas with lymphocytes [Makino, S., Kunimoto, K., Muraoka, Y., Mizushima, Y., Katagiri, K. & Tochino, Y. (1980) *Exp. Anim. (Tokyo)* 29, 1–13]. We show that the genes for tumor necrosis factor α and granzyme A, a serine protease associated with cytoplasmic granules of cytotoxic cells, are expressed during the development of spontaneous diabetes mellitus in the nonobese diabetic mouse. Granzyme A-positive cells are found both in and surrounding the islets, implying induction prior to islet infiltration. Tumor necrosis factor α expression is exclusively observed in the intra-islet infiltrate, predominantly in lymphocytes adjacent to insulin-producing beta cells, the targets of the autoimmune destruction, implying that tumor necrosis factor α expression is induced locally—i.e., in the islet. A considerable portion of cells expressing tumor necrosis factor α appear to be CD4+ T cells. This T-cell subset was previously shown to be necessary for development of the disease. Thus, these findings may be important for understanding the pathogenesis of autoimmune diabetes mellitus and potentially also for that of other T-cell-mediated autoimmune diseases.**

The autoimmune response in type I diabetes is characterized by insulitis, in which lymphocytes infiltrate the islets of Langerhans. The invasion of the pancreas islets by lymphocytes leads to a specific destruction of insulin-producing beta cells (1). Nonobese diabetic (NOD) mice spontaneously develop a diabetic syndrome resembling human insulin-dependent diabetes mellitus (2, 3). In these mice, the disease can be induced by adoptive transfer of both $CD4^+$ and $CD8^+$ T lymphocytes, thus implicating a cell-mediated autoimmune pathogenesis (4, 5).

Cytoplasmic granules of cytotoxic cells contain, in addition to the pore-forming protein perforin (6), a family of highly homologous serine esterases, designated granzyme A–H (7). These proteins are released into the intercellular space upon killer–target cell interaction. In addition, the role of cytokines in the pathogenesis of autoimmune diabetes has recently received increasing attention. It was shown that interferon γ and tumor necrosis factor α (TNF-α) can induce an aberrant expression of class II major histocompatibility molecules on pancreatic beta cells *in vitro*, suggesting a role for these cytokines in the induction of the autoimmune process (8). Furthermore, TNF-α was shown to induce interleukin 1 expression (9), which is reported to be toxic to pancreatic beta cells *in vitro* (10).

Since antibodies reliably recognizing these proteins on tissue sections are not yet available, we used the technique of *in situ* hybridization for the detection of the respective mRNAs. We found that the genes for granzyme A and TNF-α are in fact expressed *in vivo* in islet-infiltrating cells during development of autoimmune diabetes in NOD mice.

MATERIALS AND METHODS

Mice. Female NOD mice were sacrificed at 6, 12, and 18 weeks of age (20 weeks for male NOD mice), or after onset of hyperglycemia, producing Tes-Tape (Eli Lilly) values of 2+ or higher. Cumulative incidences of the onset of overt diabetes in our NOD colony are 70% in females and 5% in males at 28 weeks of age, with a mean onset in female NOD mice at the age of 22 ± 6 weeks (n = 24).

Tissue Processing. Pancreas, spleen, and mesenteric lymph nodes were excised and immersed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Tissues were immediately placed into a regular household microwave oven for 20 sec, which raised the temperature to ≈50°C. After postfixing overnight in 4% PFA, tissues were further processed for paraffin embedding by routine techniques.

Preparation of Labeled Probes. A 1108-base-pair (bp) cDNA fragment of the 5′ end of the murine TNF-α gene (position 1–1108) (11) (kindly provided by Genentech), a 775-bp cDNA fragment of the granzyme A gene (Hanukah Factor; ref. 12), and a 677-bp fragment of the murine insulin gene (position 701–1378) (kindly provided by S. Efrat, Cold Spring Harbor Laboratory) were subcloned into the pGem2 transcription vector by standard techniques. After linearization of the plasmids with the appropriate restriction endonucleases, ^{35}S-labeled antisense and sense RNA probes were prepared with T7 and SP6 RNA polymerases (both obtained from Boehringer Mannheim), respectively, as described in detail (13).

***In Situ* Hybridization.** Serial sections containing pancreas, spleen, and lymph node tissues were hybridized *in situ* with an antisense RNA probe of the insulin, granzyme A, and TNF-α gene, and the sense probe of the TNF-α gene, respectively. At least three of those series, with a minimal distance of 20 μm from each other, were hybridized per animal.

In situ hybridization was performed as described in detail (13) with modifications for paraffin sections. Tissue sections (3 μm thick) were placed on precleaned and poly-L-lysine (Sigma)-coated microscopic slides. The dried sections were deparaffinized twice in fresh xylene for 10 min and rehydrated in graded ethanol. The sections were then fixed in 4% PBS-buffered PFA for 20 min at room temperature, rinsed in PBS, and subsequently treated with proteinase K (Boehringer Mannheim) at 1 μg/ml at 37°C for 30 min. After postfixation and acetylation, hybridization was performed

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Abbreviations: NOD, nonobese diabetic; TNF-α, tumor necrosis factor α; PFA, paraformaldehyde.

with 2×10^5 cpm of ^{35}S-labeled RNA probe per μl of hybridization solution (10^5 cpm/μl for the insulin probe) for 18 hr at 48°C as described in detail (13). After digestion of single-stranded, nonhybridized RNA and extensive washing, slides were dipped into NTB2 nuclear track emulsion (Eastman Kodak). Slides were exposed for 20 days (3 days for the insulin probe) in a light-tight box at −70°C, developed, and subsequently counterstained with either hematoxylin and eosin or nuclear fast red (0.05% in 5% aluminum sulfate) by standard techniques.

Cells. Peripheral blood cells were obtained by Ficoll (Pharmacia) gradient centrifugation. Single cell suspensions of spleen and mesenteric lymph nodes were prepared by standard techniques. Cells (4×10^4) were spun onto microscopic slides and fixed for 20 min in 4% PBS-buffered PFA for subsequent *in situ* hybridization.

Pancreas tissue from five female NOD mice (between 12 and 18 weeks old) was excised and teased into small pieces. Care was taken not to include pancreatic lymph nodes. Pooled tissues were completely digested with collagenase type IV (Sigma) (0.75 mg/ml in Hanks' balanced salt solution) in eight cycles of 7 min each. The supernatants were collected, and released cells were purified over a Percoll (Pharmacia) step gradient (14). Isolated cells reacted on ice with a monoclonal fluoresceinated anti-CD4 antibody (GK1.5) for 30 min. After washing, cells were fixed in 1% PBS-buffered PFA. The viability of cells before fixing was >95% as determined by trypan blue exclusion. A FACS II (Becton Dickinson) was used for separating $CD4^+$ and $CD4^-$ cells by forward light scatter gating and by analyzing green fluorescence. The two populations were directly deflected onto poly(L-lysine)-coated microscopic slides. *In situ* hybridization on sorted cells was performed as described above for tissue sections.

Immunohistochemistry. Frozen sections containing pancreas, spleen, and mesenteric lymph node tissues were

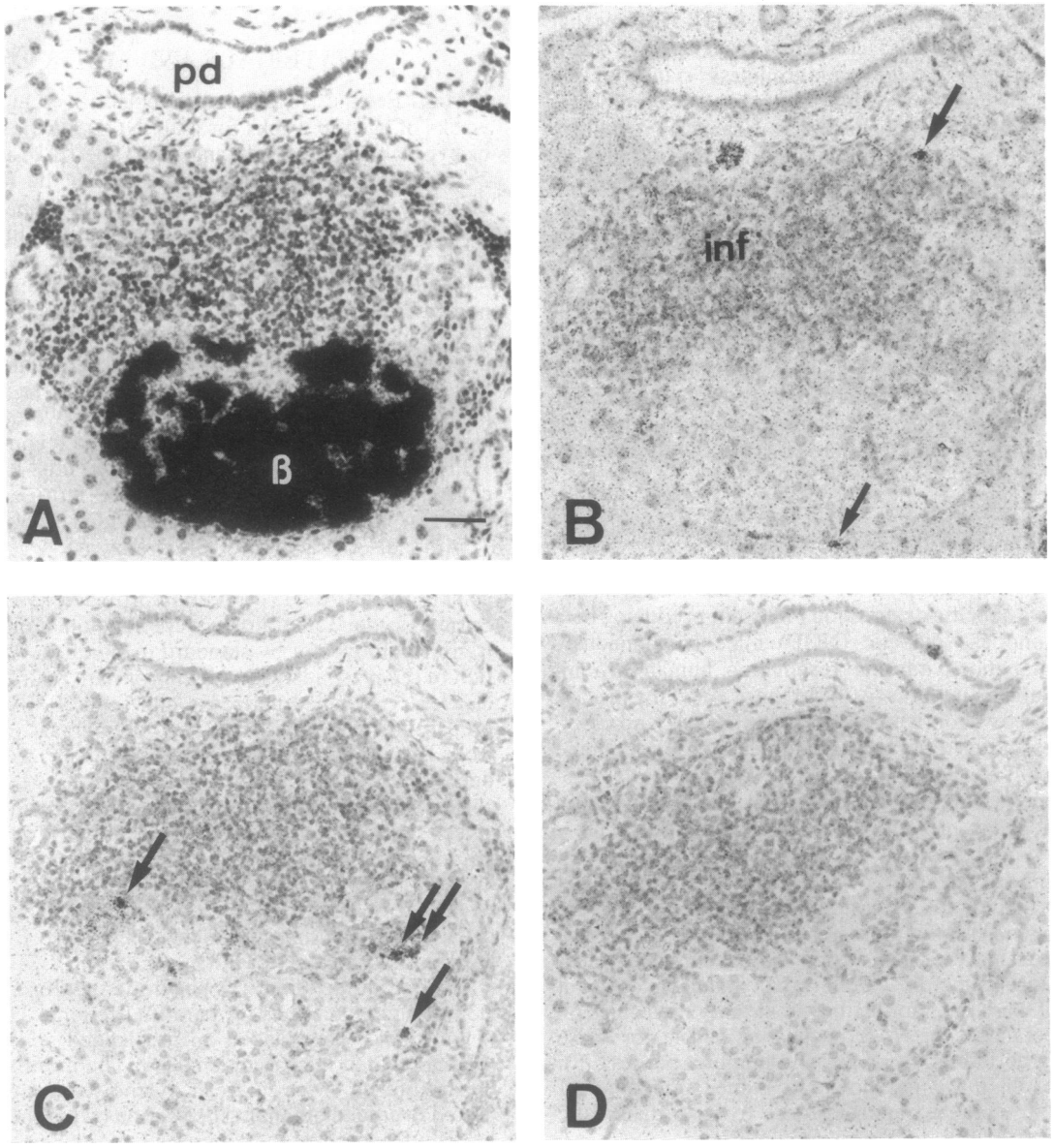

FIG. 1. Expression of the genes for insulin, granzyme A, and TNF-α in an infiltrated islet of Langerhans of an 18-week-old female NOD mouse. Photomicrographs show serial sections of paraffin-embedded pancreas hybridized *in situ* with radiolabeled antisense RNA probes of the insulin (*A*), the granzyme A (*B*), the TNF-α gene (*C*), and the sense probe of the TNF-α gene, which is identical to the cellular target mRNA and therefore was used as a negative control (*D*). The islet, located close to a pancreatic duct (pd), is heavily infiltrated (inf) and still shows an insulin mRNA-positive beta cell area (β). TNF-α mRNA-positive cells (arrows in *C*) are preferentially located adjacent to this area, whereas granzyme A mRNA-positive cells (arrows in *B*) show a less restricted localization. (Bar = 100 μm.)

stained with the rat monoclonal antibodies 53.2.1 (anti-Thy 1.2), GK1.5 (anti-CD4), 53.6.7 (anti-CD8), and M1/70 (anti-Mac1) (all obtained from American Type Culture Collection) as a first-stage reagent. Peroxidase-conjugated rabbit anti-rat immunoglobulin (Dako, Copenhagen) was used as a second stage reagent at a 1:25 dilution in 10% normal mouse serum. 3,3'-Diaminobenzidine (Sigma) was used as a substrate for peroxidase.

Evaluation of Slides. The inflammatory infiltrate was evaluated and classified for each islet according to the following grading system: 0, intact islet; 1, area of mononuclear cell infiltration within an islet was <25%; 2, 25–50%; 3, >50%; 4, end stage of insulitis characterized by small retracted islets with or without residual infiltrate (according to ref. 5). Severity of insulitis was estimated for each animal by evaluating two noncontiguous sections stained with hematoxylin and eosin, containing at least 15 islets, and including hybridized sections.

After *in situ* hybridization with a ^{35}S-labeled antisense RNA probe, cells were considered positive for gene expression when they had at least three times as many silver grains as cells hybridized with the corresponding sense RNA probe, which is identical to the cellular target mRNA and was used as a negative control.

RESULTS AND DISCUSSION

***In Situ* Hybridization of Pancreas Sections.** Pancreas sections of female NOD mice sacrificed at 6, 12, and 18 weeks of age or after onset of hyperglycemia, respectively, were hybridized *in situ* with radiolabeled antisense RNA probes of the granzyme A or the TNF-α gene. Mesenteric lymph nodes and spleens were included on each slide as positive controls. Furthermore, preservation of cellular mRNA and localization of beta cells in the pancreas was examined by hybridizing tissue sections with an antisense probe of the murine insulin gene (Fig. 1*A*).

About 55% of granzyme A-positive cells in the pancreas are located in the intra-islet infiltrate. The remaining positive cells are found in the perivascular infiltrate (20% of granzyme A-positive cells in the pancreas), the pancreas parenchyma (13%), and within blood vessels (12%). Approximately half of the granzyme A-positive cells in the intra-islet infiltrate are located adjacent to the β-cell area (Fig. 1*B*). This may imply that induction of the granzyme A gene occurs prior to islet infiltration. In contrast, TNF-α gene expression is exclusively observed in the intra-islet infiltrate. More than 80% of TNF-α-positive cells are closely associated with the β-cell area (Fig. 1*C*), which suggests an *in situ* activation of the TNF-α gene rather than an infiltration of activated cells. Controls—i.e., pancreas sections hybridized with the sense probe of the TNF-α gene—contained no positive cells (Fig. 1*D*).

Granzyme A and TNF-α Expression with Progressive Insulitis. In the pancreas of NOD mice, the first histological signs of an infiltration by mononuclear cells are found at 4–5 weeks of age. The inflammatory changes of pancreatic tissues increase with age, and overtly diabetic animals with no or drastically reduced numbers of insulin-producing beta cells were regularly seen around 22 weeks of age (Table 1).

Cells expressing TNF-α mRNA are detected on most pancreas sections of 6-week-old animals, whereas granzyme A-positive cells in the intra-islet infiltrate are less frequent (Table 1). For both genes studied, the number of mRNA-positive cells increases with increasing infiltration of lymphocytes into the islets. At every time point studied, TNF-α-positive cells are approximately twice as frequent as granzyme A-positive cells. A frequency peak is observed at 18 weeks of age with an average of more than one TNF-α-expressing cell per 3-μm islet cross-section. TNF-α mRNA-positive cells are found as long as beta cells are present but are absent in the small islet remnants typically found in hyperglycemic animals. There was no significant alteration in the expression levels of the granzyme A or TNF-α gene with age or increasing insulitis, in contrast to alloreactive cells (13). The analysis of male NOD mice revealed that the lower incidence rate of insulin-dependent diabetes mellitus, 5% in male as compared to 70% in female NOD mice at 28 weeks of age, is also reflected by a lower frequency of TNF-α- and granzyme A-expressing cells: 20-week-old male NOD mice resemble ≈10-week-old female NOD mice with respect to severity of insulitis and incidence of TNF-α-positive cells. The number of granzyme A-positive cells is even more reduced in male NOD mice (Table 1).

Frequency and Phenotype of TNF-α mRNA-Positive Cells. To determine the frequency of cells expressing the TNF-α gene in the infiltrated pancreas, we isolated infiltrating mononuclear cells by collagenase digestion and subsequent Percoll gradient centrifugation. In the pancreas of female NOD mice between 12 and 18 weeks of age, we found an average of 3.7 TNF-α mRNA-positive cells per 1000 cells (mean of two independent experiments) (Table 2). The observed frequency of positive cells is 6–7 times higher than that found in the spleen (0.5/1000), in peripheral blood (0.6/1000), or in mesenteric lymph nodes (0.5/1000) (Table 2). The relevance of even low numbers of activated inflammatory cells in the pathogenesis of an autoimmune disease has been shown during the development of experimental allergic encephalomyelitis in rats (15).

Immunostaining revealed that the lymphocytic infiltration of islets of 12- to 18-week-old NOD mice predominantly consisted of Thy 1.2-positive cells. CD4-bearing cells are more commonly found in the lesions than cells expressing the

Table 1. Expression of granzyme A and TNF-α in the intra-islet infiltrate with progressive insulitis

NOD mice	Number of mice	Islets evaluated	Severity of insulitis	mRNA-positive cells/100 islet cross-sections	
				Granzyme A	TNF-α
6-week-old female	4	138	0.3	7	15
12-week-old female	5	241	1.0	26	48
18-week-old female	5	190	2.2	50	122
Hyperglycemic female	5	132	3.4	39	52
20-week-old male	3	68	0.9	4	32

Serial pancreas sections were hybridized *in situ* with ^{35}S-labeled antisense RNA probes of the TNF-α and granzyme A genes. Severity of insulitis was evaluated and calculated for each animal according to the following system of grades: 0, intact islets; 1, area of mononuclear cell infiltration within an islet was <25%; 2, 25–50%; 3, >50%; 4, end stage of insulitis characterized by small retracted islets with or without residual infiltrates (according to ref. 5). Animals were considered hyperglycemic after producing Tes-Tape (Eli Lilly) values of 2+ or higher. Mean onset of overt diabetes was 22 weeks of age.

Table 2. Frequency and phenotype of TNF-α mRNA-positive islet-infiltrating cells

Source	Cell subset	TNF-α mRNA-positive cells	
Spleen	Unfractionated	4/8389 (0.5)	
Lymph node	Unfractionated	2/4139 (0.5)	
Blood	Unfractionated	2/3436 (0.6)	
		Exp. 1	Exp. 2
Pancreas infiltrating cells	Unfractionated	29/9539 (3.0)	20/4528 (4.4)
Pancreas infiltrating cells	$CD4^-$	15/8654 (1.7)	7/3558 (2.0)
Pancreas infiltrating cells	$CD4^+$	15/5589 (2.7)	17/4481 (3.8)

TNF-α mRNA-positive cells are indicated as positive cells/total cells. In parentheses are shown positive cells per 1000 cells. Cells infiltrating the pancreas were isolated by collagenase digestion, sorted with the FACS, and subsequently hybridized at the single cell level with a ^{35}S-labeled antisense RNA probe of the TNF-α gene.

CD8 antigen. Close analysis of the infiltration showed that infiltrating cells located near islet cells consisted preferentially of T lymphocytes, some of which had penetrated into the islets, while B lymphocytes were mainly found at the periphery of the infiltrates.

Thus, the most frequent cell types at the predominant location of TNF-α-expressing cells are T cells. Macrophages are very rarely observed at this particular site, as determined by immunostaining with a monoclonal antibody to Mac1 (M1/70). We assumed, therefore, that at least some of the infiltrating TNF-α mRNA-expressing cells are of the $CD4^+$ or $CD8^+$ phenotype. For the determination of the phenotype of cells expressing the TNF-α gene, pancreas-infiltrating cells were isolated by collagenase digestion, stained with a fluoresceinated antibody to CD4 (GK1.5), sorted with a FACS, and subsequently hybridized with the antisense RNA probe of the TNF-α gene. Since cells expressing granzyme A are not exclusively present in the intra-islet infiltrate, we did not determine the phenotype of these cells. After sorting into a $CD4^-$ and a $CD4^+$ population (60% and 40% of total, respectively) (Fig. 2*A*) in two independent experiments, we found means of 1.9 and 3.3 TNF-α mRNA-expressing cells, respectively, per 1000 cells (Fig. 2*B*; Table 2). These numbers of TNF-α mRNA-positive cells are very likely to represent an underestimate, since TNF-α mRNA, which has a short half-life (16), is probably slowly degraded during the prolonged isolation procedure and is finally undetectable by *in situ* hybridization in pancreas-infiltrating cells with a low expression level of the TNF-α gene.

The data demonstrate that TNF-α mRNA-positive cells, exclusively present in the intra-islet infiltrate, are in part of $CD4^+$ phenotype. Together with the observation that cells at the predominant location of TNF-α-expressing cells—i.e., adjacent to the β-cell area, are mostly Thy 1.2-positive and barely Mac1-positive, we conclude that a considerable fraction of cells expressing the TNF-α gene in the lesion are $CD4^+$ T cells. It was shown only recently that production of TNF-α, originally described as a product of macrophages, can be induced *in vitro* in T cells (17), particularly $CD4^+$ T cells (18), and induction of TNF-α mRNA in T cells correlates with secretion of TNF-α protein (17). A crucial role for T cells in the development of insulitis and overt diabetes was previously predicted by adoptive transfer experiments (4, 5). Elimination of $CD4^+$ T cells *in vivo* with anti-CD4 antibodies prevents the development of the autoimmune disease (19).

The kinetics of target cell destruction in the islets of NOD mice is apparently very different from that of other cell-mediated cytotoxic reactions *in vivo*, where cells recognized as nonself are eliminated within a few days (13, 20, 21). Thus, even a low incidence of granzyme A- and TNF-α-positive cells over a long period of time may be sufficient to mediate considerable tissue damage and, therefore, relevant to autoimmune insulitis in NOD mice. Granzyme A-positive cells, mainly $CD8^+$ T cells, are cytolytic *in vitro* (12) and are

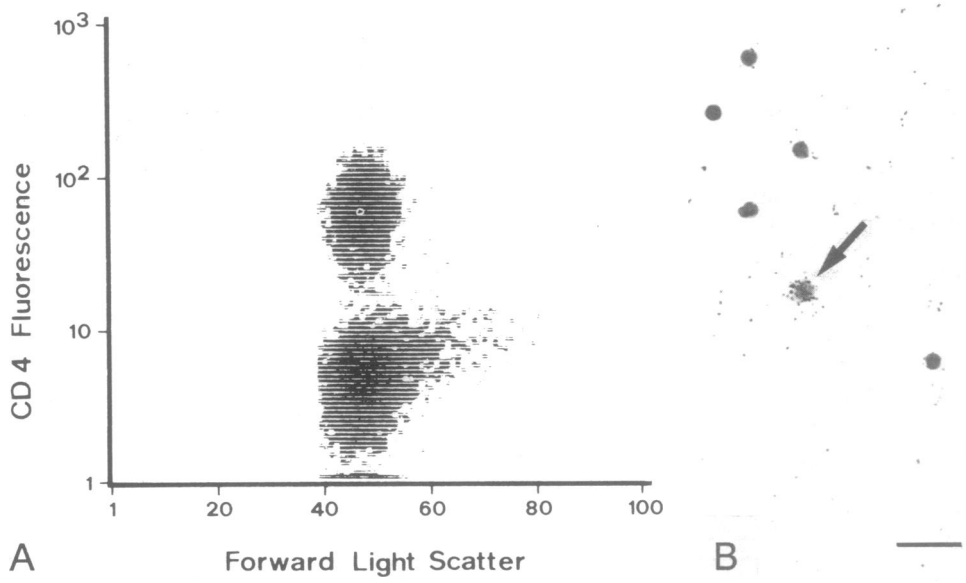

FIG. 2. Expression of the TNF-α gene in $CD4^+$ pancreas-infiltrating cells. Cells were isolated by collagenase digestion and separated into a $CD4^+$ (40%) and a $CD4^-$ (60%) population with a FACS (*A*). Separated cells were subsequently hybridized *in situ* at the single cell level with the ^{35}S-labeled antisense RNA probe of the TNF-α gene. The photomicrograph shows $CD4^+$ pancreas-infiltrating cells with one cell containing TNF-α mRNA (arrow in *B*). (Bar = 25 μm.) (See Table 2 for numerical values.)

prominent participants in allograft rejection *in vivo* (13). An important role for cells expressing TNF-α is supported by the effects of TNF-α upon islets *in vitro* (8–10), the restricted localization of TNF-α expression in the infiltrated islets *in vivo*, and expression of TNF-α partially in $CD4^+$ T cells *in vivo*, a cell subset that was found to be necessary for development of the disease (4, 5, 19). Expression of TNF-α also in $CD4^+$ T cells *in vivo* invites speculation on an involvement of this cytokine in other autoimmune diseases, which are solely mediated by $CD4^+$ T cells, such as experimental allergic encephalomyelitis.

Note. Since this work was done, Young *et al.* (22) have demonstrated immunohistochemically that perforin—another killer cell granule protein—is also present in the lymphocytic infiltrates of NOD mice developing diabetes, strengthening the interpretation that cytolytic phenotype cells are participants in these lesions.

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