
Original Articles

β -Cell Apoptosis in an Accelerated Model of Autoimmune Diabetes

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Abstract

Background: The non-obese diabetic (NOD) mouse is a model of human type 1 diabetes in which autoreactive T cells mediate destruction of pancreatic islet β cells. Although known to be triggered by cytotoxic T cells, apoptosis has not been unequivocally localized to β cells in spontaneously diabetic NOD mice. We created a model of accelerated β -cell destruction mediated by T cells from spontaneously diabetic NOD mice to facilitate the direct detection of apoptosis in β cells.

Materials and Methods: NOD.*scid* (severe combined immunodeficiency) mice were crossed with bm1 mice transgenically expressing the costimulatory molecule B7-1 (CD80) in their β cells, to generate B7-1 NOD.*scid* mice. Apoptosis in islet cells was measured as DNA strand breakage by the TdT-mediated-dUTP-nick end labeling (TUNEL) technique.

Results: Adoptive transfer of splenocytes from sponta-

neously diabetic NOD mice into B7-1 NOD.*scid* mice caused diabetes in recipients within 12–16 days. Mononuclear cell infiltration and apoptosis were significantly greater in the islets of B7-1 NOD.*scid* mice than in non-transgenic NOD.*scid* mice. Dual immunolabeling for TUNEL and either B7-1 or insulin, or the T cell markers CD4 and CD8, and colocalization by confocal microscopy clearly demonstrated apoptosis in β cells as well in a relatively larger number of infiltrating T cells. The clearance time of apoptotic β cells was estimated to be less than 6 min.

Conclusions: B7-1 transgenic β cells undergo apoptosis during their accelerated destruction in response to NOD mouse effector T cells. Rapid clearance implies that β cells undergoing apoptosis would be detected only rarely during more protracted disease in spontaneously diabetic NOD mice.

Introduction

Studies in the non-obese diabetic (NOD) mouse model of spontaneous insulin-dependent (type 1) diabetes have established a key role for cyto-

toxic CD8 T cells in effecting the destruction of insulin-producing, pancreatic islet β cells (1,2). Mediators of β -cell destruction include the contents of CD8 T-cell granules (e.g., perforin and granzymes), T-cell surface molecules (e.g., Fas-L, TNF, other TNF family members) and secreted cytokines (e.g., TNF, IFN- γ) (3–8). All these mediators are known to induce DNA fragmentation and the morphological changes of apoptosis through complex signaling cascades that involve

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the activation of cysteine proteases or caspases (9). However, there remains a paucity of direct evidence for apoptosis localized to β cells in the autoimmune lesion in vivo. O'Brien et al. (10) described morphologic changes of apoptosis in the islet lesion of spontaneously diabetic NOD mice, but they identified insulin-positive cells only in adjacent tissue sections. Kurrer et al. (11) localized DNA strand breaks, a hallmark of apoptosis (12–14), to insulin-positive cells by standard microscopy, but in a model of accelerated β -cell destruction in immunodeficient NOD.*scid* (severe combined immunodeficiency) mice expressing a single transgenic, anti-islet T-cell receptor. Apoptotic cells are rapidly removed by phagocytosis (12,15) and therefore may be difficult to detect in situ in the unmanipulated spontaneously diabetic NOD mouse if the relatively small and probably nonrenewable population of β cells is destroyed over a period of many weeks.

To directly demonstrate apoptosis in β cells undergoing destruction by normal NOD mouse T cells, we employed dual labeling and confocal scanning laser microscopy in a new transfer model of accelerated β -cell destruction. Splenocytes from spontaneously diabetic NOD mice were transferred into NOD.*scid* mice that expressed a transgene for the immune costimulator molecule B7-1 (CD80) in their β cells. This mouse was generated initially to study the influence of B7-1 on the effector phase of an autoimmune response (16). Enforced B7-1 expression in β cells has been shown to induce rejection of allogeneic islets deleted of passenger leukocytes (17), to induce autoimmune β -cell destruction in the presence of local (transgenic) TNF- α (18) or IL-2 (16), and to accelerate β -cell destruction in NOD mice (19). Therefore, we anticipated that our accelerated transfer model would facilitate detection of β -cell apoptosis in response to islet infiltration by normal NOD mouse T cells.

Materials and Methods

Mice

NOD mice (Lt/Jax) and NOD.*scid* mice were bred under specific pathogen-free conditions at the Walter and Eliza Hall Institute of Medical Research, Melbourne. Transgenic bm1 mice were generated by expressing murine B7-1 cDNA under the control of the rat insulin promoter (RIP). RIP-B7-1 NOD.*scid* mice were obtained by backcrossing RIP-B7-1 mice of the 377 lineage, which have high expression of B7-1 on all β cells, onto

the NOD.*scid* background for between four and seven generations and selecting at the second backcross generation for homozygosity at the NOD MHC locus and at the *scid* locus. Adoptive transfer of diabetes was performed by i.v. injection of 2×10^7 pooled splenocytes from newly diagnosed diabetic female NOD mice ($n = 7-10$) into male or female B7-1 NOD.*scid* mice, or transgene-negative littermates. Recipients were monitored for diabetes by measuring blood glucose in retro-orbital venous blood every 5 days, or earlier if glycosuria was detected. Diabetes was diagnosed if blood glucose exceeded 11 mM on two occasions.

Processing of Pancreatic Tissue

Pancreata ($n = 5$ mice per group) were removed 5, 10, or 15 days after adoptive transfer. Half of each pancreas was fixed in 4% neutral buffered paraformaldehyde (NB-PFA) and the other half snap-frozen in Tissue-Tek embedding compound 4583 (Miles, Elkhardt, IN) on liquid nitrogen. Sections (6 μm) were cut at six different levels of each pancreas and collected on slides coated with 3-aminopropyltriethoxy-silane (Sigma, St. Louis, MO).

Insulinitis Grading

Mononuclear cell infiltration of islets, insulinitis, was graded by scoring and then averaging a minimum of 15 separate islets in each pancreas. The grading scale was as follows: 0, no infiltration, islet intact; 1, infiltration only at the periphery of the islet; 2, peri- and intra-islet infiltration of <20%; 3, intra-islet infiltration of <50%; 4, massive infiltration of >50% of the islet.

Time Course of Apoptosis

Apoptosis was demonstrated by labeling for DNA strand breaks with the TdT-mediated-dUTP nick end labeling (TUNEL) technique. The extent of DNA strand breakage in apoptosis allows TUNEL to distinguish apoptosis from necrosis (12–14). Cryosections were postfixed in acetone for 5 min and in 2% NB-PFA for 30 min. Slides were rinsed in mouse-tonicity phosphate-buffered saline (MT-PBS) and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. After washing, sections were blocked sequentially with avidin (0.1% in 0.05 M Tris buffer) and biotin (0.01% in 0.05 M Tris buffer) at room temperature (RT). Sections were adapted to

terminal deoxynucleotidyl transferase (TdT) buffer (Promega, Madison, WI) and then incubated with the TdT-reaction mix containing 25 μ mol of CoCl_2 , 1.6 μ l TdT (19 units/ μ l; Promega) and 1 μ l biotin-conjugated dUTP (Boehringer-Mannheim, Mannheim, Germany) per 100 μ l TdT buffer for 60 min at 37°C. Sections treated with DNase I (10 ng/ml) for 10 min at RT were used as positive control for TUNEL staining. Sections were incubated in streptavidin-Texas red (Caltag; 1/500) for 1 hr to identify dUTP-positive nuclei. Apoptotic events per islet area were quantified by a calibrated eyepiece squared into 100 fields on an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany).

Detection of β Cells and Islet T Cells Undergoing Apoptosis

Insulin-positive apoptotic β cells were detected in paraffin sections of NB-PFA fixed tissue. Sections were dewaxed and digested with proteinase K (20 μ g/ml for 30 min at 37°C) and subjected to TUNEL as described above, followed by labeling for insulin with guinea pig anti-porcine insulin serum (Chemicon International Inc., Temecula, CA) diluted 1/300, for 30 min at RT. The secondary antibody was FITC-conjugated anti-guinea pig immunoglobulins (Jackson ImmunoResearch Labs, West Grove, PA).

Double immunolabeling for TUNEL and B7-1 or for TUNEL and T-cell markers was performed on cryosections postfixed with NB-PFA as described above. After TUNEL, sections were incubated with anti-CD4 (H129.19) and anti-CD8 (53-6.7) monoclonal antibodies for 1 hr at RT, followed by incubation with FITC-conjugated anti-rat IgG (Vector Lab., Burlingame, CA) diluted 1/50 and streptavidin-Texas red diluted 1/500, for 60 min at RT in the dark. B7-1 was detected with the hamster 16-10A1 monoclonal antibody (20) and FITC-conjugated anti-hamster immunoglobulins (PharMingen, San Diego, CA). The sections were mounted with fluorescence mounting medium (DAKO, Glostrup, Denmark) and analyzed by confocal microscopy.

Image Analysis for Quantification of Insulin-positive Islet Area

Paraffin sections were dewaxed and incubated with guinea pig anti-insulin serum, followed by horseradish peroxidase-conjugated rabbit anti-guinea pig immunoglobulins (DAKO) diluted 1/200, for 30 min at RT, and were color developed with 3-amino-9-ethylcarbazole (AEC)

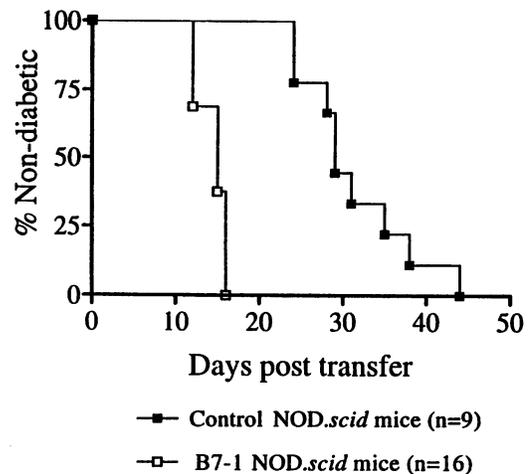


Fig. 1. Time course of diabetes development in B7-1 and control NOD.scid mice after intravenous injection of splenocytes from diabetic NOD mice. For each experiment, a litter of mice (female and male) received 2×10^7 splenocytes at day 0.

(DAKO). Analysis of the insulin-positive islet area, as a percent of total islet area, was performed with the LEICA Q 500 MC image processing and analysis system and combined with TUNEL to compare insulin-positive areas of islets with and without apoptosis.

Statistics

Data are represented as mean \pm SD. Differences among group results were analyzed by two-tailed *t* tests, with the Welsh adjustment. Kaplan-Meier survival curves were computed with Prism GraphPad Software, version 2 (San Diego, CA).

Results

Adoptive Transfer

Splenocytes from diabetic NOD mice transferred diabetes into both B7-1 and control NOD.scid mice. However, B7-1 NOD.scid mice developed diabetes within 16 days after transfer, whereas diabetes onset was delayed in control mice (Fig. 1). The mean time of diabetes onset in the B7-1 NOD.scid mice (15 ± 1.7 days) was significantly less ($p < 0.001$) than in controls (29 ± 6.2 days).

Insulinitis and β -Cell Destruction

Insulinitis was evaluated 5, 10, and 15 days after adoptive transfer. Both B7-1 and control

Table 1. Apoptotic events (mean \pm SD) and insulinitis scores (mean \pm SD) in B7-1 transgenic and control NOD.scid mice after transfer of splenocytes from diabetic NOD mice

| Days after Transfer of IDDM | Control NOD.scid Mice | | B7-1 Transgenic NOD.scid Mice | |
|-----------------------------|---|--------------------------------------|---|--|
| | Apoptotic Events/ $10^4 \mu\text{m}^2$ (<i>n</i> islets) | Insulinitis Score (<i>n</i> mice) | Apoptotic Events/ $10^4 \mu\text{m}^2$ (<i>n</i> islets) | Insulinitis Score (<i>n</i> mice) |
| 5 | 0.100 \pm 0.267 (<i>n</i> = 85) | 0.663 \pm 0.050 (<i>n</i> = 4) | 0.035 \pm 0.169 (<i>n</i> = 70) | 0.736 \pm 0.191 (<i>n</i> = 4) |
| 10 | 0.074 \pm 0.160 (<i>n</i> = 74) | 1.027 \pm 0.694 (<i>n</i> = 8) | 0.867 \pm 1.036 (<i>n</i> = 75)**.## | 2.194 \pm 0.643 (<i>n</i> = 8) ⁺⁺ |
| 15 | 0.304 \pm 0.523 (<i>n</i> = 51) | 1.500 \pm 0.551 (<i>n</i> = 3) | 1.115 \pm 2.526 (<i>n</i> = 66)*.# | 3.044 \pm 1.180 (<i>n</i> = 3) ⁺ |

* $p < 0.001$, ** $p < 0.0001$ compared to B7-1 transgenic mice at day 5.

$p < 0.05$, ## $p < 0.0001$ compared to control mice at the same time point.

⁺ $p < 0.005$, ⁺⁺ $p < 0.0001$ compared to control mice at the same time.

NOD.scid mice exhibited a progressive increase in the insulinitis score after transfer (Table 1). However, insulinitis was significantly greater by 10 days after transfer into B7-1 NOD.scid mice. At this time, B7-1 NOD.scid islets were infiltrated predominantly by CD8⁺ T cells, but confocal microscopy revealed apoptosis in both CD8⁺ and CD4⁺ T cells (Fig. 2).

At 10 days, the insulin-positive islet area in B7-1 NOD.scid mice ($61.5 \pm 24.5\%$, $n = 70$) was significantly less ($p < 0.0001$) than in control mice ($79.5 \pm 8.6\%$, $n = 63$). By day 15, when most of the B7-1 NOD.scid mice were diabetic, the respective insulin-positive islet areas were $23.2 \pm 30.6\%$ ($n = 11$) and $77.3 \pm 9.4\%$ ($n = 55$) ($p < 0.001$).

Apoptosis and β Cells

The number of apoptotic events per islet area was measured 5, 10, and 15 days after adoptive transfer. On day 5, the density of apoptotic events was low in islets of both transgenic and control NOD.scid mice, but by day 10 it was significantly higher in B7-1 NOD.scid mice (Table 1). At this time, the B7-1 NOD.scid mice were not yet diabetic but more than 70% of their islets were positive for apoptosis, compared to less than 25% in control mice. The density of apoptosis was related to the degree of β -cell destruction. For example, at day 10, the insulin-positive area of islets with apoptosis was significantly reduced ($41.9 \pm 21.7\%$, $p < 0.0001$) compared to islets without apoptosis ($75.1 \pm 14.7\%$).

B7-1 expression in B7-1 NOD.scid mice varied among individual islets, but in general mirrored inversely the degree of β -cell destruction. It was uniform and strong in islets without apoptosis (Fig. 3A) but was decreased or absent with β -cell destruction and apoptosis (Fig. 3B, C).

Double labeling for TUNEL and insulin in B7-1 NOD.scid mice clearly revealed the presence of apoptosis in insulin-positive β cells (Fig. 4A–C) and the absence of apoptosis in well-granulated, insulin-positive islets without evidence of β -cell destruction (Fig. 4D–F). Numeration revealed that 66/357 (18.5%) of the TUNEL-positive cells were insulin-positive 10 days after transfer. Occasional cells that did not stain for insulin, or for CD4 or CD8, were also observed to undergo apoptosis. Although some of these may be β cells that had degranulated and lost insulin expression, the small, uniform size of many of them suggests that they were T cells that had lost CD4 or CD8 expression in the process of undergoing apoptosis.

Our quantitative data allowed us to approximate the clearance rate for apoptotic β cells on the basis of several assumptions. If the mouse has 400 islets each containing 1000 β cells and if these β cells are destroyed over 5 days in a B7-1 NOD.scid mouse, then the rate of β -cell loss would average 56 cells/minute/mouse. On day 10, we found that the number of apoptotic cells/islet was 4.2 ± 6.51 (mean \pm SD), of which 18.5%, or 0.779, were β cells. The number of apoptotic β cells/mouse is therefore $0.779 \times 400 = 312$. If we assume that all β cells die by apoptosis and that the death rate over 5 days is

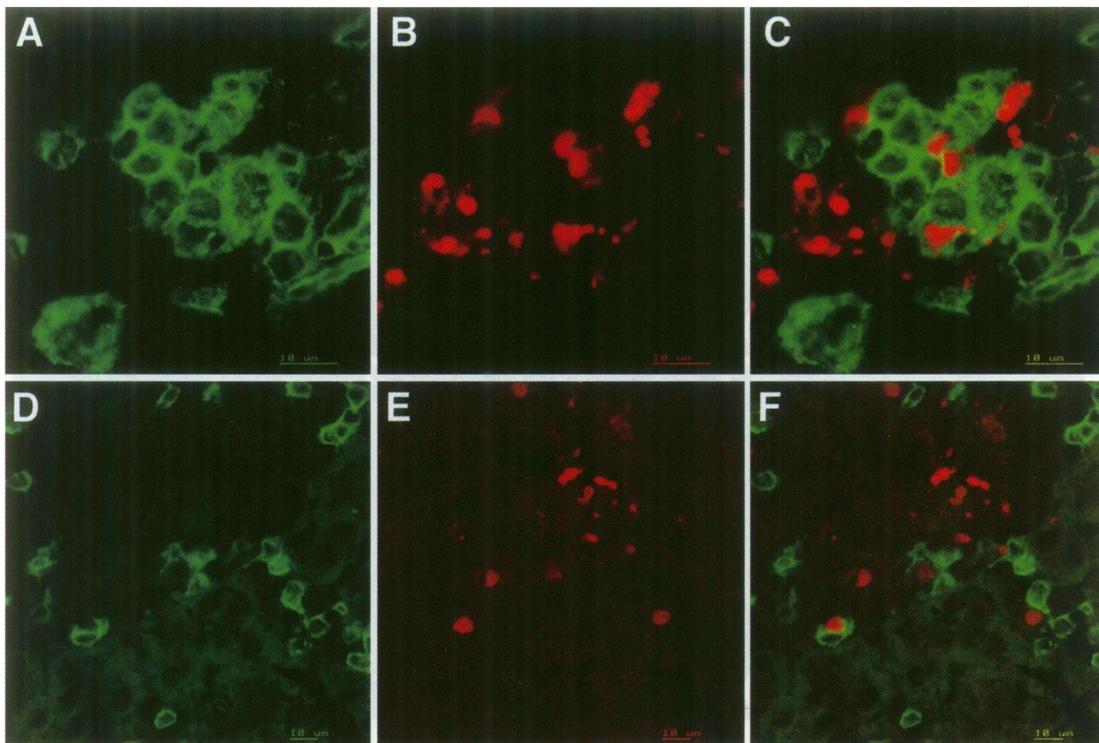


Fig. 2. Confocal microscopy of islets from a B7-1 NOD.scid mouse 10 days after transfer of splenocytes from diabetic NOD mice. Islets were stained for CD8 (green: A, C) and CD4 (green: D, F) and for TUNEL (red: B, C, E, F). Relative magnifications are indicated by the 10 μ m bars.

linear, then the clearance rate for apoptotic β cells is 312 (apoptotic β cells)/ 56 (β cells lost/minute) = 5.6 min.

Discussion

Aberrant expression of the costimulator molecule B7-1 on β cells provides a potent signal that

enhances T cell-mediated β -cell death on the NOD genetic background. Insulinitis and diabetes were significantly accelerated and synchronized when splenocytes from spontaneously diabetic NOD mice were transferred into B7-1 NOD.scid recipient mice. This represented an optimized situation in which to detect β -cell apoptosis in response to NOD mouse T cells. The onset of diabetes in B7-1 NOD.scid mice was preceded by

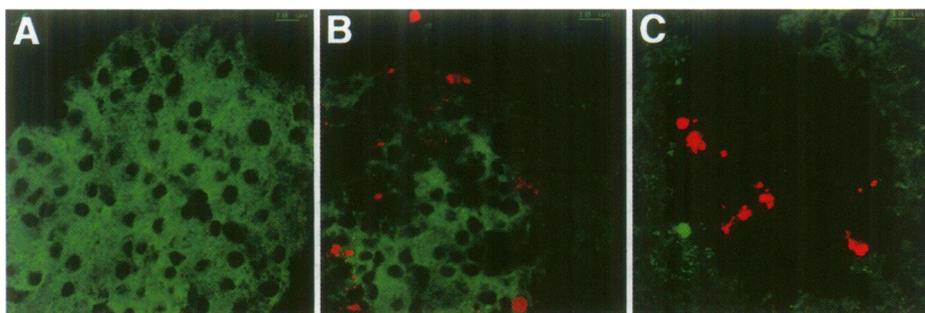


Fig. 3. Confocal microscopy of islets from B7-1 NOD.scid mice showing different degrees of β -cell destruction, 10 days after transfer of splenocytes from diabetic NOD mice. B7-1 is labeled green and TUNEL-positive nuclei are labeled red. (A) Uniform, strong B7-1 staining with no evidence of β -cell loss or TUNEL-positive nuclei. (B) Nonuniform B7-1 staining of cells also positive for TUNEL, in an islet with patchy β -cell loss. (C) Absent B7-1 staining in an islet positive for TUNEL.

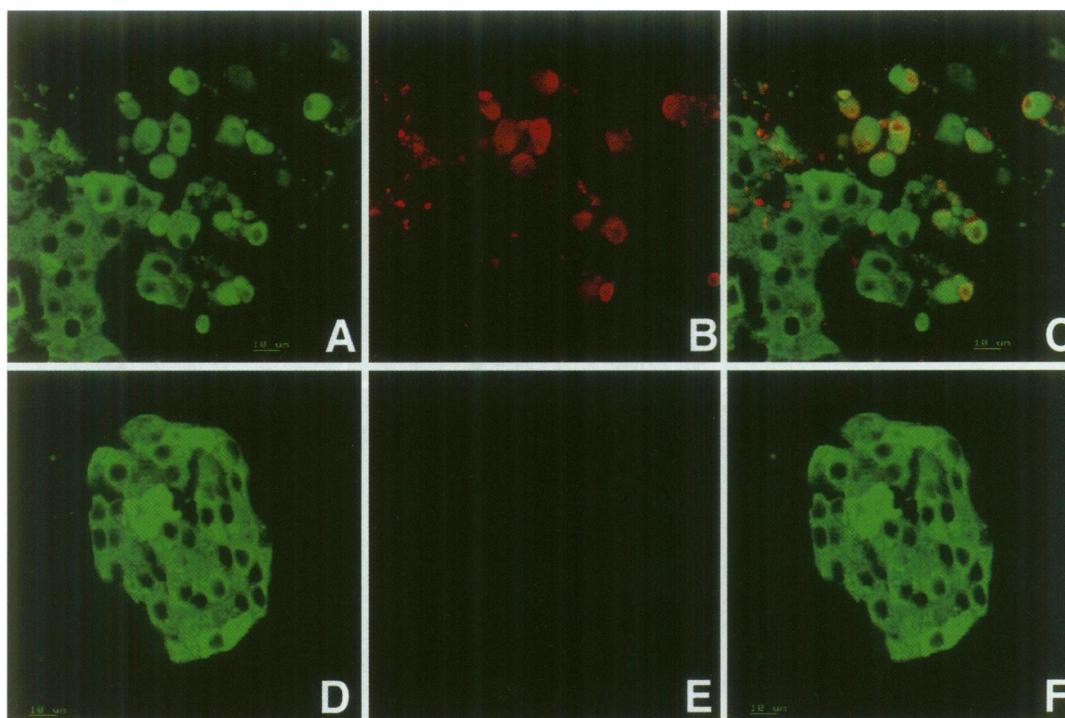


Fig. 4. Confocal microscopy of islets from B7-1 NOD.scid mice labeled for insulin (green: A, D) and TUNEL (red; B, E) 10 days after transfer of splenocytes from diabetic NOD mice. The overlaid image (C) reveals insulin-positive β cells undergoing apoptosis. Well-granulated insulin-positive islets were negative for TUNEL (D–F).

marked intra-islet infiltration and a reduced insulin-positive islet area. By 10 days after transfer we also observed an increased density of apoptotic events in the islets and a high percentage of islets positive for apoptosis. Dual labeling for TUNEL-positive cells and either β cells (insulin or B7-1) or infiltrating T cells ($CD8^+$ or $CD4^+$) and colocalization by confocal microscopy revealed that a minority of apoptotic cells were β cells and the majority were infiltrating cells.

Two groups (10,11) have recently documented apoptosis in the insulinitis lesion. In NOD mice, O'Brien et al. (10) reported infrequent β -cell apoptosis, detected by morphologic criteria in hematoxylin and eosin sections. However, they identified apoptotic β cells only by labeling for insulin in adjacent tissue sections and not by dual labeling. This indirect approach to localization may be inaccurate and could overestimate the number of apoptotic β cells, as the majority of detectable apoptotic events are in cells of the immunoinflammatory infiltrate. The use of confocal scanning laser microscopy allowed us to obtain high-resolution, high-magnification images of single optical sections of dual-labeled islets and thereby unequivocally demonstrate apo-

ptosis in β cells as well as in infiltrating T cells. Kurrer et al. (11) labeled for TUNEL and insulin to demonstrate β -cell apoptosis by standard light microscopy in a different accelerated model, in which NOD.scid mice express only transgenic anti-islet $CD4^+$ T cells. They estimated the average clearance rate of apoptotic β cells at 1.7 min. In our model, in which β -cell apoptosis was identified precisely by confocal microscopy, the estimate was 5.6 min. Given the various assumptions that have to be made to derive these values, too much should not be made of this difference. The point is that clearance occurs within minutes and therefore there is little likelihood of capturing evidence of β -cell apoptosis in spontaneously diabetic NOD mice in which β -cell destruction probably occurs over many weeks rather than days. The narrow time frame of β -cell destruction in the accelerated model facilitates detection of short-lived apoptotic events. Nevertheless, only a minority of the TUNEL-positive cells within infiltrated islets (18.5% 10 days after transfer) were β cells.

By manipulating the NOD mouse transgenically, enforced expression of either $CD4^+$ (11) or $CD8^+$ (18) T-cell receptors can be shown to kill β

cells. An advantage of the B7-1 NOD.*scid* model is that the effector T cells are derived from spontaneously diabetic NOD mice and are both CD4⁺ and CD8⁺. Adoptive transfer into the B7-1 NOD.*scid* mouse therefore more closely recapitulates, albeit in an accelerated fashion, the process of β -cell destruction in spontaneous NOD diabetes.

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