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Apoptosis and beta-cell destruction in pancreatic islets of NOD mice with spontaneous and cyclophosphamide-accelerated diabetes

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Summary Autoimmune-mediated destruction of pancreatic islet beta cells leads to insulin-dependent diabetes in non-obese diabetic (NOD) mice. Although both direct cytotoxic T cell- and indirect cytokine-, nitric oxide- or free radical-mediated mechanisms induce beta-cell apoptosis in vitro, beta-cell death in vivo in spontaneous autoimmune diabetes is not well-characterized. Furthermore, whether beta cells die gradually, or rapidly in the late pre-clinical stage, is a question of current interest. To investigate beta-cell death in vivo, we measured the frequency and intra-islet localisation of apoptosis, defined as DNA strand breaks by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) technique, during spontaneous and cyclophosphamide-accelerated diabetes in NOD mice. In spontaneous diabetes, the frequency of apoptosis in islets correlated with the progression of beta-cell destruction with age. Although apoptosis was detected at low frequency within the reduced insulin-positive islet area of pre-diabetic mice at 90 days of age, it was rarely co-localised to beta cells. After acceleration of beta-cell destruction with cyclophosphamide, the frequency of apoptosis reached maximum at 12 days, at which time 3.2 % of apoptotic cells were beta cells. Apoptosis was most frequent in the insulin-negative islet area comprised of mononuclear cell infiltrate and was localized to $CD8^+$ T cells. The rarity of detectable apoptotic beta cells in spontaneous pre-diabetic mice with pronounced insulitis and reduced insulin-positive islet areas most likely reflects the rapid clearance of apoptotic beta cells. Our findings are more consistent with gradual destruction of non-renewable beta-cells in spontaneous diabetes, than with their rapid, accelerated destruction (as after cyclophosphamide) in the late pre-clinical stage. [Diabetologia (1998) 41: 1381–1388]

Keywords NOD mouse, beta-cell apoptosis, diabetes, T cells, cyclophosphamide

Type I (insulin-dependent) diabetes mellitus in humans and non-obese diabetic (NOD) mice is the consequence of selective, autoimmune-mediated destruction of pancreatic islet beta cells [1–3]. A model has been proposed in which beta-cell function decreases progressively in the presence of islet autoimmunity [1]. It is, however, still controversial whether beta-cell destruction in the NOD mouse occurs gradually over months following mononuclear cell infiltration of islets (insulitis) at 4–6 weeks of age [4, 5] or more rapidly in the days to weeks preceding diabetes onset [6, 7]. Beta cells could be destroyed by direct interaction with cytotoxic CD8 [8–11] or CD4 [12] T cells, or indirectly via soluble cytokines or free radicals released by inflammatory cells within the islet lesion [13–16]. Beta-cell death could occur by at least two mechanisms, necrosis or apoptosis, which are biochemically and morphologically distinct. Initiated by a variety of intercellular and intracellular signals, apoptosis or programmed cell death is executed

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Abbreviations: NOD, Non-obese diabetic; NB-PFA, neutral buffered paraformaldehyde; TUNEL (TdT-mediated dUTP nick end labelling); TdT, terminal deoxynucleotidyl transferase; MT-PBS, mouse tonicity phosphate buffered saline.

through an intrinsic cascade that triggers cysteine proteases to activate endonucleases, resulting in DNA fragmentation and associated nuclear and cytoplasmic condensation. The cell or its apoptotic fragments are then rapidly phagocytosed by neighbouring cells or macrophages [17, 18]. In vivo studies of beta-cell death [11–15] strongly favour the apoptotic mechanism. Cytokines induce mainly apoptosis not necrosis in human islet cells [19] and two other molecules strongly implicated in the active process of apoptosis, perforin from cytotoxic T cells and Fas which interacts with Fas ligand on cytotoxic T cells, are necessary for diabetes development in NOD mice [20, 21].

Although experimental studies implicate apoptosis as the mechanism of beta-cell death, there is little direct evidence of beta-cell apoptosis in vivo in spontaneous autoimmune insulitis. One study reported apoptosis of beta-cells in NOD mice but did not co-localise apoptosis directly to insulin-positive islet cells [22]. Co-localisation is necessary because immuno-inflammatory cells in the islet infiltrate are likely to be a major source of apoptotic events. DNA strand breaks have been co-localized to insulin-positive cells, but in a transgenic NOD T-cell receptor model of rapidly accelerated beta-cell destruction [12]. If beta cells are destroyed over several months in spontaneous NOD mouse diabetes, apoptotic beta cells could be difficult to detect, given the rapid clearance of apoptotic cells reported in other situations [17, 23]. Therefore, to directly localise apoptosis to beta and non-beta cells within the islet lesion we studied, in addition to unmanipulated NOD mice, mice in which destructive insulitis was accelerated by cytophosphamide. The frequency and the intra-islet location of apoptotic cells was documented temporally in relation to beta-cell destruction, to elucidate further the natural course of pre-clinical disease.

Materials and methods

NOD mice. NOD mice (Lt/Jax) were bred under specific conditions free of pathogens in the animal breeding facilities of the Walter and Eliza Hall Institute of Medical Research. Protocols were approved by the Animal Ethics Committee. The incidence of diabetes is 90 % in females and 35 % in males at 250 days of age. Diabetes was diagnosed if two sequential measurements of blood glucose exceeded 11 mmol/l. Female NOD mice used in these experiments were litter mixed and housed in groups of eight. Beginning at 50 days of age, every 2 weeks four mice were killed by CO_2 inhalation for islet immunohistochemistry. To accelerate diabetes, a single dose of cyclophosphamide (CYP, Pharmacia, North Ryde, Australia) of 300 mg/kg was given i. p. to male NOD mice aged 90 days.

Insulitis grading. Insulitis was graded by scoring and then averaging a minimum of 35 separate islets in 4 % neutral buffered paraformaldehyde (NB-PFA)-fixed sections stained with haematoxylin and eosin. The grading scale was as follows: 0, no infiltration, islet intact; 1, infiltration only at islet periphery; 2, intra-islet infiltration under 20 % of islet; 3, intra-islet infiltration under 50 % of islet; 4, massive infiltration more than 50 % of islet.

Immunohistochemistry for detection of apoptotic beta cells. Pancreata from female NOD mice at 50, 64, 78 and 90 days of age and at the onset of diabetes ($n = 4$ at each time), and from male NOD mice ($n = 6$) 12 days after injection of cyclophosphamide, were removed immediately and fixed in 4 % NB-PFA. Tissue was embedded in paraffin and sections (6 μm) from six levels of the pancreas were collected on slides coated with 3-amino-propyltriethoxy-silane (Sigma, St. Louis, USA).

Apoptosis was shown by labelling DNA strand breaks with the TUNEL (TdT-mediated dUTP nick end labelling) technique, followed by staining for insulin to detect beta cells. The extent of DNA strand breakage in apoptosis allows TUNEL to distinguish apoptosis from necrosis [24, 25]. Sections were deparaffinised and digested with proteinase K (20 μg/ml for 30 min at 37 °C). Endogenous biotin or avidin was quenched by sequential incubation with avidin (0.1 % in 0.05 mol Tris buffer) or biotin (0.01 % in 0.05 mol tris (hydroxymethyl) aminomethane (Tris) buffer, (DAKO, Carpinteria, USA) for 20 min at room temperature. Sections were adapted to terminal deoxynucleotidyl transferase (TdT) buffer (500 mmol/l cacodylate buffer, 5 mmol/l $CoCl_2$; Promega, Madison, USA). TdT (19 Units/μl; Promega) was then used to label free 3′-OH DNA ends with biotin-conjugated dUTP (50nmol/l; Boehringer Mannheim, Germany) at 37 °C for 60 min. For this, TdT buffer was substituted with 25 mmol/l $CoCl_2$, 1.6 μl TdT and 1 μl biotin-conjugated dUTP per 100 μl. Slides treated with DNaseI (10 ng/ml) for 10 min at room temperature were used as a positive control. Slides were washed in mouse tonicity phosphate buffered saline (MT-PBS) and incubated with guinea pig anti-insulin serum (Chemicon International INC., Temecula, USA) diluted 1/300, for 30 min at room temperature. After washing, sections were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-guinea pig immunoglobulins (DAKO) diluted 1/200 and streptavidin-conjugated alkaline phosphatase diluted 1/100 (Calbiochem, La Jolla, USA), for 30 min at room temperature. Staining for insulin was developed with 3-amino-9-ethylcarbazole (AEC, DAKO) for 10 min, followed by staining for TUNEL-positive cells with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NPT; Sigma) for 15 min. The sections were mounted with glycerogel (DAKO). Cells showing dark blue nuclear BCIP/NPT staining (TUNEL-positive) surrounded by a distinct red-brown cytoplasmic ring of AEC precipitate were considered to be beta cells undergoing apoptosis.

A confocal laser-scanning microscope (Leica Lasertechnik, Heidelberg, Germany) was also used to co-localise insulin and TUNEL and thereby demonstrate apoptotic beta cells. The secondary reagents were fluorescein isothiocyanate (FITC)-conjugated anti-guinea pig immunoglobulins (Jackson ImmunoResearch Labs, West Grove, Pa., USA) and streptavidin-Texas red (Caltag, San Francisco, Calif., USA).

Quantification of apoptosis. Using the LEICA Q 500 MC image processing and analysis system, calibrated to 10 μm with an object micrometer (Olympus, Tokyo, Japan), we quantified apoptosis in light microscopic sections. Double-staining for apoptosis and beta cells enabled quantification of the frequency of apoptosis within three different areas: the whole islet area, the insulin-positive islet area and the insulin-negative islet area. The whole islet included all endocrine cells within the capsule and, if insulitis was present, the intra-islet infiltrate. The insulin-positive islet area represented beta cells which, in older mice, could

be surrounded by infiltrating mononuclear cells. The insulin-negative islet area consisted predominantly of infiltrating mononuclear cells and presumably some non-beta endocrine cells. Frequency was expressed as apoptotic events/10^4 μm^2 islet area. Sections stained by immunofluorescence were examined with an Axiophot fluorescence microscope (Zeiss, Oberkochen, Germany) and apoptotic events/islet area quantified using a calibrated eyepiece squared into 100 fields.

Localisation of apoptosis in cryopreserved islets after cyclophosphamide injection. After injection with cyclophosphamide, four mice were killed on days 3, 7, 9, 12 and 15 to determine the time course of islet apoptosis. Pancreata were snap frozen in isopentane in liquid-nitrogen and stored at –70 °C until use. Cryosections (6 μm) were post-fixed with acetone (5 min) and then 2 % NB-PFA (30 min) at room temperature. Slides were rinsed in MT-PBS and incubated in permeabilisation solution (0.1 % Triton X-100, 0.1 % sodium citrate) for 2 min on ice. After washing, sections were blocked with avidin or biotin and stained for TUNEL as described above.

To detect apoptotic T cells, sections were washed in MT-PBS after TUNEL and stained with anti-CD3 (KT3), anti-CD4 (H129.19) or anti-CD8 (53–6.7) monoclonal antibodies, followed by incubation with FITC-conjugated rabbit anti-rat IgG (1/50, Vector Lab. Inc., Burlingame, USA) and streptavidin-Texas red (1/500) for 60 min at room temperature in the dark. The sections were washed and mounted with fluorescence mounting medium (DAKO).

Statistical analysis. Data are presented as means ± standard deviation (SD). Group data were compared by the two-tailed Student *t*-test or the non-parametric Mann-Whitney test (for insulitis scores).

Results

Unmanipulated NOD mice. In unmanipulated female NOD mice, the frequency of apoptosis in islets was related to age. At 50 days of age, apoptosis was seldom detectable (Fig. 1 a); only 14/98 (14 %) of islets contained cells undergoing apoptosis and nearly all of these were in the insulin-negative area. At 90 days of age, the proportion of islets positive for apoptosis had increased to 56/102 (55 %) (Table 1). At this age, 53/56 (95 %) of apoptosis-positive islets contained apoptotic cells in the insulin-negative area, but 9/56 (16 %) now had apoptotic cells in the insulin-positive area (Table 1). In newly-diagnosed diabetic mice, age 126 ± 16 days, 16/17 (94 %) of islets were positive for apoptosis.

The frequency of apoptosis was fairly low at 50 and 64 days of age but thereafter increased noticeably, together with the insulitis scores (Fig. 2 A, Table 1). The maximum frequency, 1.2 ± 1.8 per 10^4 μm^2, was seen in newly-diagnosed diabetic mice. Although the frequency of apoptosis in the insulin-positive area was increased at 78 and 90 days (Fig. 2 C), co-localisation by confocal microscopy seldom showed apoptotic beta cells.

At each age, apoptosis was associated with beta-cell destruction and was detected preferentially in islets with the lowest per cent insulin-positive area or highest insulitis score. The per cent insulin-positive islet area was lower in islets with apoptosis at 50, 64, 78 and 90 days of age (Fig. 3). By 90 days of age, more than half the islets were positive for apoptosis and in these the insulin-positive area was reduced by more than 50 % (Fig. 3).

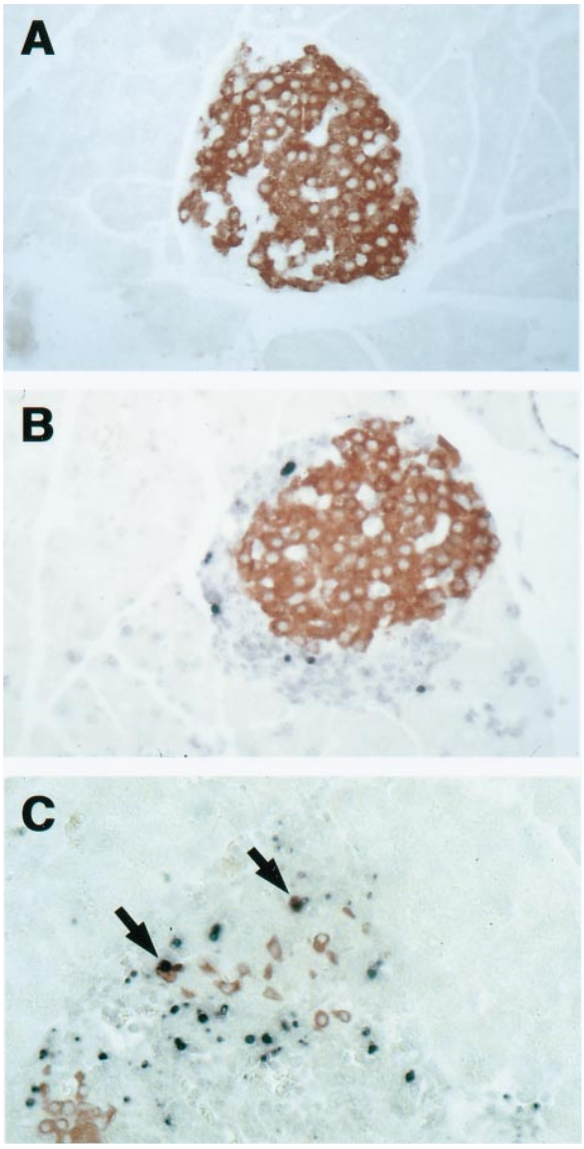

Fig. 1 A–C. Double-staining by immunohistochemistry for insulin (red-brown) and apoptosis (TUNEL-positive DNA strand breaks) (dark blue) in paraffin sections of NOD mouse pancreas. Islets of 50-day-old female mice were negative for apoptosis (**A**). Apoptotic cells were seen mainly in the insulin-negative islet area of islets from 90-day-old female mice (**B**). Apoptotic beta cells were identified by a TUNEL-positive nucleus surrounded by insulin-positive cytoplasm in male mice 12 days after cyclophosphamide injection (**C**, arrowed)

NOD mice treated with cyclophosphamide. Apoptotic events per islet were correlated with the islet area 3, 7, 12 and 15 days after injection with cyclophospha-

Table 1. Time course of apoptosis in unmanipulated NOD mice

Age (days)	Insulitis score	Whole islet area	Insulin-negative islet area	Insulin-positive islet area
		Islets with apoptosis (%)	Islets with apoptosis (%)	Islets with apoptosis (%)
50 ($n = 4$)	0.59 ± 0.874	14/98 (14)	13/98 (13)	1/98 (1)
64 ($n = 4$)	0.81 ± 0.561	10/103 (9.7)	9/103 (8.7)	1/103 (0.97)
78 ($n = 4$)	1.9 ± 1.35[a]	42/100 (42)	38/100 (38)	13/100 (13)
90 ($n = 4$)	2.2 ± 1.51[a]	56/102 (55)	53/102 (52)	9/102 (8.8)

[a] $p < 0.001$ compared with age 50 days

mide. By day 12, when all islets were positive for apoptosis, the correlation between individual islet area and number of apoptotic events was $r = 0.71$ ($p < 0.0001$). This relation allowed us to compare the frequency of apoptosis per unit islet area (10^4 μm^2) in islets of different sizes. By day 3, 33/50 (66%) of islets were positive for apoptosis but the frequency of apoptotic events per whole islet area was low; however, this increased ($p < 0.01$) by 9, 12 and 15 days. By day 12, 56/56 (100%) of islets contained apoptotic cells at maximum frequency (Fig. 4).

To show beta-cell apoptosis, further quantitative and qualitative assessment of apoptosis was done on paraffin-embedded tissue from day 12. At this time, the insulin-positive islet area averaged 37 ± 21.8% ($n = 115$ islets) and 88/115 (77%) of islets had an insulin-positive area less than 50%. There was an inverse relation between the frequency of apoptosis and the insulin-positive area ($r = –0.53$, $p < 0.0001$). In the whole islet area apoptotic events averaged 4.5 ± 2.6 ($n = 115$) and 108/115 (94%) of islets were positive. The lowest frequency, 1.9 ± 3.2, was in the insulin-positive islet area, and 78/108 (72%) of islets were positive. The highest frequency, 6.2 ± 3.3, was in the insulin-negative area, and 108/108 (100%) of islets were positive. At 12 days after injection with cyclophosphamide, 77/2455 (3.2%) of cells undergoing apoptosis in the islet lesion were also insulin-positive (Fig. 1 C). Localization of apoptosis to beta cells was confirmed by confocal microscopy (Fig. 5). The evaluation of light (Fig. 1 c) and confocal (Fig. 5) images consistently showed only a small minority of apoptotic beta cells.

Immune markers on cells undergoing apoptosis. At day 12 after injection with cyclophosphamide, the insulin-negative islet area that comprised infiltrating mononuclear cells contained the highest frequency of apoptotic cells. As cell-surface markers are sensitive to chemical fixation, we used cryosections to identify both T cells and apoptotic events. Apoptosis was observed in CD3-positive cells that were CD8$^+$ (Fig. 5 A, B) but apoptotic CD4$^+$ T cells could not be shown (Fig. 5 C).

Discussion

To analyse the relation between beta-cell destruction and apoptosis in the islet lesion of NOD mice with either spontaneous or cyclophosphamide-accelerated diabetes, we quantificated DNA strand breakage by the TUNEL technique in three islet areas: the whole islet, the insulin-positive islet and the insulin-negative islet. Although the differentiation of apoptosis from necrosis can be controversial, extensive DNA strand breakage shown by the TUNEL technique is characteristic of apoptosis [17, 24, 25]. Although it cannot be excluded that beta cells also die by necrosis, there is no evidence for this and the experimental data [11–15, 19–21] support apoptosis as the major mechanism of beta-cell death. In accordance with the litera-

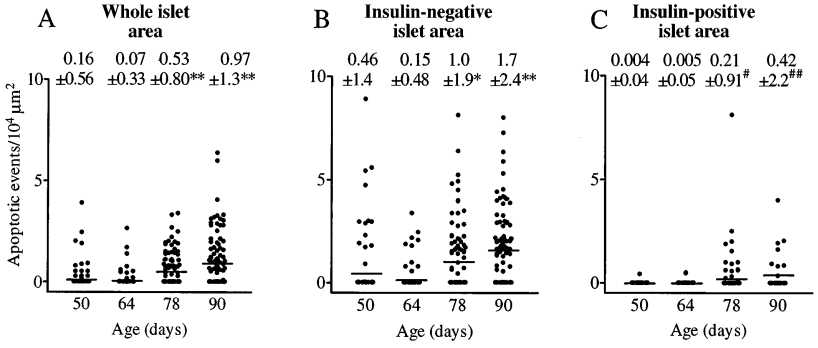

Fig. 2 A–C. Frequency of apoptosis by age in whole (**A**), insulin-negative (**B**) and insulin-positive (**C**) islet areas of unmanipulated female NOD mice. Data were obtained from 98, 103, 100 and 102 individual islets at the respective ages. The means ± SD is given above each group; *$p < 0.01$, **$p < 0.001$), #$p < 0.02$, ##$p < 0.05$ compared with day 50

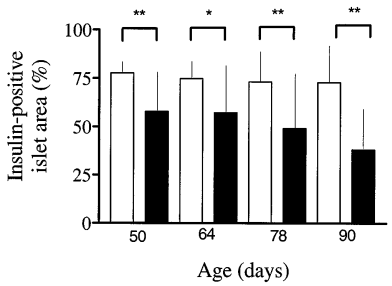

Fig. 3. Comparison of insulin-positive areas with (■) or without (□) apoptosis in islets of female NOD mice by age. The insulin-positive islet area is reduced in islets with apoptosis (*$p < 0.005$; **$p < 0.0001$). Data are represented as means ± SD

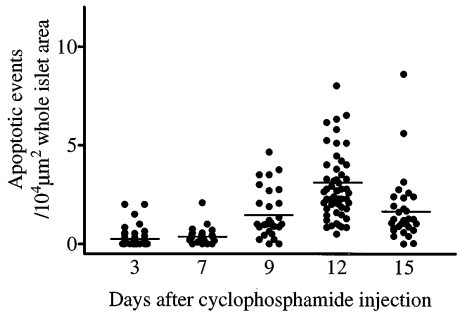

Fig. 4. Time course of frequency of apoptosis in whole islets after injection with cyclophosphamide. Islets were analysed from six mice at each time

ture [3, 5, 26], the insulitis score increased and the insulin-positive islet area decreased as female mice became older. A relation between insulitis and beta-cell destruction has been described [4]. Although it was found that beta-cell loss began with insulitis, a major reduction of beta-cell mass did not occur until insulitis was extensive, similar to what we saw at 78–90 days of age. In addition, we observed a relation between the per cent of islets positive for apoptosis and age. In islets with apoptosis, the insulin-positive islet area was decreased at all time points, and the frequency of apoptosis reflected the severity of beta-cell destruction. Apoptotic events were, however, seldom located in the insulin-positive area, being found mainly in the intra-islet infiltrate. Confocal microscopy showed that TUNEL-positive cells even in the insulin-positive islet area were usually not insulin-positive and were therefore more likely to be infiltrating cells.

Different dynamics of infiltrating mononuclear cell and beta-cell turnover could account for the higher frequency of apoptosis in the insulin-negative compared with insulin-positive islet areas. Rodent beta cells normally have a turnover of less than 3% of total cells per day [27, 28], which must be exceeded in mice with destructive insulitis. If, however, beta-cell destruction occurs over weeks to months, and the clearance of apoptotic cells is extremely rapid [17, 23], then very few beta cells are likely to be observed undergoing apoptosis at any one time. In accelerated beta-cell destruction in NOD *scid* mice mediated by a NOD mouse transgenic $CD4^+$ T-cell receptor, it was estimated that apoptotic beta cells were removed within 1.7 min and had an expected frequency of less than a single cell per 100 islets in unmanipulated NOD mice [12]. In a second accelerated model resulting from transfer of splenocytes from spontaneously-diabetic female NOD mice into NOD mice transgenically expressing the co-stimulator molecule B7–1 in their beta cells [29], we also estimated that apoptotic beta cells were cleared within minutes. Thus, it is not surprising that beta cells undergoing apoptosis in unmanipulated NOD mice are rarely detected.

During this investigation, O'Brien et al. [22] published a report of beta-cell apoptosis in unmanipulated NOD mice. They also found an increase in the frequency of apoptosis with age, reaching maximum near the onset of diabetes, and not equally distributed between islets. They reported, however, that beta-cell apoptosis was frequent, whereas we could find only the rare example. The explanation could be methodological. They identified apoptosis by morphologic criteria in haematoxylin and eosin stained sections and beta cells by insulin staining in adjacent tissue sections, whereas we examined sections that were double-stained for TUNEL and insulin and confirmed the results by confocal microscopy. Given the predominance of apoptosis in the intra-islet infiltrate, the lack of co-localisation could lead to overestimation of the frequency of beta-cell apoptosis. O'Brien et al. [22] also reported the number of apoptotic cells per islet. Individual islet areas were, however, remarkably variable and we showed the number of apoptotic events is related to islet area. Therefore, expression of apoptotic events per islet area is likely to be a more objective basis for comparison. Because the morphology of apoptotic cells is characterised by nuclear and cytoplasmic condensation it could be difficult to identify insulin by staining TUNEL-positive cells, and this would result in underestimation of the number of apoptotic beta cells. We confirmed our light-microscopical results, however, by confocal microscopy, which allowed us to separate staining of the nucleus and cytoplasm and show in most cases, insulin-negative cytoplasm in apoptotic cells.

To increase the probability of detecting apoptosis in beta cells, the onset of diabetes was accelerated by cyclophosphamide. By 12 days after injection with cyclophosphamide beta-cell destruction was widespread, shown by pronounced intra-islet infiltration and a reduced insulin-positive area, and by considerably decreased pancreatic insulin protein and mRNA [30]. At this time, double-staining for apoptosis and insulin showed that 3.2% of apoptotic cells were beta cells. Although it is not possible to accurately count the total number of individual beta cells

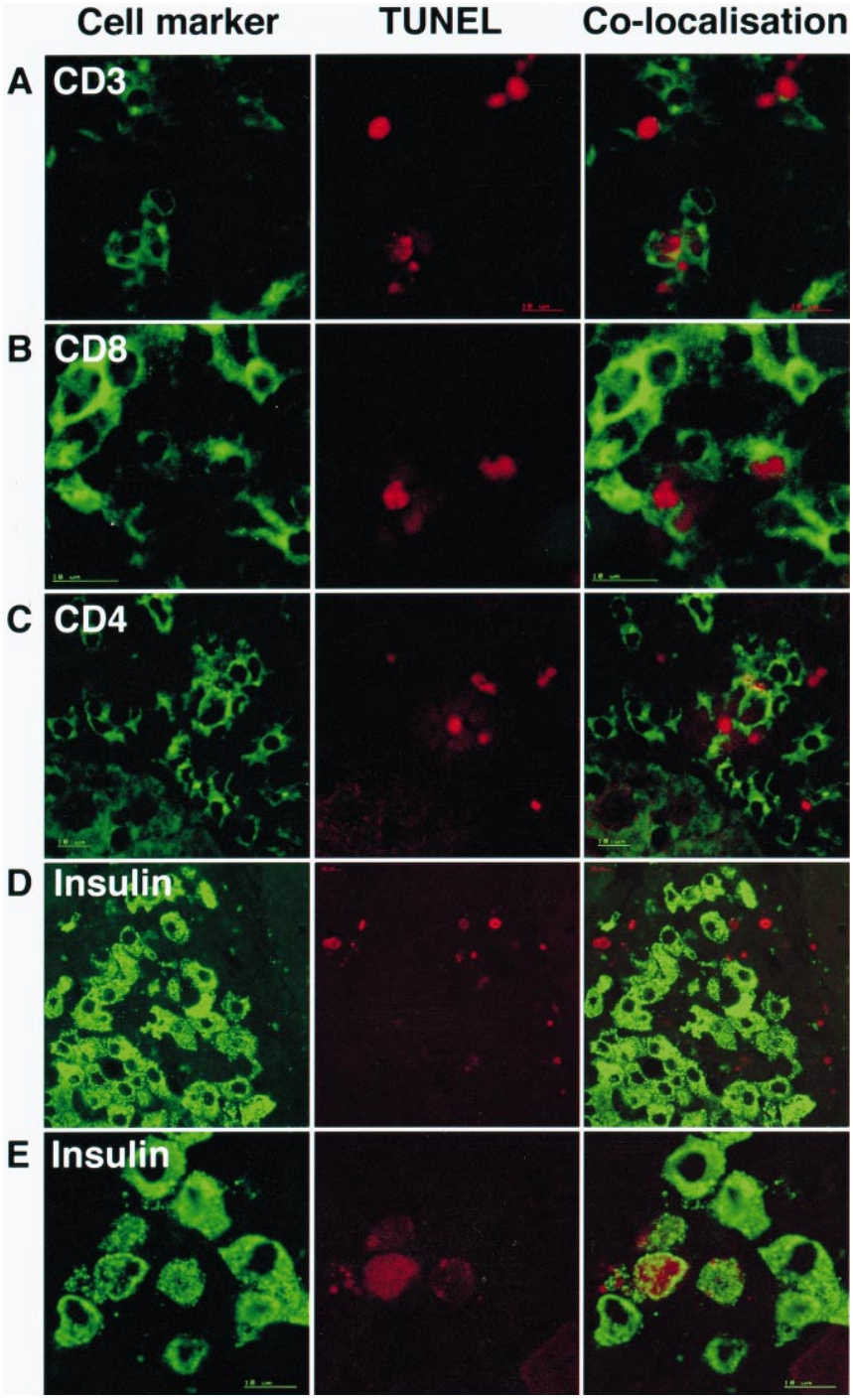

Fig. 5 A–E. Confocal microscopy of double-immunofluorescence staining for markers of islet-infiltrating T cells (**A**, CD3; **B**, CD8; **C**, CD4) and beta cells (**D** and **E**, insulin) in green, and apoptosis (TUNEL) in red, in islets of male NOD mice 12 days after injection with cyclophosphamide. The majority of apoptotic cells in the islet lesion are insulin-negative (**D**); a minority are insulin-positive beta cells (**E**)

in situ, we estimate that these apoptotic beta cells represent no more than 0.05 % of the total. This proportion is consistent with the rapid clearance of apoptotic beta cells we have discussed. In the accelerated transgenic TCR model, double-staining for insulin and TUNEL was also done and apoptotic beta cells directly identified by light microscopy [12]. Similarly, apoptotic beta cells were found only in islets infiltrated by mononuclear cells. Although we found an inverse relation between the frequency of apoptosis and the insulin-positive islet area in unmanipulated and cyclophosphamide-treated mice, apoptosis in

both was largely confined to cells within the infiltrate. At the time of maximum beta-cell destruction 12 days after treatment with cyclophosphamide, CD8 T cells, which greatly increase in frequency at this stage [30] and are required for beta-cell death [31], seem to be the predominant T cell undergoing apoptosis. We had expected some activated CD4 cells would undergo apoptosis and therefore our inability to unequivocally co-localise CD4 and TUNEL could have been partly technical.

Comparison of unmanipulated mice with mice given cyclophosphamide provides a clue to the temporal pattern and mode of beta-cell destruction. In unmanipulated mice, the insulin-positive islet area in islets with apoptosis was already decreased (compared with islets without apoptosis) at 50 days of age, in keeping with the inverse relation between apoptosis and beta-cell destruction which we assume extends further back in time. O'Brien et al. [22] reported that beta-cell apoptosis was present at 3 weeks of age before the appearance of T cells in NOD islets. Further studies are required to determine if beta-cell apoptosis precedes mononuclear cell infiltration. Around this age, however, NOD islets are first infiltrated by macrophages [32], whose products including interleukin-1 (IL-1) can induce beta-cell apoptosis [13–16]. The insulin-positive islet area in islets with apoptosis decreased further after 64 days of age; by 90 days, apoptosis was present in a majority of islets and in these the insulin-positive area had decreased by more than 50%. Despite this evidence of substantial beta-cell death, however, apoptotic events were rarely detected in insulin-positive cells. By comparison, at the height of beta-cell destruction 12 days after injection with cyclophosphamide, when apoptosis was detected in 100% of islets and a majority had an insulin-positive area less that 50%, a minority of apoptotic events were detected in beta cells. Taken together, and assuming that TUNEL is a measure of beta-cell death, our results are more consistent with the view that spontaneous beta-cell destruction in the NOD mouse occurs gradually over time rather than rapidly just before the onset of diabetes, accounting for the difficulty in securing evidence for short-lived beta-cell apoptosis.

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