CD8 T Cell Clones from Young Nonobese Diabetic (NOD) Islets Can Transfer Rapid Onset of Diabetes in NOD Mice in the Absence of CD4 Cells

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Summary

T cells play an important role in the pathogenesis of diabetes in the nonobese diabetic (NOD) mouse. CD8 cytotoxic T cell lines and clones were generated from the lymphocytic infiltrate in the islets of Langerhans of young (7-wk-old) NOD mice by growing them on (NOD × B6-RIP-B7-1)F_1 islets. These cells proliferate specifically to NOD islets and kill NOD islets in vitro. The cells are restricted by H-$2K^d$, and all bear T cell antigen receptor encoded by Vβ6. When these CD8 T cell lines and clones are adoptively transferred to irradiated female NOD, young NOD–SCID, and CB17–SCID mice, diabetes occurs very rapidly, within 10 d of transfer and without CD4 T cells.

The nonobese diabetic (NOD)[1] mouse develops spontaneous diabetes and is a good model of human type 1 or insulin-dependent diabetes mellitus (IDDM). Genes mapping to the MHC play a vital role in susceptibility to IDDM, and environmental influences can also profoundly affect the incidence of disease. IDDM is a T cell–mediated disease and, in its natural history, both CD4 and CD8 T lymphocytes are seen to infiltrate the pancreatic islets of Langerhans. Moreover, the ultimate destruction of β cells, on adoptive transfer of IDDM, requires both CD4 and CD8 T cells (1–6).

The MHC genes of the NOD mouse are a major component of the genetic susceptibility to diabetes (7). The MHC class II region has been extensively studied, and these mice express the unusual I-A^{g7} whose β chain has a histidine residue at position 56 and a serine residue at position 57 of the β chain, whereas all other haplotypes have proline and aspartic acid, respectively, at these residues. In addition, these mice fail to express I-E because of a mutation in the first exon of the Eα gene (8). If the NOD I-A^{g7} is altered by substitution of amino acid 56 (9) or by the presence of an I-E transgene (9, 10), then diabetes is greatly retarded. The impact of the MHC class I region has been less studied. However, it is clear that the relatively common MHC class I alleles are also important for development of disease in NOD mice. NOD mice bred to MHC class I–deficient, β_2-microglobulin gene knock-out mice (NOD-β_2m^{null}) develop neither insulitis nor diabetes (11–13). The particular MHC class I alleles appear to be important, as NOD mice congenic for the MHC haplotype of CTS mice, which bear NOD MHC class II alleles but different MHC class I alleles (unique to CTS), have a reduced incidence of diabetes (14).

A number of studies have shown that both CD4 and CD8 T cells from newly diabetic donors are required for adoptive transfer of disease (1–3). Studies using cloned T cells have also indicated that in adoptive transfer into irradiated recipients, both CD4 and CD8 T cells are necessary for optimal disease transfer (4–6). However, CD4 T cell clones alone can cause diabetes in irradiated recipients (5) and accelerate diabetes in young NOD mice that have endogenous CD8 cells (15). CD4 T cells from diabetic NOD donors (16) have also been shown to transfer disease into NOD–SCID mice, which are immunodeficient due to the SCID mutation, which prevents normal development of functional lymphocytes (17). It has been suggested on the basis of these observations that NOD APC process soluble antigens from β cells and present these in the context of MHC class II I-A^{g7} to CD4 cells, which can then damage islet β cells in a delayed-type hypersensitivity response (18).

Pancreatic β cells, the target of the autoimmune attack, express MHC class I but not MHC class II molecules (19, 20). CD4 T cells must therefore recognize peptides released from the β cells and presented on an APC that expresses MHC class II, perhaps resident dendritic cells. This would seem to require prior β cell damage, most likely mediated by MHC class I–restricted CD8 T cells, since there is no insulitis in β_2-microglobulin–deficient NOD

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[1] *Abbreviations used in this paper:* IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; NOD-β_2m^{null}, NOD × MHC class I–deficient, β_2-microglobulin gene knock-out mice; RT, reverse transcription.

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mice. Thus, all of these findings taken together suggest that CD8 T lymphocytes are important in the initiation of diabetes in the NOD mouse. The absence of insulitis or diabetes in these NOD-β_2m^{null} mice has been attributed to the fact that CD8 T cells require MHC class I for development. In addition, diabetic spleen cells can adoptively transfer disease into the NOD-β_2m^{null} mice, but since disease onset is delayed compared with normal NOD mice (12), this suggests that CD8 T cells also play a direct effector role in causing diabetes.

There are few reports of CD8 T cell clones reactive to islets in the literature. Nagata et al. (6) have described NOD-derived CD8 T cell clones that will only transfer disease in the presence of CD4 T cells, and Shimizu et al. (5) showed that their CD8 T cell clones would not transfer disease, even in the presence of CD4 T cells. There have been no previous reports of CD8 T cells transferring disease in the absence of CD4 T cells.

T cells recognize antigen as peptides that are complexed with MHC molecules. However, recognition of antigen alone is not sufficient to activate the cell and, instead, may induce anergy (21, 22). A second signal is required, and this may be delivered by interaction of the molecule CD28 with molecules of the B7 family of costimulatory molecules on APC (23, 24). Much evidence suggests that B7–CD28 interaction activates CD4 T cells and is required for the production of the cytokines IL-2 and IFN-γ (23, 25–28). Naive CD4 T cells require both ligand and costimulator to be present at the same APC for optimal T cell activation (29). The role of this costimulatory pathway for CD8 T cells has been more controversial, although, as with CD4 T cells, it has been shown that CD8 T cells can be activated in the presence of B7 (30). Tumors that express B7-1 can become immunogenic and activate CTL, bypassing the need for exogenous help from CD4 T cells (31–33). It appears that the costimulatory interaction is necessary for activation of the cells but not for effector function (34).

Here we report that when islet-specific CD8 T cell clones derived from islet-infiltrating cells of young NOD mice are activated in the presence of the costimulator B7-1, expressed on NOD β cells by means of the rat insulin promotor, they can very rapidly and efficiently cause diabetes in irradiated NOD, NOD–SCID, and CB17–SCID mice in the absence of CD4 T cells.

Materials and Methods

Mice. Female 7-wk-old NOD/Caj mice were used to generate T cell clones and as recipients in adoptive transfer experiments. Mice were housed in specific pathogen–free conditions. In this colony, female mice develop diabetes from 12 wk of age, reaching an incidence of 90% by 24 wk. BALB/c and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used for in vitro cytotoxicity assays. In addition, NOD-SCID (The Jackson Laboratory), CB17–SCID (H-2^d), and B6–SCID (H-2^b) mice were used as recipients for adoptive transfer.

Generation and Propagation of T Cell Clones. T cell clones were generated from the islet infiltrate of 7-wk-old female NOD mice. The infiltrating lymphocytes were grown in culture in Clicks medium supplemented with 5% fetal bovine serum and 5 U IL-2 (EL-4 supernatant) at 37°C and 5% CO_2. After 7 d, the cultures were stimulated with irradiated islets from (NOD × C57BL/6J-RIP B7-1)F$_1$ hybrid mice, which express the costimulator B7-1 on the islets of Langerhans (35) as a source of antigen. The islets were isolated by collagenase digestion as described previously (4). After further restimulation with two cycles of antigen at 2-wk intervals, the cells were then cloned by limiting dilution. These cultures were maintained on Clicks medium supplemented with 5% fetal bovine serum and 5 U IL-2. Islet antigen, in the form of irradiated islets from (NOD × C57BL/6J-RIP B7-1)F$_1$ hybrid mice, was added every 2 wk.

Flow Cytometry. Cells were stained with the following mAbs: FITC-conjugated anti-CD4 and anti-CD8 (GIBCO BRL, Gaithersburg, MD), PE-conjugated anti-α/β-TCR; FITC-conjugated anti-Vβ 2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 14, 17; FITC-conjugated anti-CD44; FITC-conjugated anti-CD69; FITC-conjugated anti–heat stable antigen; anti-CD28 (PharMingen, San Diego, CA); anti-α4 integrin or CD49d (R1-2), and anti–intercellular adhesion molecule 1 (YN/1). FITC-conjugated anti–hamster antibody (CALTAG Laboratories, South San Francisco, CA) was used with anti-CD28, and FITC-conjugated anti–rat IgG antibody (Hyclone Laboratories, Inc., Logan, UT) was used with anti-α4 integrin and anti–ICAM-1. The cells were incubated with the directly conjugated antibodies for 30 min at 4°C in PBS containing 1% FCS and 0.1% sodium azide and then washed and analyzed on FACS® IV (Becton Dickinson Immunocytometry Systems, Mountain View, CA). When unconjugated antibodies were used, there was a further incubation with the secondary antibody using the same conditions before analysis.

Cytokine Profile. Total cellular RNA was prepared from 5 × 10^6 T cells using RNAzol B (Biotecx Laboratories Inc., Houston, TX) according to the manufacturer's instructions. RNA was then primed at 55°C using oligo dT(12–18) (GIBCO BRL) and reverse transcribed at 37°C with 300 U of Maloney murine leukemia virus reverse transcriptase (GIBCO BRL) in a final volume of 40 μl containing 0.55 μl RNAsin (Promega Corp., Madison, WI), 4 μl dithiothreitol, 8 μl 5× buffer (Tris-HCl, KCl, $MgCl_2$), and 25 μmol dNTP (Pharmacia Biotech Inc., Piscataway, NJ). The reaction was terminated by heating to 70°C for 10 min, and the final volume was made to 100 μl. 2 μl of cDNA was then used for subsequent PCRs.

PCR reactions were carried out using 1 U Taq polymerase (Promega Corp.) in a reaction containing 2.5 μl 10 × PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, at 25°C, 1% Triton X-100), 1.5 mM $MgCl_2$, 0.25 μl dNTP mix (25 mM), 100 ng each primer, and 2 μl cDNA in a final volume of 25 μl. PCR primers for actin, IL-2, IFN-γ, IL-4, TNF-α, and TNF-β were originally purchased from CLONTECH (Palo Alto, CA). Primers for IL-5, IL-12, TGF-β (36), and perforin (37) were synthesized in the Keck Facility of Yale University.

PCR primer sequences were as follows. Actin 5′ primer: GTG GGC CGC TCT AGG CAC CAA, 3′ primer: CTC TTT GAT GTC ACG CAC GAT TTC; IL-2 5′ primer: ATG TAC AGC ATG CAG CTC GCA TC, 3′ primer: GGC TTG TTG AGA TGA TGC TTT GAC A; IFN-γ 5′ primer: TGA ACG CTA CAC ACT GCA TCT TGG, 3′ primer: CGA CTC CTT TTC CGC TTC CTG AG; TNF-α 5′ primer: ATG AGC ACA GAA AGC ATG ATC CGC, 3′ primer: CC AAA GTA GAC CTG CCC GGA CTC; TNF-β 5′ primer: TGA CAC TGC TCG GCC GTC TCC A, 3′ primer: GTT GCT CAA AGA GAA GCC ATG TCG; IL-4 5′ primer: ATG GGT CTC AAC CCC

CAG CTA GT, 3′ primer: GCT CTT TAG GCT TTC CAG GAA GTC; IL-5 5′ primer: GAA AGA GAC CTT GAC ACA GCT G, 3′ primer: GAA CTC TTG CAG GTA ATC CAG G; IL-12 5′ primer: ATG GCC ATG TGG GAG CTG GAG, 3′ primer: TTT GGT GCT TCA CAC TTC AGG; TGF-β 5′ primer: ACA GGG CTT TCG ATT CAG CGC, 3′ primer: CCC TTG GGC TCG TGG ATC CAC; perforin 5′ primer: GCC ACG ACC TGT CCC TGC, 3′ primer: TTG GTT CCC GAA GAG C. The PCR reaction profile used for all the cytokines except perforin was denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min for 30 cycles followed by an extension at 72°C for 7 min. For the perforin reaction, the extension temperature used was 65°C.

TCR Characterization. cDNA, synthesized as above, was amplified using primers for Cβ (CTG CTC GGC CCC AGG CCT CT) and Vβ6 (GGC GAT CTA TCT GAA GGC TA). A single band was visualized after electrophoresis on 1% agarose gel (GIBCO BRL). The sample was purified using a DNA purification column (Wizard; Promega Corp.) and sequenced on both strands using an automatic sequencer (model 737; Applied Biosystems, Inc., Foster City, CA). The α chain was cloned similarly, using a degenerate primer for Vα purchased from Operon Technologies Inc. (Alameda, CA) and a Cα primer (ATA TCT TGG CAG GTG AAG CTT GT) using conditions described (38).

Thymidine Incorporation Proliferation Assays. 10^4 T cells were incubated with 10–20 purified irradiated islets from 6-wk-old NOD or (NOD × C57BL/6J-RIP B7-1)F_1 hybrid mice in a V-bottom plate for 72 h. The cultures were pulsed with 1 μCi [^{3}H]thymidine (New England Nuclear, Boston, MA) and harvested 16 h later.

Chromium Release Assay. Islets were isolated as above from 6-wk-old NOD mice and dispersed into single cells by washing once in Ca^{2+}- and Mg^{2+}-free HBSS (GIBCO BRL) and then incubation with 1× Trypsin–EDTA (GIBCO BRL) at 37°C for 10 min. The islets were cultured overnight in Clicks medium and 5% FCS. The islet cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham Corp., Arlington Heights, IL) in 0.2 ml Clicks medium with 5% FCS at 37°C for 2 h, washed three times, and resuspended in Clicks medium with 5% FCS at 10^5 cells/ml. The cells were seeded in a round-bottom 96-well plate at 10^4 cells/well. Effector cells were added in E/T ratios of 50:1, 25:1, and 10:1 to each well in duplicate. Culture medium was added to a set of target cells for estimation of spontaneous cell lysis. Total cell lysis was determined by lysing the cells with hydrochloric acid. The plates were centrifuged at 100 *g* for 2 min and incubated at 37°C for 6, 12, and 22 h. 120 μl of supernatant was harvested from each well, and the radioactivity measured in a gamma counter (LKB, Wallac, Gaithersburg, MD). Specific lysis was calculated as percent lysis = 100 × (test cpm − spontaneous cpm)/(total cpm − spontaneous cpm). As control target cells, Con A blasts were generated by incubating spleen cells for 48 h with 2.5 μg/ml Con A, and then the cells were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 h and used in the cytotoxicity assays as above.

Adoptive Transfer. Female NOD mice (7 wk old) irradiated with 725 rad from a caesium source, 2–3-wk-old female NOD–SCID mice, 2–3-wk-old CB17–SCID (H-2^d) mice, or 3-wk-old B6–SCID (H-2^b) mice were used in the adoptive transfer experiments. In the initial experiments using transfer into irradiated NOD mice, the CD8-cloned T cells (5 × 10^6) were injected alone or admixed with CD4 T cells (5 × 10^6), purified by CD4 T cell isolation columns (Pierce Chemical Co., Rockford, IL) from the spleen cells of recently diabetic NOD mice (95% purity). As a positive control, animals were injected with 5 × 10^6 purified CD4 cells mixed with 5 × 10^6 purified CD8 T cells or with whole diabetic spleen cells. As a negative control, animals were injected with 5 × 10^6 purified CD4 T cells alone or with PBS. The irradiated mice were injected intravenously 24 h after irradiation and monitored for glycosuria using Diastix (Ames, Elkhart, IN), and diabetes was confirmed by a blood glucose measurement of >13.9 mmol (250 mg/dl). The pancreas was examined by immunohistology. In later experiments involving the various animals with the SCID mutation, only the cloned CD8 T cells were used.

Immunohistopathological Analysis. Pancreata from diabetic mice and control animals which had been irradiated and injected with saline were either fixed in 10% buffered formalin or processed for immunohistochemistry by fixation in paraformaldehye lysine periodate. After 24 h, the pancreata were sucrose infused, embedded in Tissue Tek OCT (Miles Inc., Elkhart, IL), and frozen in isopentane. The portion of the pancreas that was fixed in formalin was paraffin embedded and stained with hematoxylin and eosin. The sections were microscopically examined for the presence of insulitis—the presence of mononuclear cell infiltration in the islets of Langerhans. For immunohistochemistry, 7-μm-thick frozen sections were stained with biotinylated YT4.3 antibody recognizing CD4, TIB 105 antibody recognizing CD8, B220 antibody, which stains B cells, and anti-TCR Vβ6 antibody (PharMingen). The color was developed using diaminobenzidine tetrahydrochloride and nickel ammonium sulphate. Sections were counterstained with hematoxylin.

Cell Tracing. The cloned T cells were labeled with the fluorescent dye DiI (Molecular Probes, Inc., Eugene, OR) at 37°C for 30 min and washed three times in PBS, and 5 × 10^6 cells were transferred as above. This dye is extremely useful because sections containing cells stained with the dye can also be stained by conventional immunohistochemistry. Mice were killed at 24 h and at 5 d after becoming diabetic, and the tissue was prepared for frozen section as above. Sections were first viewed under fluorescent microscopy, photographed, and then stained with the appropriate mAbs for immunohistochemistry.

Results

NOD CD8 T Cell Lines Are Generated by Growing Cells from the Islets of Prediabetic NOD Mice on Islets from B7-1–expressing (NOD × B6)F_1 Hybrid Mice. The T cell lines isolated from pancreatic islets and stimulated repeatedly with islets from (NOD × C57BL/6J-RIP B7-1)F_1 hybrid mice were cloned by limiting dilution and were assessed for their ability to proliferate in response to islets from NOD mice and for their surface phenotype. Oligoclonal T cell lines D2, C7, B11, and F8 stained predominantly or exclusively for CD8. The T cell clones G9 and B11-5.2, derived by further limiting dilution, expressed CD8 as shown in Fig. 1.

The T cell clones G9 and B11-5.2 expressed identical surface markers by flow cytometry after staining with mAbs as illustrated for clone G9 in Fig. 1. The cells bear the activation markers CD28 and CD44 but not HSA or CD69. In addition, they express the adhesion molecules α4-integrin and ICAM-1.

The CD8 T cell clones G9 and B11-5.2 both express Vβ6. This is shown by staining with anti-Vβ mAb (Fig. 1). The expressed TCR of G9 was further characterized by sequencing after amplification by reverse transcription (RT) PCR. This revealed that the G9 TCR consists of Vβ6,

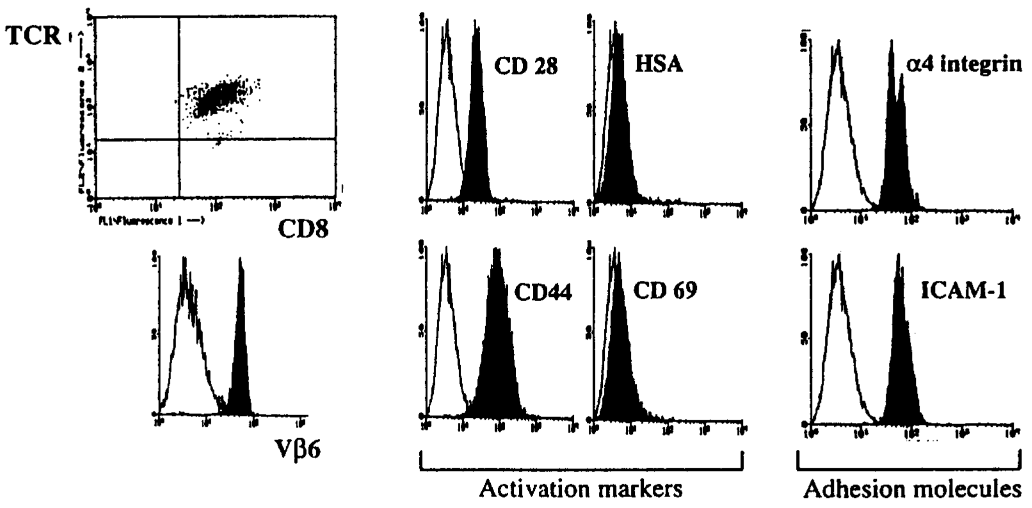

Figure 1. The T cell clone G9 has been stained with mAbs as described in the text and analyzed by flow cytometry.

Dβ1.1, Jβ2.3 and Vα15, and Jα8 (nomenclature as reported in references 39, 40), and the translated amino acid sequences are shown in Fig. 2.

CD8 T Cell Oligoclonal Lines and Clones Proliferate to NOD Islets. When tested for proliferative responses to NOD islets by thymidine incorporation, the cells respond by proliferating, with stimulation indices >10 and some considerably more, as shown in Table 1. The response of the cloned T cells to NOD islets was similar to that of the cloned T cells to the islets of (NOD × C57BL/6J-RIP B7-1)F_1 hybrid mice (data not shown).

CD8 T Cell Clones Express the Cytokines IFN-γ, TNF-α, TNF-β, and the Effector Molecule Perforin. The cytokine profile of the T cell clones was tested by RT-PCR, and the cells express typical cytokines of CD8-cytotoxic T cells: IFN-γ, TNF-α, TNF-β, and the effector molecule perforin, as shown in Fig. 3. In addition, the cells were tested for, but do not express, IL-2, IL-4, IL-5, IL-12, and TGF-β.

CD8 T Cell Clones Are Cytotoxic to NOD Islets In Vitro. The T cell clone designated G9 showed cytotoxicity toward islet cells from NOD mice when tested in a ^{51}Cr release assay after 6, 12, and 22 h. Specific lysis was demonstrated by this in vitro test as shown in Fig. 4 *A* and occurs at all time points, although the optimal time was shown to be 12 h. Spontaneous lysis was 20–30% at the earlier time points, rising to 40–50% at 22 h. Thus, subsequent ^{51}Cr release assays were performed using the optimal 12-h time point for assessment. In addition, a ^{51}Cr release assay was performed using islet cells from the NOD mouse (H-2 K^dD^b) and compared with islet cells from BALB/c (H-2^d) and C57BL/6 (H-2^b) mice. The cloned T cells showed cytotoxicity toward islet cells from NOD and BALB/c mice but not C57BL/6 mice at 12 h, indicating MHC restriction by K^d (Fig. 4 *B*). In addition, this effect was islet specific, as shown by lack of cytotoxicity towards Con A blasts used as control target cells (data not shown).

CD8 T Cell Lines Stimulated in the Presence of B7-1 Do Not Require CD4 T Cells to Cause Diabetes. To determine if the CD8 T cell lines generated by this method can transfer disease in vivo, the cloned T cell lines were injected into irradiated NOD mice either alone or mixed with CD4 T

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Vα 15
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QHPGGGPAQLILIRSNEREKRNGRLRATLDTSSQSSSLSITA

            N  Jα8               Cα
----------->  <-><---------------->  <----------
TRCEDTAVYFCATDPMGYKLTFGTGTSLLVDP NIQNPEPAVY

Vβ 6
------------------------------------>
GHLSEGYDASREKKSSFSLTVTSAQKNEMAVFLCASS

N Dβ1.1 N  Jβ2.3     Cβ
<--->< -------------> <-----------
IRDRGAETLYFGSGTRLTVLEDLRNVTPPKFSL
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Figure 2. Sequence of clone G9 TCR cDNA translated into protein and showing the junctional regions of the α and β chains.

Table 1. *CD8 T Cell Lines Proliferate to NOD Islets*

CD8 T cell line/clone	Proliferation without antigen	Proliferation with antigen
	cpm	
B11	102	10,015
C7	241	2,703
D2	184	14,418
F8	130	44,142
G9	154	14,270

Thymidine incorporation proliferation assay demonstrating proliferation of the oligoclonal CD8 cell lines C7, D2, F8, B11, and the clone G9 to NOD islets. This is shown as the mean of the proliferation in duplicate of the cells with no antigen or in the presence of antigen (10 NOD islets).

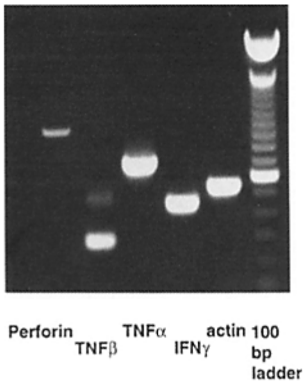

Figure 3. Agarose gel showing PCR products derived from clone G9 using primers for actin as a control, the cytokines IFN-γ, TNF-α and -β, and the effector molecule perforin.

Table 2. *Adoptive Transfer of CD8 Cell Lines to Irradiated NOD Mice*

	Days after adoptive transfer						
T cell line/clone	4	5	6	7	8	9	15
G9	3/6	6/6					
B11	0/2	1/2	2/2				
C7	0/2	2/2					
C7 + CD4	0/2	2/2					
D2	0/3	0/3	0/3	0/3	3/3		
D2 + CD4	0/3	3/3					
F8	0/2	1/2	1/2	1/2	1/2	2/2	
F8 + CD4	0/1	0/1	0/1	0/1	1/1		
CD4 alone	0/3	0/3	0/3	0/3	0/3	0/3	0/3
CD4 + CD8	0/2	0/2	0/2	0/2	0/2	0/2	2/2
Diabetic spleen	0/2	0/2	0/2	0/2	0/2	0/2	2/2
PBS	0/3	0/3	0/3	0/3	0/3	0/3	0/3

The numbers of mice developing diabetes are shown at various times after adoptive transfer to irradiated NOD mice. In addition, positive control mice were observed that had been transferred with 10^7 NOD diabetic spleen cells or 5 × 10^6 CD4 T cells + 5 × 10^6 CD8 T cells derived from diabetic splenocytes alone. Negative controls were mice that were injected with 5 × 10^6 purified CD4 T cells or mice that were irradiated but not transferred. Negative control mice did not develop diabetes until >40 d after transfer.

cells, as it had been shown previously by other investigators (4, 6) that CD4 T cells were required to cause disease. All the T cell lines tested caused disease within 9 d of injection, and the addition of CD4 T cells did not significantly alter the time course of disease, as shown in Table 2. In the subsequent experiments, only the CD8 T cell clones were used. These results have been reproduced on at least four occasions with the clone G9.

To examine further the ability of CD8 T cells to initiate and cause diabetes, their ability to adoptively transfer disease was tested in NOD-SCID animals. We observed that the G9 clone and subclones of G9 and subclones of the D2 line caused diabetes in young NOD–SCID mice in the absence of coinjected CD4 T cells up to 14 d after transfer.

To confirm the MHC restriction of the CD8 T cell clones, the cells were transferred into CB17–SCID (H-2^d) and B6–SCID (H-2^b) mice. G9 can transfer diabetes to CB17–SCID mice but not to B6–SCID mice, which is further evidence of restriction to K^d. These results are shown in Table 3.

Immunohistology of the islets taken from mice that developed diabetes at 5 d after injection of CD8 T cell–cloned lines, either alone or with CD4 T cells, in irradiated NOD mice showed extensive destruction of the islets. Irradiated control mice did not develop either insulitis or diabetes at this time. On staining with anti-CD4 and anti-CD8 antibodies, mice that received CD8 T cells had only CD8 T cells in appreciable numbers, whereas the mice that received CD8 T cells coinjected with CD4 cells had both types of cells, as might be expected. A similar picture is seen in the diabetic NOD–SCID or CB17-SCID mice transferred with CD8 T cell clones. The cells infiltrating the islets in SCID mice all bear Vβ6.

Immunohistochemistry of irradiated NOD, NOD–SCID, and CB17–SCID mice that had become diabetic is shown in Fig. 5.

When cells were labeled with the fluorescent dye DiI, it can be seen that large numbers of CD8-cloned T cells had reached and invaded the islet by 24 h, and these were still present at the time diabetes occurred 4 d later (Fig. 6). We have shown that it is possible to successfully fluorescently label cells that can also be visualized and stained with conventional immunohistochemistry. When these sections were stained using anti-CD8 and anti-Vβ6, we saw that the cells that were present in the diabetic animals were $CD8^+$ and $V\beta6^+$, as expected. No infiltration was seen in the irradiated control animals (not shown).

Thus, it has been shown that CD8-cloned T cells, when optimally activated, can home to the islet and rapidly adoptively transfer diabetes in the absence of CD4 T cells.

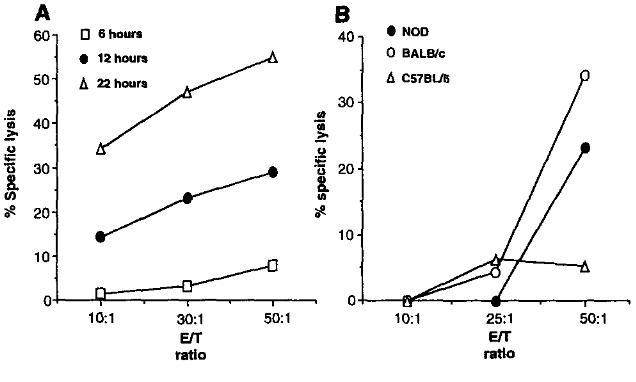

Figure 4. ^{51}Cr release cytotoxicity assay showing the cytotoxicity of clone G9 for NOD islets at three different times of incubation (*A*). MHC restriction for K^d is shown by the assay in (*B*), in which clone G9 demonstrates cytotoxicity for islets from NOD mice (K^dD^b) and BALB/c (K^dD^d) mice but not C57BL6 mice (K^bD^b). This assay was analyzed after 12 h. No specific lysis was demonstrated when control targets (Con A blasts) were used from each of these types of mice (data not shown).

Table 3. *Adoptive Transfer of CD8 T Cell Clone G9 and Subclone G9C8 to SCID Mice*

		Days after adoptive transfer										
Strain	MHC	5	6	7	8	9	10	11	12	13	14	35
NOD–SCID	K^dD^b $A^{g7}E^o$	2/10	5/10	6/10	6/10	6/10	7/10	7/10	7/10	7/10	8/10	8/10
CB17–SCID	K^dD^d A^dE^d	0/7	0/7	2/7	4/7	4/7	4/7	4/7	4/7	4/7	4/7	4/7
C57BL/6–SCID	K^bD^b A^bE^o	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

The numbers of mice developing diabetes are shown at various times after adoptive transfer to NOD–SCID, CB17–SCID, and C57BL/6–SCID mice. The numbers for transfers into NOD–SCID and CB17–SCID mice are the totals over at least three experiments. The mice that did not develop diabetes were observed for at least 35 d after transfer.

Discussion

Many lines of evidence suggest that CD8 T cells are important in the pathogenesis of IDDM. In human IDDM, CD4 and CD8 cells are found in the postmortem pancreatic sections of patients who have died at the onset of disease (41, 42) and in pancreatic biopsy specimens from newly diagnosed patients (43). Identical twins who are discordant for IDDM and who have received pancreatic transplants from the nondiabetic twin rapidly developed a recurrence of disease. Histology of the pancreatic grafts showed that the infiltrating cells were predominantly CD8 T cells (44). There is also evidence that CD8 T cells play a key role in the NOD animal model of diabetes. However, CD8 T cell clones are difficult to isolate, and, consequently, there have been few studies to date reporting these cells (5, 6, 45).

This study has shown that it is possible to isolate islet-reactive CD8 cytotoxic T lymphocytes from 7-wk-old NOD mice, a time point at which the animals have insulitis but would not become diabetic for several weeks. These cells are capable of destroying pancreatic β cells and causing diabetes in the irradiated NOD mouse without cotransferred CD4 T cells, and diabetes appears much more rapidly (5 d after transfer) than has been demonstrated in any previous study, 21 d being the earliest time reported by Nagata et al. (6). In addition, the CD8-cloned T cells could transfer disease in NOD–SCID/Lt mice as well as CB17–SCID mice (matched for the MHC class I molecule K^d), which lack functional endogenous lymphocytes. These CD8-cloned T cells differ from previously isolated diabetogenic CD8 T cell clones (4–6) in that they have been stimulated by an antigenic target that bears the costimulatory B7-1 molecule. Pancreatic β cells in the NOD mouse express MHC class I molecules (19, 20), and there is no evidence that they express the B7-1 or B7-2 costimulatory molecules under normal circumstances in vivo. One of the mechanisms of peripheral tolerance is thought to involve the induction of anergy by tissue cells, which do not express costimulatory molecules, protecting them from attack by T cells recognizing self-molecules. One of the problems that may be encountered in the isolation of CD8 cytotoxic T lymphocytes is that islet antigens presented by pancreatic islet β cells, which do not have costimulatory molecules present, cannot optimally activate the cells. Therefore, cells that are stimulated in vitro by targets that lack a costimulatory second signal may be rendered anergic and be difficult to maintain.

The CD8 T cells, can, once activated, proliferate in the absence of the costimulatory signal, as shown by the in vitro proliferation to NOD islets that do not bear B7. It is not known whether these cells would become anergic if cultured with NOD islets lacking the costimulatory signal. The cells demonstrate in vitro cytotoxicity as measured in ^{51}Cr release assays toward NOD islets, and these cells have a very marked ability to destroy the pancreatic islets in vivo. Histologic studies have shown that at the time that the animals develop diabetes, most of the β cells in islets are completely destroyed, and the architecture is very distorted. It is possible that the relatively low in vitro cytotoxicity is due to the fact that pancreatic islets are poor targets for these cytotoxic assays and, in previous studies, high specific lysis was only found for CD8 T cell lines and not for clones (6) or at high E/T ratios of 50:1 (5). The fact that the CD8 T cell lines and clones can cause rapid disease in NOD mice, which do not have B7-1 expression on the pancreas, indicates that B7-1 is not required either for specific recognition or for effector function.

It is interesting that these cells have been generated from 7-wk-old mice that are likely to have some insulitis but are a number of weeks away from the development of diabetes. It is possible that a naive subset of lymphocytes has been stimulated and induced to react by activation that includes costimulatory signals. Alternatively, it suggests that cells are already present at this time that may be able to mediate significant damaging effects on the pancreatic islets, given appropriate stimulation, and that these may well be cells that play a role in the initiation of the disease process. The presence of cells

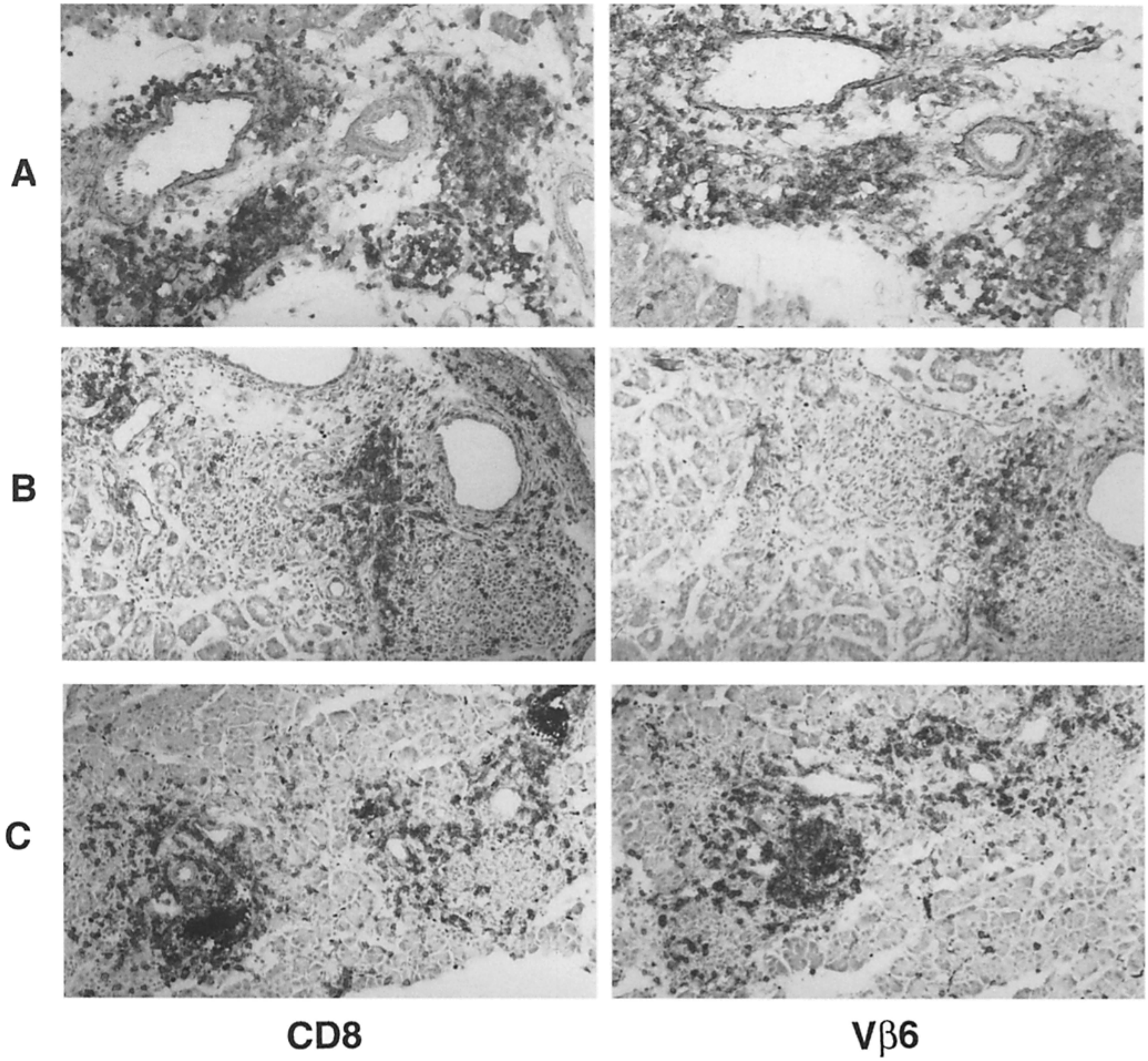

Figure 5. Immunohistochemistry showing staining with anti-CD8 and anti-Vβ6 of pancreatic sections of mice transferred with clone G9 and taken at the time diabetes occurred. An irradiated NOD mouse is shown in *A*, a NOD–SCID mouse in *B*, and CB17–SCID mouse in *C*.

in the islets of young NOD mice that are capable of inflicting damage has already been suggested by studies in which islet-infiltrating T cells from prediabetic animals can transfer diabetes to NOD–SCID animals as rapidly as the islet-infiltrating cells from diabetic mice (46).

The importance of T cells expressing TCR encoded by Vβ6 has previously been suggested by Edouard et al. (47), who showed that if a population of T cells were depleted of TCR-Vβ6–bearing cells, these cells could no longer adoptively transfer diabetes. The present study highlights the importance of Vβ6$^+$ CD8 T cells. Of a number of the initial oligoclonal CD8 T cell lines generated, only those that expressed predominantly Vβ6 were able to adoptively transfer diabetes in vivo, and subsequently, all the T cell clones generated that were capable of causing disease expressed TCR encoded by Vβ6. The TCR encoded by Vα15 (nomenclature according to reference 39) has previously only been reported by Pircher et al. (48). The TCR does not resemble the α or β chain sequences described elsewhere (6, 45), suggesting that a different islet autoantigen is recognized by the CD8 T cell clones reported here, which appear to be involved in disease initiation rather than effector function.

The strongest evidence that CD8 T cells may be important in the initiation of type 1 diabetes in the NOD mouse comes from studies in which NOD mice lacking β_2-microglobulin (and hence MHC class I and most CD8 T cells) de-

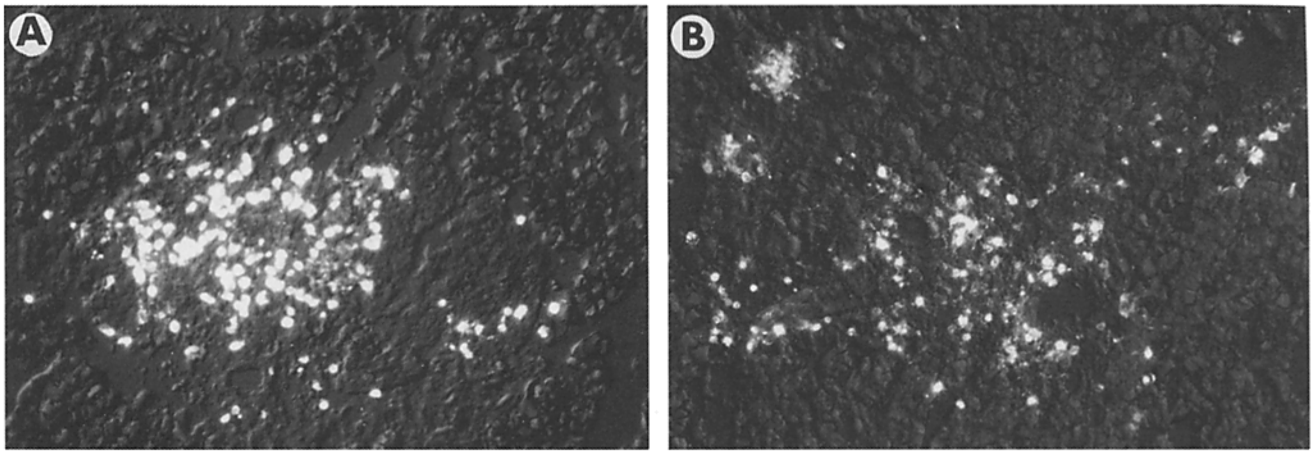

Figure 6. Pancreatic sections taken at (*A*) 1 and (*B*) 5 d after transfer of clone G9 labeled with DiI showing labelling of the cells within the islets with the lipophilic dye.

velop neither insulitis nor diabetes (11–13). Our study has indicated that CD8 T cells that have the potential to cause disease are present in the early stages of insulitis, and, given optimal activation conditions in vitro, are capable of causing diabetes, not only in the irradiated adoptive transfer model but also in young NOD–SCID or CB17–SCID mice that express the appropriate restriction element for TCR recognition of antigen. CD8 T cells, therefore, have the ability both to initiate damage in young animals as well as to perform the final effector function.

A number of models exist for the pathogenesis of type 1 diabetes that do not suggest that CD8 T cells play any role in initiation of the disease process. In light of the fact that activated CD8 T cells can cause diabetes in young SCID animals, we suggest a model for the pathogenesis of diabetes, whereby activated CD8 T cells, which may originate outside the pancreas (for instance, in the peripancreatic lymph nodes), may play an initiating role in the damage to pancreatic islets. This is possible because they have the effector molecules capable of causing damage to the β cells. This could lead to a release of soluble islet antigens, which may then be taken up by professional APC within the islet and presented to $CD4^+$ T cells, which have different regulatory and effector functions. Amplification and diversification of the antigenic response, perhaps by activation of B cells, can then lead to the growth and activation of effector CD4 cells, which predominate over any regulatory elements that may be present. Cytokines such as IL-2 produced by these cells could then play a role in recruiting further CD8 T cells and activating them. CD4 T cells can also recruit and activate macrophages to produce other effector molecules, such as nitric oxide, all of which could act together to damage sufficient numbers of islet β cells to cause diabetes.

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