Transfer of Diabetes in the NOD-scid Mouse by CD4 T-Cell Clones

Differential Requirement for CD8 T-Cells

Jeffrey D. Peterson and Kathryn Haskins

Transfer of an interleukin 2/interferon-γ-secreting islet-specific CD4+ T-cell clone, BDC-6.9, in the immunodeficient NOD-scid mouse induces destruction of pancreatic β-cells without help from host B-cells, CD4+ T-cells, or CD8+ T-cells. However, a second islet-specific T-cell clone, BDC-2.5, showing the same cytokine profile and T-cell receptor Vβ expression as BDC-6.9 was not capable of inducing diabetes or insulitis in NOD-scid mice. Even though BDC-2.5 by itself readily induces diabetes in young unmanipulated NOD mice, cotransfer of CD8-enriched T-cells was required to induce disease in NOD-scid mice. Immunohistochemical staining of pancreatic lesions in young NOD mice receiving either BDC-2.5 or BDC-6.9 showed the presence of CD4+, CD8+, Vβ4+, and MAC-1+ cells within the infiltrate, similar to infiltrates in lesions of spontaneously diabetic female NOD mice. In contrast, NOD-scid mice that received BDC-6.9 showed only the presence of CD4+Vβ4+ T-cells and a large population of MAC-1+ cells in islet lesions. NOD-scid recipients of cotransferred BDC-2.5/CD8+ splenic T-cells showed a small population of CD4+ T-cells and a larger population of CD8+ T-cells within the infiltrated islets, whereas no infiltrate was detectable in recipients of CD8+ splenocytes or BDC-2.5 alone. Our results suggest that at least two types of islet-specific CD4+ T-cell clones play a role in diabetes pathogenesis. Diabetes 45:328-336, 1996

I DDM is an immune-mediated disease that occurs in humans and in experimental rodent models such as the NOD mouse. In both the NOD mouse and in humans, there are inflammatory lymphocytic infiltrations of the pancreatic islets (insulitis) (2,3) and circulating anti-islet antibodies (4–6) and a strong genetic association with loci of the major histocompatibility complex (7,8), suggesting an autoimmune disease process. A major role for T-cells in diabetes pathogenesis is supported in that total T-cell depletion (2,9) or CD4+ T-cell depletion (10–12) of NOD mice results in decreased incidence of spontaneous diabetes and insulitis.

Earlier studies established that both CD4 and CD8 T-cells were required for successful adoptive transfer of disease with diabetic spleen cells into young (13) or irradiated adult (14,15) NOD mice, but the relative roles of the two subsets have not been well defined and remain a subject of controversy. The importance of CD4 T-cells in disease has been suggested by studies in which CD4 T-cells from donor NOD mice treated with cyclophosphamide were shown to induce diabetes in T-depleted NOD mice (16), and purified CD4, but not CD8, T-cells from diabetic donor spleens were shown to transfer disease at low efficiency in immunodeficient NOD-scid mice (17). On the other hand, there is the hypothesis that although CD4 T-cells are essential for diabetes pathogenesis, they are important mainly because they recruit CD8 T-cells as the final effectors in the destruction of islet cells (18,19). This possibility is supported by the observation that short-term CD8 T-cell lines are effective at mediating cytotoxic destruction of islets in vitro (20).

Strong evidence for a dominant effector role for CD4 T-cells has come from studies with islet-reactive T-cell clones that are diabetogenic in vivo. We have reported previously on CD4 T-cell clones isolated from NOD mice that could rapidly induce diabetes in young NOD (21) or NOD F1 (22) recipients. In contrast with the adoptive transfer studies with diabetic splenocytes (13), our studies demonstrated unequivocally that effective acceleration or transfer of disease could occur through administration of CD4 T-cells only. Although CD4 T-cell clones were sufficient for induction of diabetes in young NOD or NOD F1 recipients, we could not rule out a requirement for participation by host CD8 T-cells in these mice. We therefore decided to investigate disease transfer by T-cell clones in the NOD-scid mouse, and we report here that at least one of the diabetogenic CD4 T-cell clones from our panel can cause diabetes in the absence of any other T-cells. A second clone, however, required the cotransfer of CD8 T-cells.

RESEARCH DESIGN AND METHODS

Mice. NOD/Jt-scid/scid mice, 6–8 weeks old, and newborn NOD/Bdc mice were obtained from the breeding colony at the Barbara Davis Center for Childhood Diabetes. All mice were maintained on standard laboratory food and water ad libitum and housed in microisolator cages in specific pathogen-free facilities. NOD-scid mice were handled under sterile conditions in a laminar flow hood and housed in sterile microisolator cages.

Propagation and assay of T-cell clones. Diabetogenic T-cell clones were cultured and assayed as described previously (23,24). Cultures...
TABLE 1  Cytokine profiles of islet-specific T-cell clones

<table>
<thead>
<tr>
<th>T-cell clone</th>
<th>Antigen</th>
<th>[3H]Tdr incorporation (cpm)</th>
<th>IL-2</th>
<th>TNF-α/β</th>
<th>IL-3 + GM-CSF</th>
<th>IL-4 (ng/ml)</th>
<th>IFN-γ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDC-2.5</td>
<td>25,000 NOD islet cells/ml</td>
<td>79,341</td>
<td>++</td>
<td>+</td>
<td></td>
<td>&lt;0.02</td>
<td>29.5</td>
</tr>
<tr>
<td>(Vβ4αVα1)</td>
<td>None</td>
<td>400</td>
<td>−</td>
<td>−</td>
<td>&lt;0.02</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td>BDC-6.9</td>
<td>25,000 NOD islet cells/ml</td>
<td>29,913</td>
<td>−</td>
<td>−</td>
<td>&lt;0.02</td>
<td>34.5</td>
<td></td>
</tr>
<tr>
<td>(Vβ4βVα13.1)</td>
<td>None</td>
<td>70</td>
<td>−</td>
<td>−</td>
<td>&lt;0.02</td>
<td>&lt;0.1</td>
<td></td>
</tr>
</tbody>
</table>

IL-4 and IFN-γ were quantitated by comparison to recombinant cytokine control. *Cytokine levels >50% of concanavalin A supernatant (CAS) control. †Cytokine levels undetectable (<1% of CAS control).

RESULTS

For the experiments reported here, we have used two T-cell clones, BDC-2.5 and BDC-6.9, that were isolated from spleen and lymph nodes of newly diabetic NOD mice and selected for reactivity to islet cells as antigen in the presence of irradiated NOD splenocytes as APCs. (24). The antigens recognized by these T-cell clones are not yet defined but are apparently localized in the membrane of the β-granules (25). Neither clone reacts to any of a variety of putative diabetes autoantigens, including insulin, GAD, heat shock proteins, peripherin, and carboxypeptidase H (25). The TCRs of these two clones share the same Vβ8 (Vβ4), but have different Vα regions (27). Both clones are diabeticogenic in unirradiated 2-week-old NOD recipients (21,28) and in several 8- to 11-day-old NOD F1 strain combinations (22).

Two diabetogenic CD4 T-cell clones are of the Th1 phenotype. To further characterize the T-cell clones BDC-2.5 and BDC-6.9, we carried out a comparative analysis of cytokines in culture supernatants from antigen-stimulated clones. The results of these assays are summarized in Table 1 and show that both BDC-2.5 and BDC-6.9, when stimulated with NOD islet cells and irradiated APCs, produced high levels of IL-2, IFN-γ, TNF, and IL-3/GM-CSF, but no IL-4—a cytokine profile consistent with the phenotype of Th1 T-cells.

were renewed on a biweekly basis with 1 × 10^6 responding T-cells, 2.5 × 10^7 irradiated (3,500 rad from a 60Co source) NOD spleen cells as antigen-presenting cells (APCs), 5 × 10^9 NOD islet cells as antigen, and 2.5% EL-4 supernatant as a source of interleukin (IL-2) in culture medium (Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum). Islet cells used as antigen were prepared by making cell suspensions of fresh islets obtained by collagenase digestion of pancreases isolated from donor mice (23,25). Culture flasks were incubated at 37°C and 10% CO₂, and at the end of each 2-week period, the T-cell clones were counted, harvested, and assayed for islet reactivity in T-cell proliferation assays. To expand cultures for disease transfer experiments, 1 × 10^7 T-cell clones were harvested from 4-day cultures and placed into 120-mm culture medium supplemented with 6,000 U of human recombinant IL-2 (AMGEN, Thousand Oaks, CA) for another 4 days. This procedure typically resulted in an 8- to 15-fold expansion of T-cells. Upon harvesting, the T-cells were washed three times in Hank's balanced salt solution and checked for monoclonality by analyzing a sample for T-cell receptor (TCR) Vβ expression.

**Cytokine assays.** Islet-specific T-cell clones were cultured in duplicate (1 × 10^5 cells/well) with 2.5 × 10^5 irradiated splenocytes as APCs in the presence or absence of 25,000 NOD islet cells as antigen. Supernatants were harvested at 24 and 48 h for analysis of IL-2, IL-4, interferon (IFN)-γ, tumor necrosis factor (TNF) α/β, and IL-3/gp130 (colony-stimulating factor) (CSF). IL-2 was detected by incubating serial dilutions of culture supernatants (1:2-1:2,048) with IL-2-sensitive HT2 cells (20,000 cells/well) for 24 h at 37°C in 10% CO₂. After the incubation, 50 μg of a vital dye (MTT, Sigma, St. Louis, MO) was added to each well for 2 h at room temperature, followed by 125 μl HCl-isopropanol to dissolve the MTT crystals. Absorbance (570 tests/630 references) was read on a microtiter plate reader. Culture supernatants were quantitated by comparison of dilution curves to a concanavalin A supernatant standard.

TNF was detected by incubating serial dilutions of culture supernatants (1:2-1:2,048) with TNF-α-sensitive WEHI-164 cells (5,000 cells/well) for 48 h at 37°C in 10% CO₂. IL-3/GM-CSF was detected by incubating serial dilutions of culture supernatants (1:2-1:2,048) with IL-3/GM-CSF-sensitive FD cells (5,000 cells/well) for 24 h at 37°C in 10% CO₂. After the incubation, assays were developed with MTT and quantitated as described above.

IL-4 was detected by sandwich enzyme-linked immunosorbent assay (ELISA) using two anti-mouse IL-4 antibodies. IFN-γ secretion was measured by the InterTest ELISA kit (Genzyme, Boston, MA).

**Disease transfer.** For each separate experiment, litters of young NOD mice (11-14 days old) or adult NOD-scid mice (8-10 weeks old) received intraperitoneal or intravenous injections with 1 × 10^6 islet-specific clones once a week for 3 weeks. Experimental and control mice were monitored regularly after the second injection of clones, for 3 weeks in young NOD or for up to 3 or 4 months in NOD-scid, for elevations in urine glucose by using Tes-Tape (Lilly, Indianapolis, IN). Mice with elevated urine glucose levels were tested for high blood glucose levels by spectrophotometric methods (Megavision, Cambridge, MA). Overt diabetes was defined by elevated urine glucose levels for 2 consecutive days and a final blood glucose reading >15 mmol/l, at which point the animals were killed. Pancreatic samples from each mouse were saved for histological analysis.

In NOD-scid cotransfer studies in which the T-cell clone BDC-2.5 was administered in combination with CD8 splenocytes, the first intravenous injection was made with 10⁵ clones and 10⁹ CD8 splenocytes, whereas subsequent injections were with BDC-2.5 alone. Purification of CD8 T-cells was performed by passing spleenocytes from diabetic female NOD mice over negative selection columns (anti-CD4-conjugated Cellulose columns, Biotex, Edmonton, Alberta, Canada), followed by lysis of residual CD4 T-cells with monoclonal antibody GK1.5 and complement (Low Tox guinea pig complement, Cedarlane). This double depletion of CD4 T-cells yielded 90% pure CD8 T-cells as analyzed by flow cytometry.
In the absence of islet-cell antigen, no cytokine production was detectable. Although assays with BDC-2.5 in general showed higher levels of IL-2 and TNF production (two- to threefold) than those carried out with BDC-6.9, this result appears to correlate with the levels of antigen-specific in vitro proliferation of the two clones. Both clones produced comparable levels of IL-3/GM-CSF and IFN-γ and showed no detectable IL-4 secretion.

**Transfer of diabetes to NOD-scid mice.** To investigate the importance of an intact host immune system on the diabeticogenicity of CD4 T-cell clones, BDC-2.5 and BDC-6.9 were administered to adult NOD-scid mice or to young NOD mice included as controls for T-cell clone diabetes activity. In experiments with NOD-scid recipients, injections of spleen cells from diabetic NOD mice were also included as positive controls. Negative controls were NOD and NOD-scid littermates that received no treatment. After receiving the second injection, all mice were closely monitored for clinical onset of diabetes and at the end of the observation period, were killed to obtain pancreatic samples for histological analysis.

Figure 1 shows results from disease transfer experiments in which young NOD and NOD-scid mice were treated according to our previously established protocol in which two or three injections of T-cells, spaced 1 week apart, were made intraperitoneally. In Fig. LA, diabetes was induced in young NOD mice; four of four mice receiving clone BDC-6.9 and two of four mice receiving BDC-2.5 became diabetic within 11 to 21 days after the first injection. These results were consistent with earlier experiments in young NOD mice in which we observed close to 100% disease incidence with single injection of splenocytes from diabetic NOD mice. No disease induction was observed with the T-cell clone BDC-2.5.

The slow and variable rate of diabetes induction by BDC-6.9 in NOD-scid mice suggested three possibilities: 1) inefficient trafficking of the clones to the target organ, 2) a threshold dose requirement for islet-reactive cells, or 3) a need for help supplied by host CD4 or CD8 T-cells to cause rapid β-cell destruction. To test whether variable disease incidence was due to poor trafficking of the clones to the pancreas and/or influenced by the number of injections administered, we repeated disease transfer experiments with both T-cell clones, using varying numbers of intravenous injections. The results of these studies are represented in Fig. 2. Three successive intravenous injections of clone BDC-6.9 to NOD-scid mice resulted in 100% diabetes incidence, whereas a single injection of the clone was much less efficient, causing disease in one-third of the recipients (Fig. 2A). However, under the same experimental conditions, the T-cell clone BDC-2.5 did not transfer disease to NOD-scid recipients.

Representative histology samples from these mice are shown in Fig. 3 and confirm the clinical diabetes observations. Aldehyde fuchsin staining of formalin-fixed paraffin-embedded pancreas sections from healthy unmanipulated young NOD and adult NOD-scid mice showed normal β-cell granulation and a complete absence of lymphocytic infiltration (insulitis). Figure 3C and D are representative sections from mice that received BDC-6.9; in both young NOD and NOD-scid recipients, extensive intra-islet infiltration and β-cell destruction were indicated. In young NOD mice treated with BDC-2.5, the lesions were much like those of mice receiving BDC-6.9, showing severe insulitis and β-cell degranulation (Fig. 3E). In contrast, NOD-scid recipients of BDC-2.5, all of which remained normoglycemic (Fig. 1), showed no evidence of islet destruction and only rare signs of mild nondestructive insulitis in ~10% of islets examined.

**CD8 T-cells restore diabeticogenic activity of BDC-2.5.** Experiments by us (Fig. 1B) and by others (17) have
demonstrated that adoptive transfer of whole splenocyte populations from diabetic NOD mice to NOD-scid recipients results in rapid (3–4 weeks) and reproducible induction of diabetes. Because our clones are CD4⁺ and CD4 T-cells isolated from diabetic spleens can transfer disease at low efficiency into NOD-scid mice (17), we investigated whether CD8 T-cells might have a role in facilitating disease induction in NOD-scid mice by clone BDC-2.5. Figure 4 represents pooled data from two experiments in which NOD-scid mice received 1) three intravenous injections of BDC-2.5, 2) a single injection of CD8⁺ CD4-depleted T-cells purified from a newly diabetic female NOD spleen, or 3) one injection of combined BDC-2.5 T-cells and CD8 splenocytes, followed by two more injections, 1 and 2 weeks later, of BDC-2.5 alone. No overt diabetes was seen after injection of CD8 splenocytes or BDC-2.5 alone in either experiment. In contrast, four of six mice became overtly diabetic after administration of CD8 splenocytes in combination with BDC-2.5.

To determine whether transfer of BDC-2.5 or CD8 splenocytes alone were capable of inducing preclinical disease states in recipient mice, we examined frozen pancreatic sections for the presence of infiltrating cells. In the photomi-
NOD-scid mice received intravenous injections of BDC-2.5, $10^7$ FIG. 4. Reconstitution of BDC-2.5 diabetogenicity by CD8-enriched splenocytes. In two separate experiments, litters of 8- to 10-week-old NOD-scid mice received intravenous injections of BDC-2.5, $10^7$ CD4-depleted diabetic female NOD splenocytes. Mice were monitored daily for elevated urine glucose levels, and those with elevated levels were measured for high blood glucose. Mice were considered diabetic when they showed elevated glucose levels, and those with elevated levels were measured for high blood glucose. Mice were considered diabetic when they showed elevated glucose levels for 2 consecutive days and blood glucose $>$ 15 mmol/l. The lines represent the cumulative incidence of diabetes postinjection; numbers in parentheses indicate the final number of diabetic mice per total number of mice.

To investigate the nature of the cells infiltrating the pancreatic islets, we examined pancreatic sections for the presence of T-cell and macrophage surface antigens by immunohistochemical staining with antibodies to CD4, CD8, Vb4, and MAC-1. Pancreatic samples from NOD-scid mice were compared with those obtained from young NOD recipients, and the results are summarized in Table 2. The islets of BDC-6.9 NOD-scid recipients showed an extensive infiltrate predominantly composed of Vb4$^+$ CD4$^+$ T-cells as well as a significant population of macrophages (MAC-1$^+$). As would be expected, no evidence of CD8$^+$ T-cells was detected in NOD-scid mice receiving BDC-6.9.

In the islets of BDC-2.5/CD8$^+$ splenocyte recipients, we observed a large population of CD8$^+$ T-cells and slightly smaller populations of cells staining for Vb4, CD4, or MAC-1. Although the vast majority of the CD4 T-cells in these cotransfer recipient mice also stained for Vb4, there were some Vb4$^+$ CD4$^+$ T-cells in the islets because of expansion of small numbers of contaminating CD4 T-cells in our CD8-enriched splenocyte population. Negative control NOD-scid mice and recipients of BDC-2.5 or CD8 splenocytes alone showed no lymphocytic infiltrate (Fig. 5 and Table 2) and, thus, no staining for any T-cell or macrophage markers.

The NOD-scid recipients of BDC-6.9 or BDC-2.5/CD8 splenocytes differed from young NOD recipients of the clones primarily in that the transferrred clones are a dominant component of the infiltrate in the NOD-scid mice, whereas they are barely detectable after transfer in the young NOD mice. The islets of young NOD recipients show CD4 T-cells, CD8 T-cells, and a significant macrophage population with few Vb4 T-cells apparent in the lesions and are similar to the infiltrates seen in spontaneously diabetic female NOD mice. These results have been confirmed in a more quantitative manner using flow cytometric analysis and suggest that in the immunocompetent recipient, few initiating T-cells are required to trigger diabetes (J.D.P., K.H., unpublished observations).

**DISCUSSION**

We have reported on the induction of diabetes in the NOD-scid mouse by two CD4$^+$ Th1 islet-specific T-cell clones that were derived from two different NOD donors. The clones, BDC-2.5 and BDC-6.9, are similar in many respects: both proliferate in response to islet cells as antigen in the presence of NOD irradiated spleen cells as APCs; they also make Th1-type cytokines, including IL-2, IFN-$\gamma$, TNF, and IL-3/GM-CSF, but they do not produce the Th2 cytokine, IL-4. Both clones are diabeticogenic in young NOD (21,28) and NOD F1 mice (22) $\pm 2$ weeks in age. The antigens to which these clones respond are not yet defined but are localized in the membrane of the $\beta$-granules and do not appear to be among the various candidate autoantigens from islets (29) such as insulin and GAD. The only obvious differences in these two clones, detectable by in vitro assay, are in the V$\alpha$ regions of their TCR and in their response patterns to islet cells as antigen. Both clones respond to the insulin secretory granule membrane fraction isolated from NOD $\beta$-tumor cells, but they respond differently in assays with whole islet cells: BDC-2.5 reacts to islet antigen from all mouse strains we have tested, whereas BDC-6.9 responds only to islet cells isolated from NOD, its related strains (NOR and NON), and SWR (30). However, as shown in this report by the clone transfer experiments in NOD-scid mice, the requirements for effective induction of disease by the two clones are appar-
FIG. 5. Hematoxylin and eosin staining of insulitis in 8- to 10-week-old NOD-scid mice after diabetes transfer. Photomicrographs (×100) were taken of snap-frozen 5-μm pancreas sections from BDC-6.9, BDC-2.5/CD8 splenocyte, and CD8 splenocyte recipients as well as from a 4-month-old control NOD female mouse that spontaneously developed diabetes. Diabetic mice were killed at the time of diabetes onset; nondiabetic mice and control mice were killed 10 weeks after the first injection of T-cell clones or splenocytes (as described in the legend to Fig. 4). Tissue sections were stained using hematoxylin and eosin to detect the small dense nuclei of infiltrating cells indicative of the presence of disease.
TABLE 2
Characterization of cells in islet infiltrates in NOD versus NOD-scid mice

<table>
<thead>
<tr>
<th>Scoring</th>
<th>Intravenous BDC-6.9</th>
<th>Intravenous BDC-2.5</th>
<th>Intravenous CD8 splenocytes</th>
<th>Intravenous BDC-2.5 + CD8 splenocytes</th>
<th>Intrapерitoneal BDC-6.9</th>
<th>Intrapерitoneal BDC-2.5</th>
<th>Spontaneous diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Insulitis</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD4 cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CD8 cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V84 cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>MAC-1 cells</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Recipient animals received 3 injections of 10⁷ clones (one per week) with or without a single cotransfer of purified CD8 splenocytes from diabetic female NOD mice at the time of the first injection. Diabetes was defined as 2 consecutive days of elevated urine glucose levels and hyperglycemia (blood glucose > 15 mmol/l). The degree of insulitis was determined on hematoxylin and eosin stained frozen pancreas sections. A score of 0 indicates no detectable infiltration, 1 indicates a small number of infiltrating cells next to the islet, 2 indicates a large peri-islet infiltrate, and 3 indicates extensive intra-islet infiltration. The type of infiltrating cells in the islet lesions was determined by immunohistochemical staining with antibodies to CD4, CD8, V84, and MAC-1. A single + indicates that positively staining cells were <50% of the infiltrating cells; ++ indicates that positively staining cells comprised >50% of the infiltrating cells; +/− indicates that positively staining cells were clearly detectable at low numbers; and − indicates that positively staining cells were not detectable.

ently quite different. The BDC-6.9 clone provides a demonstration that a CD4 T-cell clone, in the absence of any host lymphocytes, can cause diabetes to occur in NOD-scid mice rapidly and consistently. In contrast, BDC-2.5 transfers disease only in the presence of CD8 T-cells.

It can be difficult to compare spontaneous diabetes, which appears to be mediated by heterogeneous T-cell effector populations, with disease induced by cloned T-cells because spontaneous diabetes may differ from experimentally transferred diabetes in fundamental ways that we do not yet understand. However, our present approach with two diabetogenic T-cell clones has provided us with a simple model to address important questions in diabetes pathogenesis. Are both (or is either) of these clones representative of the autoreactive T-cells involved in the initiation and propagation of the spontaneous disease process? There are several reasons why they may be. First, because the two clones, BDC-2.5 and BDC-6.9, were isolated from spleen and lymph nodes (and not the islets) of newly diabetic mice, we think it likely that they represent memory T-cells that encountered β-cell antigen early in the disease process. Second, the clones are highly diabetogenic, and it takes only a small number of these T-cells within the lesion to accelerate diabetes. Third, BDC-2.5 and BDC-6.9 belong to a panel of clones, all of which react with antigen(s) closely associated with the membrane of the β-granule, suggesting that there may be an immunodominant protein selecting this T-cell response.

In advanced stages of autoimmune disease after a variety of self-antigens have been generated, it might well be predicted that there would arise a large number of autoreactive T-cells with diverse specificities. We would hypothesize, however, that the autoreactive T-cells involved in initiation of pathogenesis are quite rare. For one thing, they are difficult to isolate. Also, we have been unable to detect bulk population spleen or lymph node T-cell responses above background to islet cell antigen (or any other antigen, including GAD and insulin) from unimmunized NOD mice, suggesting that there may be a low precursor frequency for islet-reactive T-cells. On the other hand, if NOD mice are given a low-dose immunization of islet cells (i.e., a dose that does not prime T-cells in a BALB/c mouse), T-cell responses to islet cells are readily detected.

Another reason for thinking that these T-cell clones represent rare autoreactive T-cells is that it apparently takes only small numbers to initiate disease, as indicated by the fact that they are barely detectable in islet infiltrates. In young NOD recipients, as few as 1 x 10⁷ T-cell clones can induce disease, and trace-labeling studies in NOD islet-grafted (CBA x NOD)F1 (26) and, more recently, in young NOD mice (J.D.P., K.H., unpublished observations) have shown that after transfer, the T-cell clones comprise <5% of the pancreatic infiltrate. Furthermore, preliminary results from FACS analysis of T-cells found in the islets after clone transfer indicates that when compared with T-cells in lymph nodes of NOD and BALB/c mice, the IL-2 receptor is not upregulated on T-cells in the lesion, suggesting that activated autoreactive T-cells are low in number, and the vast majority of T-cells present are a result of the inflammatory state.

It has been assumed by many investigators that the islet lesion is the most likely source of disease-relevant T-cells, but by the time that insulitis is histologically obvious, T-cells in the pancreas are diverse in TCR (31–34) and perhaps also in antigen specificity (35,36). We would argue that memory T-cells should be present in the lymphoid tissues of the mouse throughout the course of disease. Indeed, after a mouse has become diabetic, the islets may not be a good source of autoreactive T-cells because the infiltrating cells may decrease in number or disappear altogether in late stages of disease. For example, we have noted that in some older animals that develop spontaneous disease or in mice that are not immediately killed after becoming hyperglycemic in diabetes caused by the T-cell clones, there are islets that contain no infiltrate despite being devoid of granulated β-cells. This might suggest that as β-cell destruction reaches the terminal stages, the antigen is no longer present.

In transfer experiments with diabetogenic clones into young NOD mice, both CD4+ and CD8+ T-cells from the host are recruited to the site of inflammation. However, our results in the NOD-scid mouse show that at least one of our islet-specific Th1 clones, BDC-6.9, can induce disease in the absence of host B-cells, CD4+ T-cells, or CD8+ T-cells. A
second clone, BDC-2.5, requires the addition of CD8 T-cells. The difference between the two clones in their requirements for inducing disease may provide an explanation for results of others with bulk population transfer systems in which it generally has been observed that both CD4 and CD8 T-cells are needed, but in some instances, CD4 T-cells alone instigate diabetes. In studies with the transfer of diabetic spleen cells into NOD-scid mice, Christianson et al. (17) found that although the highest incidence of disease occurred with transfer of unfractonated diabetic spleen cells, >50% of recipients became diabetic with transfer of purified CD4 T-cells from diabetic animals. Similar to results we have obtained, these authors found that CD8 T-cells alone were unable to transfer disease. Diabetes could not be transferred with prediabetic CD4 populations in combination with anti-CD8 treatment in vivo, suggesting the importance of CD8 T-cells in earlier inductive stages of the disease. This possibility of a CD8 role in the initiation of diabetes is supported by recent observations that β2-microglobulin knockout NOD mice develop neither diabetes nor insulin (37,38). In light of these findings, the BDC-6.9 clone may represent a phenotype of T-cell that arises later in the disease process, whereas the BDC-2.5 clone may be representative of the earliest diabetogenic CD4 T-cells that require help from CD8 T-cells. Alternatively, both types of T-cell may be present early in the disease process, but BDC-6.9 may be a relatively rare type of CD4 T-cell that, only by the latest stage of the disease, expands to sufficient numbers within the CD4 compartment to transfer diabetes.

Another reason why our T-cell clones may be representative of autoreactive T-cells in spontaneous disease is that they appear to be reacting with an immunodominant antigen in the β-granule membrane. There is a growing list of candidate autotigens in type I diabetes (heat shock protein, GAD, or insulin) for T-cells as well as B-cells (29). However, each of the seven diabetogenic clones in our panel was selected on whole islet cells, and of those tested, there is greatly enhanced activity on fractions enriched in the β-granule membrane (25). A narrow antigen restriction pattern is further suggested by preliminary results indicating that the clones BDC-2.5 and BDC-6.9 react to the same protein fraction collected from anion exchange and gel filtration columns (B. Bergman, K.H., unpublished observations). The latter findings with these two clones are particularly interesting in view of the fact that their response patterns to whole mouse islet cells are different, indicating that there is a difference at least at the fine specificity level, perhaps due to different epitopes on the same protein. In addition to the restricted antigen specificity suggested by the responses of the clones to β-granules, we have identified two clones with identical antigen response patterns. The clone BDC-6.9 described in this report and a second clone, BDC-9.25, were derived from different animals and use different TCR Vβ regions. However, the two clones show exactly the same pattern of responses when tested on the first-generation backcross of (BALB/c × NOD)F1 to BALB/c mice, where islets from ~50% of backcross progeny were found to have the BDC-6.9 antigen (30). BDC-9.25 also can induce diabetes in NOD-scid mice, like the BDC-6.9 clone, and in the absence of any added cells. The isolation of two such clones from the peripheral T-cells of different animals with identical antigen specificities and diabetogenic activities may indeed be an indication of an important autoreactive immune phe-

ACKNOWLEDGMENTS

This research was supported in part by National Institutes of Health Award PO1 DK40414, Juvenile Diabetes Foundation Career Development and American Diabetes Association research awards (to K.H.), and by Juvenile Diabetes Foundation and National Institutes of Health postdoctoral fellowship awards (to J.D.P.).

The authors thank Bobbi Pike, Mary Portas, Duane Wambaath, and Kevin Gosselin for technical assistance in conducting this study. Human recombinant IL-2, used in T-cell clone expansion cultures, was the generous gift of AMGEN (Thousand Oaks, CA). We also wish to express our appreciation to E.H. Leiter for supplying breeding pairs of NOD-scid mice and to D. Lo and A. Kuper for advice on immunohistochemistry protocols.

REFERENCES


38. Serreze DV, Leiter EH, Christianson GJ, Greiner D, Roopenian DC: Major histocompatibility complex class I-deficient NOD.B8m<sup>−/−</sup> mice are diabetes and insulitis resistant. *Diabetes* 43:595–598, 1994


48. Serreze DV, Leiter EH, Christianson GJ, Greiner D, Roopenian DC: Major histocompatibility complex class I-deficient NOD.B8m<sup>−/−</sup> mice are diabetes and insulitis resistant. *Diabetes* 43:595–598, 1994