Pancreatic islet-specific T-cell clones from nonobese diabetic mice
(diabetes/islet antigens)

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Communicated by David W. Talmage, June 30, 1989

ABSTRACT We have produced a panel of islet-specific T-cell clones from nonobese diabetic (NOD) mice. These clones proliferate and make interleukin 2 in an antigen-specific manner in response to NOD antigen-presenting cells and islet cells. Most of the clones respond to islet-cell antigen from different mouse strains but only in the presence of antigen-presenting cells bearing the class II major histocompatibility complex of the NOD mouse. In vivo, the clones mediate the destruction of islet, but not pituitary, grafts. Furthermore, pancreatic sections from a disease transfer experiment with one of the clones showed a pronounced cellular infiltration and degranulation of islets in nondiabetic (CBA × NOD)F1 recipients.

Type I or insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease that affects 0.1–0.5% of the general population in the U.S., with clinical onset occurring most commonly before 18 years of age. The disease is a chronic, destructive process and is characterized by a long latent period. As the insulin-producing beta cells in the pancreatic islets have undergone extensive damage by the time of diagnosis, patients face a lifetime of dependency on insulin. Thus, better techniques for early diagnosis and possible rescue of beta-cell function are greatly needed. One way in which these goals may be met would be through the identification of the antigenic molecules on islet cells so that more effective prevention and immunotherapeutic procedures could be developed. Another approach is through investigation of the immunological mechanisms involved in disease pathogenesis with the view to discovering agents that may more specifically prevent beta-cell destruction. We are pursuing both lines of investigation through the generation and characterization of T-cell clones specific for islet-cell antigen.

The nonobese diabetic (NOD) mouse is a widely used animal model of IDDM, and the course of the disease in these mice closely parallels the progression of IDDM in humans. The cellular infiltration into the pancreas that leads to insulitis and beta-cell destruction can begin as early as 4 weeks of age in the NOD mouse, and, in 3–4 months, the animal may be overtly diabetic. A number of studies have indicated a role for T lymphocytes in the development of autoimmune diabetes, both in the NOD mouse and in other animal models. Harada and Makino (1) obtained data indicating a role for the thymus and demonstrating the effectiveness of antithymocyte antibodies in the prevention of overt diabetes in the NOD mouse. The protective effect of antibodies to T cells in autoimmune diabetes has also been reported in studies of the BB rat (2), in experiments with mice treated with the diabetogenic drug streptozotocin (3), and, more recently, in studies with anti-CD4 antibodies in the NOD mouse (4–6). Disease transfer studies in both BB rats (7) and NOD mice (8) or NOD F1 mice (9), in which injections of spleen cells from diabetic animals have induced diabetes in nondiabetic recipients, have further implicated a role for T cells in the disease process. More recent reports have shown that both CD4- and CD8-positive T cells are required for transfer of the disease (10, 11).

To learn more about the T cells involved in IDDM and about their antigen specificity, we have produced a panel of islet-specific T-cell clones from diabetic NOD mice. The clones have been selected on the basis of their ability to proliferate in an antigen-specific manner to whole islet-cell antigen in vitro. We have also tested these cell lines in an in vitro transplantation system to determine whether they can specifically mediate islet graft destruction. We have previously reported on an islet-specific T-cell clone derived from the NOD mouse (12). In this paper, we report on the isolation of additional islet-specific T-cell clones and the in vitro investigation of their specificities with regard to the islet antigen and major histocompatibility complex (MHC) restriction. We also describe their ability to cause specific islet damage in vivo.

METHODS

Isolation and Cloning of Islet-Specific T-Cell Lines. T-cell lines were derived from the spleens and lymph nodes of newly diabetic NOD mice as previously described (12). The tissue was homogenized, and the cells were placed directly into a culture of Click's medium containing fresh islet cells as antigen and 0.5% normal mouse serum. After 5 days in the primary culture, the cells were harvested and placed into 20-ml cultures containing fresh islet-cell antigen, NOD antigen-presenting cells (APC), and 10% (vol/vol) fetal bovine serum in a complete culture medium supplemented with interleukin 2 (IL-2) (2.5% of a phorbol 12-myristate 13-acetate-induced EL-4 supernatant). We have routinely used modified Mishell-Dutton culture medium for growing these cell lines.

Cultures were maintained by combining 1 × 10^6 responding T cells with 2.5 × 10^7 irradiated (2000 rad (1 rad = 0.01 Gy) from a 60Co source) NOD spleen cells as APC, 5–10 × 10^6 NOD islet cells as antigen, and 2.5% EL-4 supernate in a total volume of 20 ml of culture medium. Culture flasks were kept in an upright position in a 37°C CO2 incubator for 2 weeks, at which time the T cells were counted and harvested for assay and restimulation in a culture of fresh APC, antigen, and IL-2-containing medium. After two or three cycles of restimulation, there were usually sufficient cell numbers to begin cloning. Cloning of the T-cell lines was carried out by limiting dilution: cells were seeded at 10 and 5 cells per well into 96-well culture plates containing NOD APC (5 × 10^4 per well), NOD islet-cell antigen (1000–5000 cells per well), and IL-2. After 2 weeks, wells showing growth were expanded into 1-ml cultures; cells from these cultures were assayed and those that showed the best islet-specific responses were transferred into 20-ml cultures. For use in phenotypic anal-

Abbreviations: NOD, nonobese diabetic; APC, antigen-presenting cell; IL-2, interleukin 2; MHC, major histocompatibility complex; IDDM, insulin-dependent diabetes mellitus.

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ysis and in vivo, the T cells were expanded in medium containing IL-2 3 or 4 days following restimulation.

**Islet-Cell Antigen.** Antigen for normal maintenance and assay cultures was in the form of whole NOD islet cells, as described previously (12). To obtain an islet-cell suspension containing on the order of 3–5 × 10^6 individual islet cells, the pancreases of four NOD mice were digested in a collagenase-containing medium. Whole islets (300–500 islets represent a typical yield from four animals) were then picked clean of nonendocrine tissue and digested into single cells in a medium containing 0.05% trypsin and DNase at 2 μg/ml for 30 min at 37°C with intermittent agitation. The digest was spun through a layer of Eagle’s minimal essential medium/4% bovine serum albumin at 500–600 rpm (in a Jouan E4 swinging bucket rotor) for 15 min, after which the pellet was resuspended in complete culture medium. The islet cells were typically 70–90% viable at this point and were added immediately to cell cultures.

**In Vitro Assays for Proliferation.** The lines and clones were initially characterized and were continually monitored by assaying them for proliferation in response to islet-cell antigen and APC in a thymidine incorporation assay. Assays were carried out in triplicate by culturing 2 × 10^4 responder T cells, 5 × 10^6 irradiated spleen cells as a source of APC, and various numbers of islet cells, in a total volume of 200 μl of medium without IL-2, in 96-well microtiter plates for 3–5 days. For routine monitoring, the clones were incubated with syngeneic APC and antigen (2500 islet cells per well) for 3 days. The cultures were then pulsed with 1 μCi (1 Ci = 37 GBq) of [3H]thymidine and harvested 5 hr later, and their radioactivity was determined.

**Transplantation Procedures.** Transplantation of NOD islet or pituitary tissue was carried out as described (12). Islets were obtained from NOD mice and prepared in the usual manner by collagenase digestion of the pancreas, followed by removal of exocrine tissue. Pituitary tissue from the same NOD donors was used as the nonsuture control. Tissue to be grafted was added to 5–8 μl of blood from the (CBA × NOD)F<sub>2</sub> recipient. The blood was allowed to clot, and then the tissue and clot were inserted under the kidney capsule. Control grafts consisted of islet (75 uncultured islets) or pituitary tissue only and were placed under the capsule of the right kidney. Experimental grafts contained 1 × 10<sup>5</sup> test T cells in addition to islet or pituitary tissue and were transplanted on the opposite kidney. After 2 weeks, the kidneys were removed, and the grafts were examined macroscopically. Kidneys, spleens, and pancreases were then fixed in 10% (vol/vol) buffered formalin. Histological slides were made of sectioned material and stained with hematoxylin and eosin and, in the case of islet tissue, also with aldehyde fuchsin.

**Disease Transfer Studies.** Disease transfer studies were carried out by making consecutive injections of test T cells into 3-month-old (CBA × NOD)F<sub>2</sub> recipients three times a week for 4 weeks. Injections were made i.p. with 2 × 10<sup>6</sup> T cells per injection. Recipient mice were monitored throughout the period for blood glucose levels, and, at the end of 4 weeks, the pancreas from each animal was removed for histological examination.

**RESULTS**

**Response of T-Cell Clones to NOD Islet-Cell Antigen.** We have isolated a panel of islet-specific T-cell clones from NOD mice. Table 1 lists these clones and provides a summary of their reactivity characteristics. In vitro, the islet-specific clones proliferate and produce IL-2 in response to APC and islet-cell antigen from the NOD mouse. A typical proliferative response is illustrated in Fig. 1 with the results from an assay with the T-cell clone BDC-2.5, in which the islet-cell antigen concentration was varied. This experiment shows that the magnitude of the proliferative response of the islet-specific T-cell clones increases with the titer of islet-cell antigen. Similar results are obtained with BDC-2.5 or the other clones if the response measured is IL-2 production (data not shown). Fig. 1 also presents representative controls: the clones do not proliferate in the absence of antigen and APC or with APC alone. When sufficient antigen was available, we included controls for responding T cells and NOD islet cells in the absence of APC. In some assays, we did note a response above background with NOD islet cells. These values were quite variable from one experiment to another and were always much lower (e.g., 10-fold) than responses obtained in the presence of APC.

**Response of T-Cell Clones to Antigen from Other Mouse Strains.** Our islet-specific clones were derived, initially characterized, and subsequently maintained by culturing the T cells with syngeneic APC and islet-cell antigen. We started with this system because we did not know whether the antigen recognized by the NOD-autoreactive T cell would be unique to the NOD islet cell. The data in Fig. 2 indicate that this is not the case and that the clones, with one exception, can respond to antigen present on islet cells from other mouse

![Table 1. Responses of islet-specific T-cell clones](image)

<table>
<thead>
<tr>
<th>Clone</th>
<th>CD4/CD8</th>
<th>No Ag</th>
<th>NOD IC</th>
<th>Non-NOD IC</th>
<th>Rat IC</th>
<th>E4TC</th>
<th>Islet graft damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDC-2.4</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>BDC-2.5</td>
<td>-/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>BDC-4.6</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
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</table>

Ag, antigen; IC, islet cells. The circles indicate instances in which the reactivity patterns of clones are different.

![Fig. 1. Proliferative response of the islet-specific T-cell clone BDC-2.5 to NOD APC and increasing numbers of NOD islet cells. Responding T cells were incubated with APC and islet-cell antigen for 3 days at 37°C, after which the cultures were pulsed with [3H]thymidine (1 μCi per well) and harvested. Results shown are average values of triplicate samples. Controls included in this experiment were responding T cells alone and responders plus APC in the absence of islet-cell antigen. Levels of thymidine incorporation for controls shown in this graph are representative of background levels (typically between 100 and 1000 cpm) in these proliferation assays.](image)
strains. Results are from one experiment in which four islet-specific T-cell clones were tested for proliferation in response to NOD APC and islet-cell antigen obtained from three different mouse strains. The T-cell clones BDC-2.5, BDC-4.6, and BDC-5.10 consistently reacted with antigen from both NOD and other mouse strains. However, one clone, BDC-6.9, was found to react only with islet cells from the NOD mouse.

In addition to testing islet cells from non-NOD mouse strains, we also tested a mouse beta-cell tumor line, betaC3, as a source of antigen. betaC3 (kindly provided by S. Efrat, Albert Einstein College of Medicine, Bronx, NY) is one of a group of pancreatic beta-cell lines established from beta-cell tumors that were derived from transgenic mice carrying an insulin-promoted simian virus 40 tumor antigen gene (13). As indicated in Table 1, the clones could also proliferate in the presence of this tumor line. It was interesting to note that a second beta-cell tumor, RIN-m5F, an insulinoma derived from a rat islet cell tumor (14), did not serve as antigen for these clones.

Response of T-Cell Clones to Antigen from Rat or Human Islets. We have also tested the T-cell clones for reactivity to islet-cell antigen derived from rat and human pancreatic tissue. Of the clones screened to date, we have found one, BDC-5.2, that, in the presence of NOD MHC, responds to normal rat (DA and Lewis) islet cells, as well as to islet cells obtained from NOD or other mouse strains (data not shown). None of the clones tested reacts to human islet-cell antigen, at any concentration tested, up to 20,000 islet cells per test culture.

Phenotype and MHC Restriction of T-Cell Clones. Flowcytometric analysis of the islet-specific T-cell clones, carried out with antibodies to the murine CD4 and CD8 T-cell markers, established that all of the clones tested were of the CD4 phenotype. To determine the MHC restriction characteristics of the clones, we performed assays with APC from various mouse strains. Fig. 3 illustrates the results obtained with two of the T-cell clones in experiments in which the proliferative response to BALB/c islet-cell antigen was measured in the presence of APC from five mouse strains: NOD, CBA, (CBA × NOD)F1, B6, and BALB/c. Due to variable low responses sometimes obtained with NOD islet-cell antigen in the absence of APC, it was necessary to carry out the MHC restriction assays with antigen obtained from a non-NOD source. In Fig. 3A are typical results obtained with clone BDC-2.5: a good proliferative response to BALB/c islet-cell antigen was seen only in the context of APC from the NOD mouse or from the (CBA × NOD)F1. Results obtained in a separate experiment with a second clone, BDC-6.3, are shown in Fig. 3B. This clone was observed to proliferate in response to BALB/c islet-cell antigen with APC from NOD, (CBA × NOD)F1, or BALB/c mice. However, the islet antigen-specific response of BDC-6.3 occurs only with APC from the NOD or F1 mouse strains. The response observed with BALB/c APC takes place with or without addition of antigen, suggesting that BDC-6.3 may exhibit allorreactivity with the MHC of the BALB/c. Responses to alloantigens by antigen-specific T-cell clones or hybrids are not an unusual occurrence (15), and we observed allorreactivities with other T-cell clones. However, with every islet-specific clone, the islet antigen response occurs only in the context of the MHC of the NOD, and these clones do not respond to NOD APC in the absence of islet-cell antigen.

islet-specific T-cell clones destroy islet grafts in vivo. To determine whether the T-cell clones that demonstrated islet
antigen specificity in the in vitro assays for T-cell proliferation and IL-2 production could also lead to effects in vivo, we investigated the action of the clones on grafted islet tissue. Transplants of NOD islet or pituitary tissue into (CBA × NOD)F₁ recipients were made in the absence of test T-cell clones under the capsule of one kidney and in the presence of the T cells on the contralateral kidney. After 2 weeks, the mice were sacrificed, and the grafts were examined for signs of destruction.

These experiments have been carried out on six of the eight clones described here, and the results, along with those we have obtained from in vitro assays, are summarized in Table 1. As indicated in Table 1, in every case in which a clone had been demonstrated to show an islet antigen-specific response in vitro, that clone, upon testing in a transplant experiment, was also observed to cause islet antigen-specific destruction of graft tissue in vivo. Transplants containing pituitary tissue, obtained from the same donors as the islets, showed no signs of graft rejection whether they were made in the presence or absence of the T-cell clones. Analysis of the islet control grafts (made in the absence of T cells) showed that, with the exception of results obtained with clone BDC-6.9, the islet tissue of these grafts was healthy and intact. In contrast, islets grafted in the presence of the islet-specific T cells were totally destroyed. In the case of BDC-6.9, not only were the islets in the experimental graft site completely destroyed, but we also observed massive infiltration and considerable degranulation in the islets of the control graft site, indicating a migration of reactive cells from the test kidney. Furthermore, inclusion of the BDC-6.9 clone in the grafts led to infiltration and degranulation of islets in the pancreas of the recipient animal. In another transplant experiment with BDC-6.9, we found that the NOD-specific pattern of this clone's response that had been observed in the proliferation assays was confirmed in the in vivo experiments. If BDC-6.9 was added to CBA islet grafts, there was no tissue destruction.

One other clone tested in the in vivo transplant system exhibits behavior different from that of the islet-specific clones listed in Table 1. The nonspecific clone BDC-2.4 has been maintained as a negative control for the islet-specific clone BDC-2.5. BDC-2.4 is derived from the same T-cell line, BDC-2; this clone is not specific for islet antigen, but it proliferates and makes IL-2 in response to APC (with or without antigen) from the NOD mouse. When tested in the in vivo transplant system, BDC-2.4 did not damage grafted tissue (Table 1).

Disease Transfer with T-Cell Clones. We have also attempted to transfer disease with the islet-specific T-cell clones. Other experiments of this type have generally been made with single injections of T cells from diabetic mice into irradiated nondiabetic recipients (8, 9). In one of our first experiments, the recipient animals were irradiated (750 rad) prior to the injection of T cells. All of the recipient animals exhibited widespread, nonspecific pancreatitis that made interpretation of the results difficult. Subsequent studies were therefore carried out on unirradiated recipients. The mice received repeated injections of T-cell clones i.p. for 4 weeks. (CBA × NOD)F₁ mice were chosen as recipients since they do not develop diabetes nor do they exhibit any signs of insulitis in the pancreas.

The first disease transfer experiment was carried out with the uncloned line BDC-2, and, in subsequent experiments, the islet-specific clones BDC-2.5 and BDC-6.9 were tested. After 4 weeks, none of the animals (each experimental group consisted of four mice) treated in this fashion showed signs of overt diabetes as measured by blood glucose levels. However, upon histological examination of pancreatic sections, periductular and perivascular inflammation was observed in islets of most of the mice tested. When this inflammation was adjacent to islet tissue, the islets were partially degranulated, as illustrated in Fig. 4A. In the experiments with BDC-2 and BDC-2.5, there was some indication in most of the recipients of infiltration and degranulation of islet tissue, although damage was not extensive. Injections of the BDC-6.9 clone, however, led to more considerable infiltration and some islet destruction in all of the recipient mice. A typical pancreatic section from one of these experiments can be seen in the photograph in Fig. 4A. There is an extensive cellular infiltrate around ducts and vessels, and the aldehyde fuchsin staining indicates the degree of degranulation that could be seen in many islets. Although all of the sections examined from the BDC-6.9 animals showed similar signs of infiltration and islet degranulation, there were also islets that appeared healthy and intact. Fig. 4B shows pancreatic islets from a control F₁ animal; two islets are shown and are darkly stained with aldehyde fuchsin, indicating the presence of normal, well-granulated tissue.

DISCUSSION

We have described a panel of cloned murine T-cell lines derived from NOD mice that are specific for islet-cell antigen both in vitro and in vivo. These clones are of the CD4

Fig. 4. Disease transfer with BDC-6.9. (A) Pancreatic section from a (CBA × NOD)F₁ mouse that received multiple injections of BDC-6.9 (2 × 10⁶ cells per injection) over a 4-week period. Periductular and perivascular infiltration by mononuclear cells is evident in this section, which was stained with aldehyde fuchsin. Letters denote pancreatic structures in the section: V, blood vessels; D, ducts; I, islets. Note lighter areas around the periphery of the islet, which illustrate degranulation; the darker areas toward the middle of the islet have stained with aldehyde fuchsin, indicating that there is still some relatively undamaged beta-cell mass in this islet. The appearance of sections cut serially into the sample block did not change, indicating that the section shown here was representative of tissue located centrally in the islet. (B) Control (CBA × NOD)F₁ mouse pancreas stained with aldehyde fuchsin. Note the presence of well-granulated islets and the absence of inflammatory cells. (×125.)
phenotype, and they proliferate and produce IL-2 in an MHC-restricted manner in response to whole islet-cell antigen. In general, the clones react to islet cells from various mouse strains but only in the presence of APC from the NOD mouse; they do not react to islet cells or NOD APC alone. Our results suggest that the T-cell clones react with islet antigens common among mouse strains and also that more than one antigenic determinant may be recognized. Although most of the clones react with islet cells from all of the mouse strains we tested, one clone (BDC-6.9) reacts only with NOD islet-cell antigen, and a second clone (BDC-5.2) is the only one we found that responds to rat islet cells. Another indication of multiple antigen specificities is provided by the various patterns of alloreactivity seen with the clones. The islet specificity of the clones was demonstrated not only by the responses to islet cells and lack of reactivity to other cell types (lymphoid, thyroid, and pituitary) in vitro but also by the tissue-specific destruction in the transplant experiments. The fact that a number of the islet-specific T-cell clones respond to a beta-cell tumor line implicates recognition of beta-cell antigens.

Experiments carried out to determine the MHC restriction characteristics of the islet-specific T-cell clones indicate that antigen recognition by the clones occurs only in the presence of NOD APC and that the MHC origin of the islet cell is not involved. Class II restriction of the clones is suggested by their CD4 phenotype and by their lack of reactivity in the presence of APC bearing the same class I MHC products as the NOD (H-2Kd or H-2Dd) mouse. The NOD mouse does not express I-E. Therefore, the clones are presumably restricted by the I-A of the NOD mouse, which, although it is essential of the d haplotype, contains several unique sequences (16). It is possible that we have in some way selected for the CD4 phenotype in the isolation of these T-cell clones, although this was not intentional. It is also possible that the phenotype reflects the kind of T cells that are involved in the disease process, the expression of which has been shown to be dependent on CD4 T cells (4-6).

Further evidence for the relevance of the T-cell clones to diabetes was provided by the results of our experiments in vivo in which the islet-specific T-cell clones were found to mediate islet but not pituitary graft destruction. These findings were extended by studies in which we attempted to adoptively transfer the disease with repeated injections of the T-cell clones. The mice in these experiments did not become hyperglycemic over the 4-week test period; however, in each group, there were animals that exhibited clear signs of cellular infiltration and islet pathology in the form of beta-cell degradation. The infiltration and beta-cell damage was especially dramatic in the experiment in which the T-cell clone BDC-6.9 was used: every animal in this group showed islet damage. We did note in all of the disease transfer animals that every pancreas had both damaged islets and intact islets that appeared healthy and free of infiltrating cells. This could explain why none of the mice became hyperglycemic; their insulin production was not sufficiently impaired to cause an elevation in blood sugar levels. Nevertheless, in terms of their relevance to diabetes, we feel that the results of these disease transfer experiments are encouraging, particularly since they were carried out in (CBA × NOD)F1 mice that do not normally manifest signs of insulitis and diabetes and were not treated in any way (e.g., irradiation) to cause them to become more susceptible to development of islet damage.

The studies described here have indicated that islet-specific T-cell clones hold promise for further investigation into the role of T cells in the pathogenesis of type I diabetes and the nature of the antigen that activates them. Biochemical analyses of islet preparations in the attempt to identify molecules with antigenic determinants that stimulate the T-cell clones should prove helpful. Another approach to learning more about the antigen specificities of the clones may be through the examination of their T-cell receptors. It has been reported that in experimental allergic encephalomyelitis, another murine model of autoimmune disease, there may be limited heterogeneity of receptors used by T cells in the recognition of myelin basic protein, and, in addition, antibodies to the Vβ regions of these receptors may prevent or reverse disease (17). We have carried out preliminary cytofluorographic experiments with a number of antibodies to mouse Vβ determinants, and our initial results do not indicate that the islet-specific T-cell clones bear antigen receptors predominantly of one Vβ type. However, this does not preclude the possibility that there may be other shared structural regions among T-cell receptors on such clones.

We thank Peter Stecha for help in preparing the graphics for this manuscript and Drs. Don Bellgrau, Marcia McDuffie, and Dale Wegmann for review and commentary. This work was supported by National Institutes of Health Program Project Grant PO1 DK40144 and by a Diabetes and Education Research Foundation award to K.H.