Prevention of Diabetes in NOD Mice by Injection of Autoreactive T-Lymphocytes

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The nonobese diabetic (NOD) mouse develops a high incidence of autoimmune diabetes and is believed to be a good model for insulin-dependent diabetes mellitus (IDDM) in humans. We isolated T-lymphocyte lines from islets of newly diabetic NOD mice, some of which are autoreactive to NOD spleen cells. Because autoreactive T-lymphocytes have been implicated in immune suppression, we injected NOD mice with an autoreactive T-lymphocyte line. The injected mice had a marked decrease in incidence of IDDM compared with control mice. Moreover, their islets showed no insulitis at 1 yr of age. We conclude that autoreactive T-lymphocytes can prevent the development of IDDM in NOD mice. This result suggests that 1) islets contain both effector cells capable of damaging pancreatic β-cells and cells able to regulate this autoimmune response, and 2) development of IDDM depends on the balance between these opposing forces. Diabetes 38:1647-51, 1989

Insulin-dependent diabetes mellitus (IDDM) is believed to be an autoimmune disease in which activated T-lymphocytes destroy the insulin-producing β-cells of the pancreatic islets (1,2). This view is strongly supported by various studies in nonobese diabetic (NOD) mice, a high percentage of which spontaneously develop IDDM that closely resembles the human disease (3,4). The characteristic pathological lesion when IDDM first appears in both NOD mice and human diabetic subjects is insulitis, a mononuclear cell infiltration of the islets consisting mostly of T-lymphocytes. To better understand the pathogenesis of IDDM, we isolated T-lymphocytes directly from the islets of recently diabetic NOD mice (5). Among the cloned lines we obtained from this source, some show strict specificity for islets, whereas others are autoreactive to NOD spleen cells as well. The cloned islet-specific T-lymphocyte lines have been shown to produce insulitis and are described elsewhere (5). In this article, we describe the ability of an autoreactive T-lymphocyte line to prevent IDDM in NOD mice.

Autoreactive T-lymphocytes respond to syngeneic but not unrelated spleen cells in mixed-lymphocyte responses (6,7). In humans, the absence of autoreactive T-lymphocytes has been correlated with the presence of autoimmune disease (8). This has led to the suggestion that autoreactive T-lymphocytes play a critical role in maintaining immunologic homeostasis; some studies have indicated that autoreactive T-lymphocytes are potent inducers of suppressor T-lymphocyte activity. Indeed, Sano et al. (9) have shown that cloned strain A autoreactive T-lymphocyte lines injected into (AxB)F1 hybrid mice suppress T-lymphocyte responses to all antigens presented by the MHC of the A genotype. These studies led us to inject an autoreactive T-lymphocyte line isolated from NOD islets into young nondiabetic female NOD mice in hopes of inducing immunosuppression and preventing IDDM. Such treatments not only inhibit development of IDDM but also inhibit development of insulitis.

RESEARCH DESIGN AND METHODS

NOD/Caj mice were bred under pathogen-free conditions from stock kindly provided by Masakazu Hattori (Joslin Clinic, Boston, MA) and housed in the animal facilities at Yale University (New Haven, CT). The NOD mice were tested weekly for urinary glucose (Diastix, Ames, Elkhart, IN). Animals showing positive test values were tail clipped and analyzed for blood glucose with a Beckman glucose analyzer (Fullerton, CA); ≥250 mg/dl glucose was classified as overtly diabetic. Diabetic animals were treated with 2 U insulin (Ultralente, kindly provided by Lilly, Indianapolis, IN) for 5 days/wk. The incidence of spontaneous diabetes in the
colony by 8 mo of age was 85% in the females and 45% in the males. Other strains of mice were bred at Yale from stocks provided by D.B. Murphy (New York State Dept. of Health, Albany, NY).

The autoreactive T-lymphocyte line (PR-3) was generated from islets obtained from newly diabetic (24–48 h) NOD female mice. Pancreatic islets were isolated with the method of Prowse et al. (10). Single islets were cultured in Click's EHAA medium supplemented with 5% fetal calf serum (FCS) and 5 U/ml recombinant interleukin 2 (rIL-2). The autoreactive T-lymphocyte line PR-3, grown out of the islets, was maintained on the following schedule: day 0, the cells were placed into Click's medium supplemented with 5% FCS; day 2, mitomycin C (MMC)-treated syngeneic NOD spleen cells were added to the cultures; and days 4–21, the cells were maintained in Click's FCS-supplemented medium with the addition of 5 U/ml rIL-2.

**Characterization of autoreactive T-lymphocytes.** Proliferation assays were used to determine the specificity of the islet-derived T-lymphocytes. Syngeneic and allogeneic MMC-treated splenocytes (10⁵) were used as stimulators and cultured with the responding islet-derived T-lymphocytes (10⁴) in 0.2 ml Click's medium and 5% FCS in 96-well microtiter plates. Forty-eight hours later, rIL-2 (5 U/ml) was added to the wells, and at 96 h of incubation, each well was pulsed with 1 μCi [³H]thymidine and harvested 16 h later. Thymidine incorporation was calculated as mean counts per minute from duplicate cultures.

The T-lymphocytes were phenotyped by staining with rat monoclonal antibodies (MoAbs) specific for Thy-1 (Y-19; 11), CD3 (YCD3-1; 12), CD8 (53.6.7; 13), and CD4 (GK1.5; 14) in staining buffer (phosphate-buffered saline, pH 7.4, with 2% FCS and 0.1% NaN₃). T-lymphocytes (10⁶) were incubated with 100 μl MoAb on ice for 30 min. Excess MoAb was removed by washing with staining buffer, and bound antibody was detected by incubation for 30 min with 100 μl fluorescein-conjugated rabbit anti-mouse Ig that cross-reacts with rat Ig. After washing, immunofluorescence analysis was performed (FACS 440, Becton Dickinson, Mountain View, CA).

Lymphokine production by the T-lymphocytes was determined by dot-blot mRNA analysis with [³²P]-labeled probes for the various lymphokines as described previously (15) and bioassay of culture supernatants from NOD islet-derived T-lymphocytes (10⁴) cultured with NOD splenocytes (10⁵) in Click's medium supplemented with FCS. Supernatants were added at 25% to the lymphokine-dependent cell lines HT2, CTLL, and Wehi-3 with and without the addition of the anti-IL-2 (S4B6; 16), anti-IL-4 (11B11; 17), and anti-interferon-γ (INF-γ) (H1; 18) antibodies. The cultures were pulsed 36 h later and harvested as described previously (15).

Nondiabetic female NOD littermates aged 5 wk were divided into treatment groups and received T-lymphocytes or saline intraperitoneally according to two schedules (single dose or multiple injections).

At the end of the in vivo study, four nondiabetic mice that had received multiple injections of T-lymphocytes or saline were killed. The pancreatic tissue was removed and fixed in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

**RESULTS**

The NOD mouse develops insulitis followed by β-cell destruction leading to IDDM. To study the lymphocytes infiltrating the islet, we isolated islets from newly diabetic female NOD mice and derived T-lymphocyte lines from these islets. The proliferative response of the islet-derived T-lymphocyte line PR-3 is shown in Fig. 1, with splenocytes or islets from strains of the following MHC genotypes used as stimulators: NOD (KdFad-Eb-Dd), BALB/c (KdFad-Eb-Dd), B10.BR (KbFad-Eb-Dd), and B10.GD (KbFad-Eb-Dd), where E~ means no expression of I-E molecules. PR-3 only proliferated in response to NOD spleen or islet cells. The failure of PR-3 cells to respond to strain B10.GD, which differs from NOD only

**FIG. 1.** Proliferative response of nonobese diabetic (NOD) mouse islet-derived PR-3 T-lymphocytes to spleen cells (open bars) and islets (solid bars). Stimulator cells (y-axis) were obtained from B10.GD, B10.BR, BALB/c, and NOD mice; proliferative response of PR-3 was measured by [³H]thymidine incorporation. cpm, Counts per minute.

**FIG. 2.** Effect of in vivo treatment with autoreactive T-lymphocytes on onset of insulin-dependent diabetes mellitus (IDDM) in female nonobese diabetic (NOD) mice. Percentage of female NOD mice developing IDDM is shown measured over time; days refers to age of recipient animals. Three treatment groups were used in 1-yr study: saline (n = 38; open bars), autoreactive PR-3 T-lymphocytes (n = 41; hatched bars), and NOD splenic T-lymphocytes (n = 5; solid bars).
FIG. 3. Histopathology of pancreas from representative saline- and PR-3-treated nonobese diabetic (NOD) female mice. Pancreatic sections from NOD mice that had received 1 yr of monthly treatments of saline (A) or autoreactive PR-3 T-lymphocytes (B) were removed and stained with hematoxylin and eosin.
at I-\(A\) within the MHC, suggests that PR-3 is specific for I-A\(^{AnO}\). To confirm that PR-3 cells were autoreactive, stimulator spleen cells from mice of 11 independently derived H-2 haplotypes were tested. An equivalent response to B10.RIII (H-2\(^b\)) and NOD splenocytes was observed (data not shown), demonstrating cross-reactivity to H-2\(^b\), presumably to I-A\(^b\).

To further characterize the autoreactive T-lymphocyte line PR-3, it was stained with MoAbs directed at various cell surface molecules. PR-3 stained homogeneously with the antibodies to CD3 and CD4, but not with anti-CD8, classifying it as a CD4 T-lymphocyte (not shown). Although the PR-3 cells used in these experiments were not deliberately cloned, they were derived from a single culture well. We believe the cells are homogeneous, because PR-3 cells frozen before the initiation of these experiments stain homogeneously with antibody to the V\(\beta_3\) portion of the T-lymphocyte receptor. This antibody stains only a small percentage of normal T-lymphocytes in NOD mice. Furthermore, all PR-3 subclones and this PR-3 line have identical characteristics.

CD4 T-lymphocytes can be further divided into T-inflammatory (T\(_i\)) and helper (T\(_h\)) T-lymphocytes by the pattern of lymphokines they produce (15,16). Lymphokine production by PR-3 on stimulation by NOD spleen cells or mitogens was determined from mRNA analysis and biological assays; PR-3 produces IL-2 and IFN-\(\gamma\) but not IL-4. These results demonstrate that the PR-3 T-lymphocyte line is composed of autoreactive CD4 T-lymphocytes belonging to the T\(_h\), T-lymphocytes.

In vivo treatment of prediabetic NOD mice with the autoreactive T-lymphocyte line PR-3. To determine whether injection of autoreactive T-lymphocytes could prevent diabetes in young prediabetic NOD mice, recipients were injected with PR-3 cells. In an initial in vivo experiment, groups of 15 nondiabetic 5-wk-old female NOD mice received a single injection of 10\(^7\) PR-3 cells or saline. In the control group, which had received saline, animals first developed IDDM at 90 days of age, and by 210 days, 80% had the disease. This is similar to the incidence of IDDM in untreated NOD mice in our colony. In the experimental group, which received a single PR-3 treatment, the onset of IDDM was delayed to 180 days, and only 30% of the mice developed the disease by 1 yr (\(P < .01\) at 180 days; \(P < .05\) at 1 yr).

In the second study, groups of animals received monthly treatments with 10\(^7\) PR-3 cells, saline, or 10\(^7\) young NOD splenic T-lymphocytes (Fig. 2). Again, we observed that IDDM appeared at \(\sim 90\) days in the saline-treated control mice and reached an incidence of 85% at 240 days. Three of the five mice that were injected with splenic T-lymphocytes from NOD female mice had developed IDDM at 150 days. However, in the experimental mice treated with the PR-3 autoreactive T-lymphocyte line, disease was virtually absent; 10% of the mice developed IDDM after 1 yr (\(P < .01\) at 1 yr).

To evaluate the effect of the in vivo PR-3 treatment on the development of insulitis in the NOD mice, the pancreas was sectioned and stained at the end of the treatment schedule. Representative sections from saline- and PR-3-treated mice are shown in Fig. 3. Nearly all of the islets from the saline-treated nondiabetic mice showed the typical insulitis observed in the NOD mouse, with many mononuclear cells infiltrating the islets (Fig. 3A). By contrast, all islets examined from nondiabetic NOD mice treated monthly with the autoreactive PR-3 T-lymphocyte line had no lymphocytes inside or surrounding the islets (Fig. 3B).

**DISCUSSION**

We previously documented that the islets of recently diabetic NOD mice contain islet-specific CD4 and CD8 T-lymphocytes (5). A combination of islet-specific CD4- and CD8-cloned T-lymphocytes can transfer insulitis and \(\beta\)-cell destruction to irradiated NOD mice (5), demonstrating that the \(\beta\)-cell destruction observed in the NOD mouse can be mediated by islet-specific T-lymphocytes alone. In this study, we show that autoreactive T-lymphocytes are also present in NOD islets by isolating such cells as T-lymphocyte lines. It is intriguing that autoreactive T-lymphocytes are present with islet-specific effector T-lymphocytes in NOD islets, because autoreactive T-lymphocytes have been suggested to be involved in activating suppression. Their presence in islets implies that the disease process involves both effector and regulatory T-lymphocytes. To determine whether the autoreactive T-lymphocytes isolated from NOD islets were capable of modulating the disease process, we asked whether injection of such autoreactive T-lymphocytes could inhibit diabetes and insulitis in young NOD mice. The data presented herein show that the autoreactive T-lymphocyte line PR-3 significantly prevents the development of diabetes and insulitis, particularly if cells are injected monthly. Thus, the islets of recently diabetic mice contain both islet-specific T-lymphocytes capable of \(\beta\)-cell destruction (5) and cells capable of inhibiting the development of diabetes as exemplified by PR-3.

Earlier studies have strongly suggested the presence of regulatory T-lymphocytes in the lymphoid tissues of the NOD mouse. Boitard et al. (19) showed that CD4 T-lymphocytes can inhibit the development of diabetes in adoptive transfer models. The specificity of these cells has not been determined nor have they been isolated and grown in vitro. Thus, it is not clear whether such cells are similar to PR-3 or distinct.

The specificity of the inhibitory effect induced by PR-3 is not known. Cohen (20) and Lohse et al. (21) proposed two classes of suppressor cells that can inhibit autoimmune diseases. One class is anti-idiotypic, putatively specific for the receptor on the inducing T-lymphocyte (20), and the second class is described as anti-ergotypic, putatively specific for the activated state (21). Our data are not consistent with an anti-idiotypic mechanism of suppression, because PR-3 bears a receptor distinct from that found on islet-specific T-lymphocytes as shown by differences in specificity and differences in V\(\beta\) expression. Diabetogenic, islet-specific cloned T-lymphocyte lines express V\(\beta_3\)-encoded T-lymphocyte receptors (5), whereas PR-3 cells express only V\(\beta_2\)-encoded T-lymphocyte receptors (not shown). Whether PR-3 induces anti-ergotypic suppressor cells is not known. A third possibility, consistent with the earlier studies by Bell-grau et al. (22), Sano et al. (9), and Katz et al. (23), is that autoreactive T-lymphocytes induce suppression for any T-lymphocyte that recognizes antigen presented by the target MHC molecule, here presumably I-A\(^{AnO}\). We propose that autoreactive T-lymphocytes bind class II MHC molecules, internalize them, and present processed peptides from these
ligands in association with cell surface class I MHC molecules. Similar peptide–class I MHC complexes would be presented by T-lymphocytes recognizing autoantigens presented by self class II MHC molecules. This may or may not be equivalent to Cohen’s antiergotypic T-lymphocytes; further studies are required to determine the mechanism of suppression in both cases. Finally, our results are consistent with the studies by Serreze et al. (24) showing that immunostimulation, which potentiates the autologous mixed lymphocyte response in NOD mice, prevents diabetes.

In summary, our studies have shown that the islets of recently diabetic NOD mice contain both islet-specific and autoreactive T-lymphocytes. Injection of the autoreactive T-lymphocytes, isolated as a T-lymphocyte line from these islets, into young nondiabetic NOD mice profoundly inhibits (90%) the development of diabetes and almost eliminates insulins. Thus, the islet of a diabetic mouse apparently contains both effector cells and cells capable of inhibiting these effector cells. This raises the hope that this immunoregulatory balance can be shifted in favor of suppression and thus impede β-cell destruction, as has been achieved artificially in this study by repeated injection of autoreactive T-lymphocytes.

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