Comparing the Relative Role of Perforin/Granzyme Versus Fas/Fas Ligand Cytotoxic Pathways in CD8+ T Cell-Mediated Insulin-Dependent Diabetes Mellitus

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CD8+ cytotoxic T cells play a critical role in initiating insulin-dependent diabetes mellitus. The relative contribution of each of the major cytotoxic pathways, perforin/granzyme and Fas/Fas ligand (FasL), in the induction of autoimmune diabetes remains controversial. To evaluate the role of each lytic pathway in β cell lysis and induction of diabetes, we have used a transgenic mouse model in which β cells expressing the influenza virus hemagglutinin (HA) are destroyed by HA-specific CD8+ T cells from clone-4 TCR-transgenic mice. Upon adoptive transfer of CD8+ T cells from perforin-deficient clone-4 TCR mice, there was a 30-fold increase in the number of T cells required to induce diabetes. In contrast, elimination of the Fas/FasL pathway of cytotoxicity had little consequence. When both pathways of cytolysis were eliminated, mice did not become diabetic. Using a model of spontaneous diabetes, which occurs in double transgenic neonates that express both clone-4 TCR and Ins-HA transgenes, mice deficient in either the perforin or FasL/Fas lytic pathway become diabetic soon after birth. This indicates that, in the neonate, large numbers of autoreactive CD8+ T cells can lead to destruction of islet β cells by either pathway. The Journal of Immunology, 1999, 163: 4335–4341.

Insulin-dependent diabetes mellitus (IDDM) is a T cell-mediated autoimmune disease characterized by the destruction of insulin-producing pancreatic β cells located in the pancreatic islets of Langerhans (1–3). Such destruction requires both CD4+ and CD8+ T cell subsets (4–10). Several findings have suggested a crucial role for the CD8+ T cell subset in the early induction phase of IDDM (11–15). It is proposed that, during this phase, β cells are destroyed by cytotoxic CD8+ T cells, leading to the release of pancreatic Ags. These Ags are picked up and presented on professional class II-positive APCs, which can subsequently activate CD4+ T cells. Although CD4+ T cells may not damage the class II-negative β cells directly (16), they can recruit other effector cells, such as activated macrophages, that can damage β cells by secreting IL-1β and NO (17–19), or possibly other cytokines, such as TNF and IFN-γ (20–24). In addition, CD4+ T cells can expand or recruit other CD8+ T cells. Thus, cytotoxicity by CD8+ T cells is believed to be critical to the onset of diabetes.

Two major molecular pathways of CD8+ T cell-mediated cytotoxicity have been defined: 1) the exocytosis of granules containing perforin and granzyme molecules, and 2) the ligation of Fas ligand (FasL) on T cells with the apoptosis-inducing Fas molecule on target cells (25–29). Several laboratories have investigated the contribution of each of these cytotoxic mechanisms in autoimmune diabetes. Recent studies using perforin-deficient mice suggest that perforin-dependent cytotoxicity is a crucial effector mechanism for pancreatic β cell elimination in both a transgenic model of virus-induced autoimmune diabetes (30) and the nonobese diabetic (NOD) model of spontaneous diabetes (31). However, other studies have implicated the Fas/FasL cytotoxic pathway in this disease. Although normal pancreatic β cells do not express Fas (32), it can be up-regulated by exposure to IL-1β (33–36). Also, protection from spontaneous and CD8+ T cell-transferred diabetes was shown in Fas-negative NOD lpr/lpr mice, suggesting an important role for the Fas/FasL pathway in this particular model of spontaneous autoimmune diabetes (37, 38). More recently, the role of Fas in IDDM has been questioned, since so far all the experiments implicating this lytic pathway were performed in Fas-mutant lpr/lpr recipients, which have an abnormal immune system. Indeed, it was demonstrated that, although such mice do not develop diabetes, pancreata from NOD lpr/lpr mice were destroyed upon transfer into diabetic NOD mice, suggesting that Fas-deficient islets are susceptible to autoimmune destruction (39, 40). In one such study, the Fas-deficient islets were somewhat more resistant to destruction, suggesting a minor role for Fas-induced lysis in diabetes (39).

To reconcile these apparently contradictory findings, we have directly compared both molecular pathways of CD8+ T cell-mediated cytotoxicity in one model of CD8+ T cell-mediated IDDM. In this model, mice that express the influenza virus hemagglutinin (HA) under the control of the rat insulin promoter (Ins-HA mice), develop diabetes soon after the introduction of activated HA-specific CD8+ T cells derived from clone-4 TCR transgenic mice (41–43). By comparing the degree of islet destruction following...
transfer of the same number of normal clone-4 TCR CTLs, perforin-deficient clone-4 TCR per−/− CTLs or FasL mutant clone-4 TCR gld/gld, it was observed that elimination of the perforin/granzyme cytotoxic pathway had a more profound impact on the degree of β cell destruction than did the Fas/FasL pathway. However, if given sufficiently high numbers of clone-4 TCR per−/− CTLs, Ins-HA recipient mice became diabetic even if the perforin/granzyme pathway was blocked. Only by blocking both pathways of lysis were Ins-HA mice completely protected from diabetes. In agreement with these results, the elimination of either lytic pathway did not affect the initiation of spontaneous diabetes that occurs in double transgenic neonates, expressing both clone-4 TCR and Ins-HA transgenes.

Materials and Methods

Mice

BALB/c mice were purchased from the breeding colony of The Scripps Research Institute (TSRI). Ins-HA and clone-4 TCR-transgenic mice were generated and characterized as previously described (41, 42) and bred onto the BALB/c background for at least ten generations. Perforin-deficient mice were kindly provided by Drs. W. R. Clarke (UCLA) and J. T. Harty (University of Iowa) and were bred to the clone-4 TCR-transgenic mice (clone-4 TCR per−/−). BALB/c lpr/lpr and BALB/c gld/gld were kindly provided by Dr. Chisari (TSRI) and were bred to the Ins-HA-transgenic mice (Ins-HA lpr/lpr) and clone-4 TCR-transgenic mice, respectively (clone-4 TCR gld/gld).

The perforin, Fas, and FasL genotype were determined by PCR using genomic DNA prepared from tails. Primers for the perforin gene (5′-TGGCCT AGGGTCTACATCCAG-3′, 5′-CTGGAAGGATGATGATCT-3′, and 5′-ATATTGGGCTAGGGTGCCTC-3′) yield a 500-bp fragment for the wild-type and a 350-bp fragment for the mutated perforin allele. Primers for the Fas gene (5′-GGTACAAAAAGGTTACCGAT-3′, 5′-TTAACCTTGGACAGATATC-3′, and 5′-GGGAACACTATTGAGCA TAG-3′) yield a 200-bp fragment for the wild-type and a 440-bp fragment for the mutated allele.

Primers for the FasL gene (5′-TCCTGATCAATTGAGGAACTCTA AAGCC-3′ and 5′-CATGAGGCTTTGTTGGCCTAGTA-3′) yield a 178-bp fragment. This PCR fragment was subsequently digested with 5 U of the restriction enzyme Stu I (New England Biolabs, Beverly, MA) and separated on a 3% low melting agar gel (Promega, Madison, WI). The restriction enzyme Stu I recognizes the wild-type allele but not the mutated allele. Wild-type sequence yields a 178-bp fragment, whereas the mutated allele yields a 200-bp fragment. All mice were bred and maintained under specific pathogen-free conditions in the Scripps Research Institute vivarium. All experimental procedures were conducted according to the guidelines laid out in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell lines

L1210 Fas+ (H-2b) and L1210 Fas− (H-2b) were a gift from Drs. R. Dutton and L. Carter (Trudeau Institute, Saranac Lake, NY) and were maintained in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 2 mM glutamine, 5 × 10−5 M β-mercaptoethanol, 50 mg/ml gentamicin (Gemini Bio-products, Califasbasas, CA), and 200 μg/ml of the neomycin analogue G418 (Life Technologies, Gathersburg, MD). Cells were cultured in a humidified incubator at 37°C with 5% v/v CO2. After 5 days, KβHA-specific CTL activity of effector cells was assessed in a 5-b, 1Cr release assay using L1210 Fas+ and L1210 Fas− target cells in the absence or presence of 5 μg/ml KβHA peptide. Relative cytotoxic activity (%) was calculated as follows: 100 × (sample release − spontaneous release)/maximum release − spontaneous release. Four days after activation in vitro, effector T cells were adoptively transferred i.v. into the tail of sublethally irradiated (750 rad) 8-wk-old Ins-HA and Ins-HA lpr/lpr recipients.

Analysis of blood glucose level

The glucose concentration in blood obtained from the retroorbital plexus of mice was measured using the Accu-ChekIII (Boehringer-Mannheim, La Jolla, CA). Animals were considered diabetic if blood glucose levels were above 250 mg/dl.

Flow cytometry

Thy 1.2+/− recipient mice were injected i.v. with 3 × 106 clone-4 TCR Thy1.1+/− CTLs in 200 μl PBS. Four days later, animals were sacrificed, and single cell suspensions were made from spleen and lymph nodes. The presence of adoptively transferred clone-4 TCR Thy1.1+/− CTL was detected by double staining with FITC-conjugated anti-CD8 and PE-conjugated anti-Thy1.1+/− Abs (PharMingen, La Jolla, CA). Cells were analyzed using a FACScan and CELLQuest software (Becton Dickinson, Mountain View, CA).

Immunohistochemistry

Spleen and pancreas were excised and fixed overnight in 10% (v/v) formalin solution (Sigma, St. Louis, MO) and paraffin sections were stained with the perforin/granzyme and the Fas/FasL cytotoxic pathways, respectively. To confirm that clone-4 TCR CTLs are able to kill through both pathways (Fig. 1, A and B), target cells pulsed with or without cognate peptide (KβHA), in the presence or absence of EGTA. EGTA inhibits the exocytosis of CTL granules containing perforin and granzymes, which is a calcium-dependent process. However, it does not inhibit the Fas/FasL cytotoxic pathway. Clone-4 TCR CTLs are able to kill through both cytotoxic pathways (Fig. 1, A and D); clone-4 TCR per−/− kill only through the Fas/FasL cytotoxic pathway (Fig. 1, B and E); and clone-4 TCR gld/gld only through the perforin/granzyme cytotoxic pathway (Fig. 1, C and F), as expected. When the perforin/granzyme pathway is blocked by adding EGTA (Fig. 1, D–F), there is substantial Fas/FasL killing observed (Fig. 1, D and E). There is no residual cytosis left when both cytotoxic pathways are blocked (Fig. 1F), suggesting that the blocking is complete and that the perforin/granzyme and
Fas/FasL pathways are the major cytotoxic pathways for lysis by these CTL. No other cytotoxic pathway is observed (e.g., TNF-mediated cytotoxicity), under the conditions tested in this CTL assay.

**Clone-4 TCR CTLs can destroy islet β cells in vivo through both perforin/granzyme and Fas/FasL cytotoxic pathways**

To investigate the relative contribution of perforin/granzyme vs Fas/FasL-mediated cytotoxicity in the induction of diabetes mediated through recognition of HA expressed by the pancreatic islets, we used an experimental protocol that involved adoptive transfer of HA-specific clone-4 TCR cells into Ins-HA mice. To evaluate the role of perforin/granzyme-mediated cytotoxicity, clone-4 TCR cells deficient in perforin were used. To evaluate the role of Fas-mediated cytotoxicity, two different strategies were employed. Either clone-4 TCR gld/gld T cells deficient in Fas, were used for adoptive transfer into Ins-HA recipients, or normal clone-4 TCR T cells were transferred into Ins-HA lpr/lpr recipients deficient in Fas. The latter protocol required that recipients undergo sublethal irradiation to eliminate the abnormal CD4+ CD8− B220+ T cells that accumulate in the Fas-deficient Ins-HA lpr/lpr mice, since such cells express high levels of FasL and could affect the viability of transferred cells (46−51). Varying numbers of clone-4 TCR, clone-4 TCR per−/−, and clone-4 TCR gld/gld CTLs were adoptively transferred i.v. into sublethally irradiated Ins-HA or the Fas-deficient Ins-HA lpr/lpr mice (Fig. 2). When both cytotoxic pathways were intact (clone-4 TCR gld/gld T cells deficient in Fas), transfer of as few as 0.07 × 10^6 clone-4 TCR CTLs could cause disease. However, transfer of similar numbers of perforin-deficient clone-4 TCR CTLs resulted in a decrease in the incidence of diabetes (clone-4 TCR per−/− → Ins-HA). However, by increasing the number of perforin-deficient cells 30-fold, diabetes was observed. Moreover, if Fas was eliminated in the recipient pancreas, by using Fas-deficient Ins-HA lpr/lpr mice (clone-4 TCR → Ins-HA lpr/lpr), more than a 100-fold clone-4 TCR cells were required to cause diabetes.

When both cytotoxic pathways were blocked (clone-4 TCR per−/− → Ins-HA lpr/lpr), no diabetes was observed. These results suggest that elimination of either cytotoxic pathway profoundly debilitated the ability of the clone-4 TCR cells to cause diabetes. However, when Fas was lacking on the T cell (clone-4 TCR gld/gld → Ins-HA), autoimmune diabetes was quickly induced in 100% of all recipient mice at a cell number of 0.2 × 10^6 or higher. Thus, depending upon whether the Fas/FasL cytotoxic pathway is blocked at the level of the T cell (Fas-deficient cells), or at the level of the islet (Fas-deficient pancreata), a different conclusion could be made regarding the contribution of this killing pathway in CD8+ T cell-mediated diabetes.

**Clone-4 TCR cells are rejected following adoptive transfer into sublethally irradiated Ins-HA lpr/lpr recipient mice**

One possible explanation for the contradictory results described above may involve the pleiotropic effects of Fas deficiency in the recipient mice. Although Ins-HA lpr/lpr recipients were sublethally irradiated to prevent lymphadenopathy, it was possible that the Fas deficiency in the recipient may have affected the activity or viability of adoptively transferred clone-4 TCR CTLs. To test this hypothesis, activated clone-4 TCR CTLs, expressing the Thy1.1 allele, were adoptively transferred into Thy1.2-positive, sublethally irradiated BALB/c, BALB/c lpr/lpr, Ins-HA, and Ins-HA lpr/lpr recipient mice. Four days later, the presence of these transferred cells in spleen and peripheral lymph nodes was determined by flow cytometry using anti-Thy1.1 and anti-CD8 Abs (Fig. 3). There was consistently an approximately 2-fold decrease in the numbers of CTLs recovered from BALB/c or BALB/c lpr/lpr mice, compared with BALB/c recipients. There was also a small decrease in the numbers of cells recovered from Ins-HA mice compared with BALB/c recipients, suggesting that deletion was attributed to the presence of HA in the pancreas. Interestingly, there was more than a 6-fold decrease in the number of cells recovered from Ins-HA recipients.
pressed the HA Ag, were killed. Glucagon-positive (Fig. 4, F) of all recipients. To provide information early on during the process of cell destruction, mice. This number of CTLs was sufficient to cause diabetes in 1 cytotoxic pathways of CD8+ (clone-4 per-2) CTLs (Fig. 4, B) or clone-4 TCR gld/gld CTLs (A) required to cause diabetes following adoptive transfer into irradiated Ins-HA (■, ○, △) and Ins-HA lpr/lpr (●, □) recipients. Animals were considered diabetic if blood glucose levels were above 250 mg/dl. All diabetic animals died within 7–10 days. Every group represents at least nine animals divided over three independent experiments.

Immunohistochemical analysis of β cell destruction by different cytotoxic pathways of CD8+ T cells
To study whether there are morphological differences between the mechanism of islet destruction by the perforin/granzyme or Fas/FasL cytotoxic pathways, 2 x 10^6 activated clone-4 TCR, clone-4 TCR per-2, or clone-4 TCR gld/gld CTLs were adoptively transferred into sublethally irradiated Ins-HA or Ins-HA lpr/lpr recipient mice. This number of CTLs was sufficient to cause diabetes in all recipients. To provide information early on during the process of β cell destruction, mice were sacrificed 4 days after adoptive transfer, and sections of pancreata were analyzed by immunohistochemistry using anti-insulin and anti-glucagon Abs (Fig. 4). The extent of β cell destruction and islet infiltration was similar in the Ins-HA mice, which had received either normal clone-4 TCR CTLs (Fig. 4B) or clone-4 gld/gld CTLs (Fig. 4E). This suggested that FasL, contributed little to the process of β cell destruction. Fig. 4C (clone-4 per-2 → Ins-HA) and Fig. 4D (clone-4 → Ins-HA lpr/lpr) show sections of pancreata of Ins-HA and Ins-HA lpr/lpr, respectively. As anticipated, based on the fact that greater numbers of cells were required to cause disease under these two experimental conditions, more insulin-positive β cells are present, and the islet appears more intact with less infiltration, as compared with Fig. 4, B and E. Serial sections were also stained for glucagon (Fig. 4, F–J) to examine whether other pancreatic cells, not expressing the HA Ag, were killed. Glucagon-positive α cells remained intact in recipients in which the Fas/FasL pathway was blocked (Fig. 4, I and J), suggesting that perforin cytotoxicity was highly specific for the β cells. Some bystander killing of α cells may have occurred when the Fas/FasL pathway was operative (Fig. 4, G and H). However, extensive analysis of sections of different regions of the pancreas would be necessary to determine the significance of this apparent difference in numbers of α cells.

Redundancy of perforin/granzyme and Fas/FasL cytotoxic pathways in spontaneous neonatal autoimmune diabetes
Previous studies from our lab have shown that neonatal double transgenic (clone-4 TCR × Ins-HA)F1 mice develop spontaneous diabetes after birth and die within 7–14 days (42). To investigate which cytotoxic pathway is responsible for this spontaneous diabetes, neonatal double transgenic mice were bred that lacked either the perforin pathway or the Fas/FasL cytotoxic pathway. Regardless of which cytotoxic pathway was blocked, double transgenic neonates still developed spontaneous diabetes and died within 7–14 days. These data suggest that either cytotoxic pathway is able to induce spontaneous neonatal autoimmune diabetes in a model in which large numbers of maturing CD8+ T cells recognize Ag expressed by islet β cells.

Discussion
The purpose of this study was to evaluate the relative significance of the perforin/granzyme and Fas/FasL cytotoxic pathways on initiation of autoimmune diabetes. Conflicting data exist within the literature concerning the relative contribution of each pathway in the destruction of islet β cells. It is difficult to reconcile these studies since they were performed under a variety of different conditions and in different models. We initiated the current study to directly evaluate both cytolytic pathways in a single model. By using clone-4 TCR deficient in either pathway, and titering the numbers of cells transferred into Ins-HA recipients, it was possible...
to quantitatively compare the efficiency of each cytolytic pathway in β cell destruction. The incidence of diabetes decreased substantially when the perforin/granzyme pathway was blocked and the number of adoptively transferred HA-specific CTLs was decreased to less than two million. Thirtyfold more CD8+ T cells were required to cause diabetes if the perforin/granzyme pathway was eliminated. This suggests that the Fas/FasL pathway is 30-fold less efficient in β cell destruction than the perforin/granzyme pathway, yet can still lead to diabetes. In agreement with these results, we showed that, when either pathway is blocked in double transgenic (clone-4 TCR+ CD8+) F1 neonates, diabetes is still induced. The conclusion from both protocols is that, when large numbers of autoreactive T cells are present, either cytotoxic pathway is sufficient to cause diabetes.

A major role for perforin in diabetes was observed by Kägi et al., in both a virus-induced (lymphocytic choriomeningitis virus (LCMV)) and the NOD model of spontaneous disease. In the latter study, a minor role for Fas induced lysis was proposed, since some perforin-deficient NOD mice still developed diabetes, albeit with slower kinetics. However, in the LCMV-induced model, diabetes was not induced following transfer of 5 × 10⁶ perforin-deficient T cells (30). Diabetes could be induced in this model if dendritic cells pulsed with Ag were used to immunize perforin-deficient LCMV-glycoprotein (GP) mice (52). It is possible that the differences between the two models, such as amount of transgene expression in the islets or the affinity of the TCR, may be responsible for the different outcomes.

The role of the Fas-mediated cytolsis in IDDM has been the subject of a number of conflicting reports. Initial studies used NOD lpr/lpr mice to assess the contribution of this lytic pathway to diabetes and concluded that Fas was critical to β cell destruction and diabetes development (37, 38). However, there were significant concerns about the numerous immunological abnormalities manifested by lpr/lpr mice and how this might affect T cell function through mechanisms unrelated to the issue of the mechanism of β cell lysis. Indeed, two different groups have shown that Fas-deficient pancreata from NOD lpr/lpr hosts can be readily destroyed by diabetogenic T cells (39, 40). These conflicting findings could be explained by the fact that rejection of activated T cells occurred in irradiated or nonirradiated lpr/lpr hosts, as originally demonstrated by Allison et al. (39), and confirmed in this study. We have extended these studies by demonstrating that such elimination of clone-4 T cells requires the presence of Ag within the host. Thus, following transfer into lpr/lpr mice, activated clone-4 TCR cells become eliminated only if HA is expressed by the host. This indicates that in vivo activation is required in order for the T cells to become subject to Fas-mediated activation-induced cell death (53–57).

The mechanism of rejection of T cells by lpr/lpr hosts remains ill defined. One possibility could be that the CD4+CD8+ B220+ T cells that preferentially accumulate in lpr/lpr mice express high levels of FasL and can eliminate adoptively transferred T cells (50–51). However, the recipients Ins-HA lpr/lpr used in our experiments were 8 wk of age and therefore had few such double negative T cells. Another possibility could be that residual lpr/lpr CD4+ or CD8+ T cells, which are more radioresistant to gamma irradiation than conventional lymphocytes (58), eliminate adoptively transferred T cells (59, 60). In addition, recent data show that nonlymphoid organs such as liver and small intestine are capable of FasL expression and can mediate peripheral deletion of activated T cells (61). This may explain why rejection of adoptively transferred cells occurs in Fas mutant lpr/lpr mice.

In conclusion, the perforin/granzyme cytolytic pathway is 30-fold more effective in causing autoimmune diabetes than the Fas/FasL pathway. When enough anti-HA CTLs are transferred, either pathway is able to destroy HA-expressing pancreatic β cells in this
specific model of autoimmune diabetes. In the HA model, T cells were activated in vitro using optimal concentrations of peptide. It has been shown previously that, under conditions of partial T cell activation, the Fas/FasL cytotoxicity pathway may be preferentially triggered (62–64). It is possible that, in spontaneous models of autoimmune diabetes such as the NOD mouse, the Fas/FasL pathway contributes predominantly in the early stages of autoimmunity when Ag may be limiting. As disease progresses and Ag becomes more plentiful as the result of destruction of β cells, perforin-mediated islet destruction may dominate.

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References


