Fas/Fas Ligand Interactions Play an Essential Role in the Initiation of Murine Autoimmune Diabetes

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Apoptosis via Fas/Fas ligand (FasL) interactions has been proposed to be a major T-cell–mediated effector mechanism in autoimmune diabetes. To elucidate the role of Fas/FasL interactions in NOD diabetes, the effects of neutralizing anti-FasL antibody on autoimmune responses were evaluated. Islet-specific CD8+ and CD4+ T-cells expressed FasL upon activation and mediated FasL-dependent cytotoxicity against Fas-expressing target cells in vitro, although their cytotoxicity against islet cells was not blocked by anti-FasL antibody. Moreover, administration of anti-FasL antibody failed to inhibit diabetes in vivo in the CD8+ T-cell adoptive transfer model. On the other hand, blockade of Fas/FasL interactions significantly inhibited CD4+ T-cell–dependent diabetes in adoptive transfer models. These results suggest a substantial contribution of Fas/FasL interactions to CD4+, but not CD8+, T-cell–mediated destruction of pancreatic β-cells. When anti-FasL antibody was administered to NOD mice between 5 and 15 weeks of age, the onset of diabetes was slightly delayed but the incidence was not decreased. However, administration of anti-FasL antibody at 2–4 weeks of age completely prevented insulitis and diabetes. These results suggest that Fas/FasL interactions contribute to CD4+ T-cell–mediated β-cell destruction and play an essential role in the initiation of autoimmune NOD diabetes. *Diabetes* 51:1391–1397, 2002

Human type 1 diabetes is a chronic autoimmune disease in which T-cells play a crucial role (1–3). Evidence suggests that pancreatic β-cell destruction is caused by autoreactive T-cells (4–6). Recent studies have shown that cyotoxic T-cells kill target cells via two major cytolytic pathways: one perforin/granzyme B–mediated and the other Fas/Fas ligand–mediated (7,8). Fas-expressing target cells are lysed by activated T-cells that express Fas ligand (FasL) on their surface (9). However, which mechanism is responsible for the destruction of insulin-producing β-cells by autoreactive T-cells in human type 1 diabetes remains largely unknown.

In NOD mice, a model of human type 1 diabetes, the involvement of Fas/FasL-mediated cytotoxicity in the destruction of pancreatic β-cells by autoreactive T-cells has been frequently reported. Fas-deficient NOD-lpr/lpr mice did not develop any insulitis or diabetes, and adoptive transfer of diabetogenic T-cells to NOD-lpr/lpr mice also failed to induce disease (10,11). FasL-deficient NOD-gld/gld mice also developed neither insulitis nor diabetes (12). These results suggest that the Fas/FasL interaction is indispensable for autoimmune diabetes. Furthermore, transgenic NOD mice in which β-cells overexpressed FasL developed accelerated diabetes, possibly owing to suicide or fratricide of β-cells (10). Survival of NOD islets grafted into diabetic NOD mice was prolonged by administration of anti-FasL antibody (13). These findings support the notion that Fas/FasL-mediated cytotoxicity is a major effector mechanism of pancreatic β-cell destruction in NOD diabetes. On the other hand, Fas-deficient islet grafts from NOD-lpr/lpr mice failed to survive in diabetic NOD mice (14), and cyclophosphamide-induced NOD diabetes was not suppressed by anti-FasL antibody (15). Furthermore, <5% of pancreatic β-cells expressed Fas in prediabetic NOD mice (16). These recent findings suggest that Fas/FasL interactions play only a minor role in pancreatic β-cell destruction in NOD diabetes. Therefore, the exact role of Fas/FasL-mediated cytotoxicity in autoimmune diabetes is still controversial.

To resolve these disparate results, the first objective of the present study was to evaluate Fas/FasL-mediated cytotoxic activity of autoreactive CD4+ and CD8+ T-cells in vitro. Second, because both CD4+ and CD8+ effector T-cells could transfer diabetes, we assessed the ability of anti-FasL antibody to prevent autoimmune diabetes caused by adoptive transfer of these cells. We found that CD4+ but not CD8+ T-cell–mediated β-cell destruction was significantly attenuated by administration of anti-FasL antibody. This suggests that Fas/FasL-mediated cytotoxicity contributes to, but is not indispensable for, the destruction of β-cells. However, it remains to be explained why Fas- or FasL-deficient NOD mice developed neither insulitis nor diabetes. To further address this problem, we administered anti-FasL antibody to NOD mice at different stages during the development of spontaneous NOD dia-

betes. Surprisingly, administration of anti-FasL antibody to young NOD mice resulted in complete prevention of autoimmune diabetes, suggesting a critical role for Fas/FasL interactions in the induction phase.

RESEARCH DESIGN AND METHODS

Mice. NOD/Shi/Kbe mice were maintained in the Institute for Experimental Animals, Kobe University School of Medicine, Kobe, Japan. In our NOD colony, insulitis becomes noticeable in most mice at 5–7 weeks of age and becomes greatly enhanced with advancing age. The cumulative incidence of diabetes is 85% in females and 30% in males at 40 weeks of age. NOD-scid/scid mice were purchased from Clea Japan (Osaka, Japan). NOD-lpr/lpr mice were provided by Dr. N. Itoh (Osaka University, Osaka, Japan). All animals were handled under the Guidelines for Animal Experimentation of Kobe University School of Medicine.

Cells. Islet-derived CD8$^+$ T-cells (CD8$^+$ CTL [cytotoxic T lymphocytes]) were generated from lymphocytes infiltrating islets in 20-week-old female NOD mice (17). Isolated islets containing infiltrating lymphocytes were cultured in RPMI 1640 medium supplemented with 10% FCS and 3 U/ml recombinant interleukin-2 (rIL-2) (Takeda Pharmaceutical, Osaka, Japan) in a humidified incubator at 37°C. After 7-day culture, proliferating CD8$^+$ CTL were purified by deletion of CD4$^+$ T-cells using an anti-CD4$^+$ monoclonal antibody (GK1.5; ATCC, Rockville, MD) and anti-rat IgG-conjugated magnetic beads (BioMag; Advanced Magnetics, Cambridge, MA). An islet-specific CD4$^+$ T-cell clone (YNK7.3) was generated from islet-infiltrating lymphocytes in 20-week-old female NOD mice. Isolated islets were cultured in RPMI 1640 medium containing 1% NOD mouse serum and 0.5 U/ml rIL-2. After 7-day culture, 1×10^4 proliferating lymphocytes were restimulated with 20–30 mitomycin C (MMC)-treated NOD islets and NOD spleen cells in 200 μl RPMI 1640 medium in each well of round-bottomed 96-well microculture plates. After 2-day stimulation, 0.5 U/ml rIL-2 was added to expand the lymphocytes. After two to three cycles of restimulation, T-cell lines were cloned by limiting dilution. T-cells were seeded at 1 cell per well in 96-well culture plates containing 10 MMC-treated NOD islets in RPMI 1640 medium supplemented with 10% FCS and 0.5 U/ml rIL-2. A clone (YNK7.3) was repeatedly stimulated, expanded with rIL-2, and checked for CD4$^+$ expression by flow cytometry. YNK7.3 cells (1×10^4/well) showed significant proliferative responses to MMC-treated NOD islets in the presence of MMC-treated NOD splenocytes ([^{3}H]thymidine uptake without islet cells, 750 ± 26 cpm; with NOD islet cells, 6,380 ± 720 cpm). YNK7.3 cells (2×10^5) produced >30 ng interferon (IFN)-γ, <5 pg IL-4, and 146 ± 12 pg tumor necrosis factor (TNF)-α after 12-h incubation with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) and 500 ng/ml ionomycin (Calbiochem-Novabiochem, La Jolla, CA).

Jurkat cells were obtained from ATCC. L1210 and Fas-transfected L1210 cells were provided by P. Golstein (Centre National de la Recherche Scientifique, Marseille, France).

Monoclonal antibodies. The hamster hybridoma secreting anti-FasL antibody (MFL1) of IgG subtype has been described previously (18). MFL1 cells were injected into CD-1 nu/nu mice primed with tetramethylpentadecane (Aldrich Chemical, Milwaukee, WI), and MFL1 antibody was harvested as ascites, followed by purification on a protein G column.

Flow cytometric analysis. FasL expression on islet-specific T-cells was analyzed by flow cytometry. CD8$^+$ CTL or YNK7.3 cells (1×10^6) were cultured in the presence of a metalloproteinase inhibitor (19) with or without 10 ng/ml PMA and 500 ng/ml ionomycin for 6 h. The cultured cells were incubated with 1 μg MFL1 for 60 min at 4°C, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti–hamster IgG antibody (Cedarlane, Hornby, Canada). After washing with PBS, the cells were analyzed on a flow cytometer (FACS 440; Becton Dickinson, San Jose, CA).

^{51}Cr-release assays. Islets were isolated from 7- to 10-week-old NOD-scid/scid or NOD-lpr/lpr mice by collagenase digestion. Islet cells were dispersed to a single-cell suspension using 0.125% trypsin and 3 mmol/l EGTA. Single islet cells were incubated with 200 ng/ml recombinant murine IL-1α (Genzyme Diagnostics, Cambridge, MA) for 16 h. Jurkat cells, L1210 cells, Fas-transfected L1210 cells, and single-cell suspensions of islet cells were labeled with [^{51}Cr]sodium chromate for 90 min at 37°C and resuspended in 100 μl RPMI 1640 containing 10% FCS at 1×10^4 cells per well of round-bottomed 96-well microculture plates for use as target cells. Islet-specific CD8$^+$ CTL and YNK7.3 cells unstimulated or stimulated with 10 ng/ml PMA and 500 ng/ml ionomycin were used as effector cells. The effector cells were added to each well in triplicate at different effector:target (E:T) ratios (1:1, 5:1, 20:1, and 50:1). To block Fas/FasL interactions, 100 μg/ml MFL1 antibody or control hamster IgG (Southern Biotechnology Associates, Birmingham, AL) was added to the culture. The plates were incubated at 37°C for 8 h, and the supernatants were collected for determination of ^{51}Cr release. Culture medium alone or with 1%

Triton X-100 was added to target cells for determination of spontaneous and total cell lysis, respectively. Specific ^{51}Cr release was calculated as follows: specific lysis = 100 × (test cpm – spontaneous cpm)/(total cpm – spontaneous cpm).

Adoptive T-cell transfer. Whole splenocytes (6×10^7) from diabetic NOD mice or CD8$^+$ CTL (1×10^7) were injected intraperitoneally into 7-day-old NOD or NOD-lpr/lpr mice. YNK7.3 cells (1×10^7) were injected intraperitoneally at days 7 and 14. To block Fas/FasL interactions, 250 μg MFL1 in 200 μl PBS or 250 μg control hamster IgG in 200 μl PBS was injected intraperitoneally three times a week from 1 day before to 3 weeks after the transfer. Mice were monitored for the onset of diabetes by testing urine glucose levels every other day. Mice were considered diabetic when they had random plasma glucose levels of >16.7 mmol/l on 2 consecutive days. Pancreata were removed from five mice in each group and fixed in 10% formalin solution 3 weeks after transfer of diabetic splenocytes, 36 h after transfer of CD8$^+$ CTL because CD8$^+$ CTL-induced diabetes develops very rapidly (17), and 4 weeks after transfer of YNK7.3. Paraffin-embedded sections were stained with hematoxylin and eosin and scored for insulitis using the following criteria: 0 (–), no cellular infiltration; 1 (±), periinsulitis; 2 (+), intraislet infiltration in <25% of islets; 3 (++), 25–50% intraislet infiltration; 4 (+++), >50% intraislet infiltration. Histological examination for insulitis was performed on at least 20 islets in each pancreas.

Prevention of spontaneous diabetes and insulitis. Female NOD mice were injected intraperitoneally with 200 μl PBS containing 500 μg MFL1 antibody twice a week at ages 2–4 weeks, 2–30 weeks, and 5–15 weeks. Control group mice were treated with 200 μl PBS with or without 500 μg of control hamster IgG. Mice were monitored for the onset of diabetes until 35 weeks of age. At 35 weeks of age, pancreata from nondiabetic mice were removed for histologic examination.

Statistical analysis. Statistical analysis of diabetic incidence was performed by the χ^2 test. P values <0.05 were considered statistically significant.

RESULTS

FasL expression on islet-specific T-cells. To determine whether islet-specific T-cells express FasL on their surface, CD4$^+$ and CD8$^+$ T-cells isolated from diabetic NOD islets were stained with anti-FasL antibody (MFL1). Islet-derived CD8$^+$ T-cell lines, representing effector CD8$^+$ cells which we previously reported to respond to and lyse pancreatic β-cells with MHC class I restriction in vitro (20), were shown to express FasL on their surface without stimulation, i.e., there was no significant difference between cells stimulated with PMA and ionomycin and unstimulated cells (mean fluorescence intensity [MFI] 24.77 and 25.03) (Fig. 1*A* and *B*). Because unstimulated splenic CD8$^+$ T-cells did not express FasL (data not shown), these CD8$^+$ T-cells were likely already activated by islet antigen stimulation and IL-2 in vitro. On the other hand, the YNK7.3 clone, isolated from 20-week-old female NOD islets and responding to islet cell antigen with MHC class II restriction and representing effector CD4$^+$ T-cells, did not express FasL without stimulation (MFI 19.23) (Fig. 1*C*). However, after stimulation, FasL expression was observed (MFI 27.22) (Fig. 1*D*). These results indicate that both islet-specific CD8$^+$ and CD4$^+$ effector T-cells could express FasL upon activation.

Fas/FasL-mediated cytotoxicity of islet-specific T-cells in vitro. To determine whether islet-specific T-cells can destroy Fas-expressing target cells, cytotoxic activities of islet-specific CD8$^+$ CTL and the CD4$^+$ T-cell clone (YNK7.3) were tested against Fas-positive Jurkat target cells in the ^{51}Cr-release assay (Fig. 2*A* and *B*). Islet-derived CD8$^+$ CTL were strongly cytotoxic even without stimulation, and this was enhanced by ~60% after stimulation with PMA and ionomycin. The addition of MFL1 antibody significantly suppressed specific lysis by stimulated CD8$^+$ CTL. On the other hand, islet-specific CD4$^+$ YNK7.3 cells could not lyse Jurkat cells without stimulation. However,

CD8

CD4

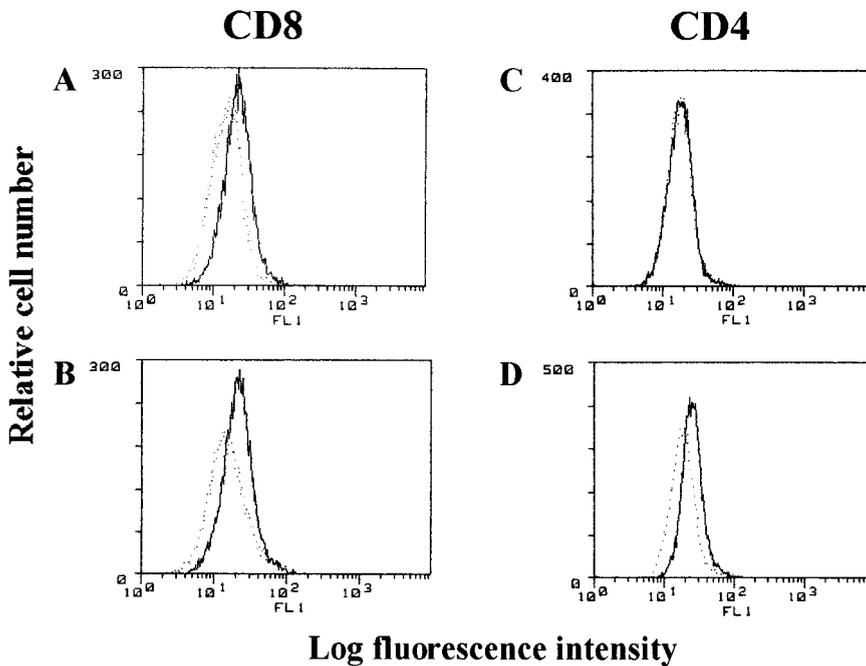**Relative cell number**

Log fluorescence intensity

FIG. 1. FasL expression on islet-specific T-cells. Islet-derived CD8$^+$ CTL and an islet-specific CD4$^+$ T-cell clone (YNK7.3) were stained with anti-FasL antibody (MFL1) followed by FITC-conjugated anti-hamster IgG (solid line). Controls were stained only with second antibody (dotted line). Islet-derived CD8$^+$ CTL were examined without stimulation (*A*) and after stimulation with PMA and ionomycin (*B*). The islet-specific CD4$^+$ T-cell clone (YNK7.3) was examined without stimulation (*C*) and after stimulation with PMA and ionomycin (*D*). One representative of four separate experiments is shown.

when stimulated by PMA and ionomycin, YNK 7.3 cells exerted significant cytotoxicity, which was completely abolished by the addition of MFL1 antibody. These results indicate that both islet-specific CD8$^+$ and CD4$^+$ T-cells can exert Fas/FasL-mediated cytotoxicity.

To confirm that the cytotoxicity of effector T-cells was Fas/FasL-mediated, Fas-transfected L1210 cells were also used as target cells (Fig. 2*C* and *D*). Stimulated CD8$^+$ CTL showed 85% specific lysis against Fas-transfected L1210 cells. The addition of MFL1 antibody reduced the cytotoxicity to the level seen on Fas-negative L1210 controls. Again, stimulated YNK7.3 cells significantly lysed Fas-transfected L1210 cells, but not Fas-negative L1210 cells.

To examine whether Fas/FasL-mediated cytolysis contributes to destruction of NOD islet cells by CD4$^+$ and CD8$^+$ T-cells, freshly isolated NOD-scid/scid islet cells were used as targets to avoid contamination by islet-infiltrating T-cells. Because overexpression of Fas was observed on the islet cell surface after 16-h exposure to IL-1α (data not shown), we used IL-1α–treated NOD-scid/scid islet cells as targets. Stimulated CD8$^+$ CTL and CD4$^+$ YNK7.3 cells exerted significant cytotoxicity against IL-1α–treated NOD-scid/scid islet cells, but this was not blocked by MFL1 antibody in vitro (Fig. 2*E* and *F*). Consistent with these results, both CD4$^+$ and CD8$^+$ T-cells could kill NOD-lpr/lpr islet cells. These results suggested that although both CD4$^+$ and CD8$^+$ effector T-cells can exert Fas/FasL-mediated cytotoxic activity, this ability apparently does not play a major role in the destruction of pancreatic islet cells in vitro.

Effect of anti-FasL antibody on diabetes caused by adoptive transfer of CD4$^+$ and CD8$^+$ T-cells. To examine whether Fas/FasL interactions are nonetheless involved in the destruction of β-cells in vivo, we performed adoptive transfer experiments (Table 1). When splenocytes obtained from diabetic NOD mice were transferred to 7-day-old NOD mice, all recipients became diabetic within 30 days. Administration of MFL1 antibody significantly inhibited the incidence of diabetes without

suppressing insulitis. Similarly, NOD-lpr/lpr recipient mice showed significant inhibition of overt diabetes. When islet-derived CD8$^+$ CTL were injected into NOD and NOD-lpr/lpr mice, all recipient mice became diabetic within 15 days. Administration of MFL1 antibody did not significantly suppress the development of overt diabetes or insulitis. On the other hand, in the case of diabetes caused by transferring islet-specific CD4$^+$ T-cells, administration of MFL1 antibody significantly decreased the incidence of diabetes without apparent suppression of insulitis, compared with the control mice. Moreover, when CD4$^+$ YNK7.3 cells were injected into NOD-lpr/lpr mice, none of the recipient mice became diabetic, and they suffered only marginal insulitis. These results suggested that Fas/FasL interactions significantly contribute to CD4$^+$, but not to CD8$^+$, T-cell–mediated diabetes.

Effect of anti-FasL antibody on spontaneous diabetes. To elucidate the role of Fas/FasL interactions in spontaneous diabetes, the MFL1 antibody was injected at various stages of NOD diabetes development (Figs. 3 and 4). When MFL1 antibody was administered during the period from 2 through 30 weeks of age, no overt diabetes was observed (Fig. 3), just as is seen in Fas-deficient NOD-lpr/lpr mice. In contrast, administration of MFL1 antibody from 5 through 15 weeks of age, when cellular infiltration of pancreatic islets usually becomes apparent in our NOD colony, resulted in a 4- to 5-week delay in the onset of overt diabetes, compared with control IgG-treated mice (Fig. 3). These results suggest that the Fas/FasL interaction plays a substantial but nonessential role in pancreatic β-cell destruction after the development of insulitis.

To explore the role of Fas/FasL interactions in the early phase of autoimmune diabetes, MFL1 antibody was administered from 2 to 4 weeks of age (Fig. 4). This blockade of Fas/FasL interaction in young NOD mice strongly inhibited the development of overt diabetes (Fig. 4*A*). Surprisingly, all except two of these nondiabetic animals (no. 4 and no. 13 in Fig. 4*B*) lacked cellular infiltration of the pancreatic
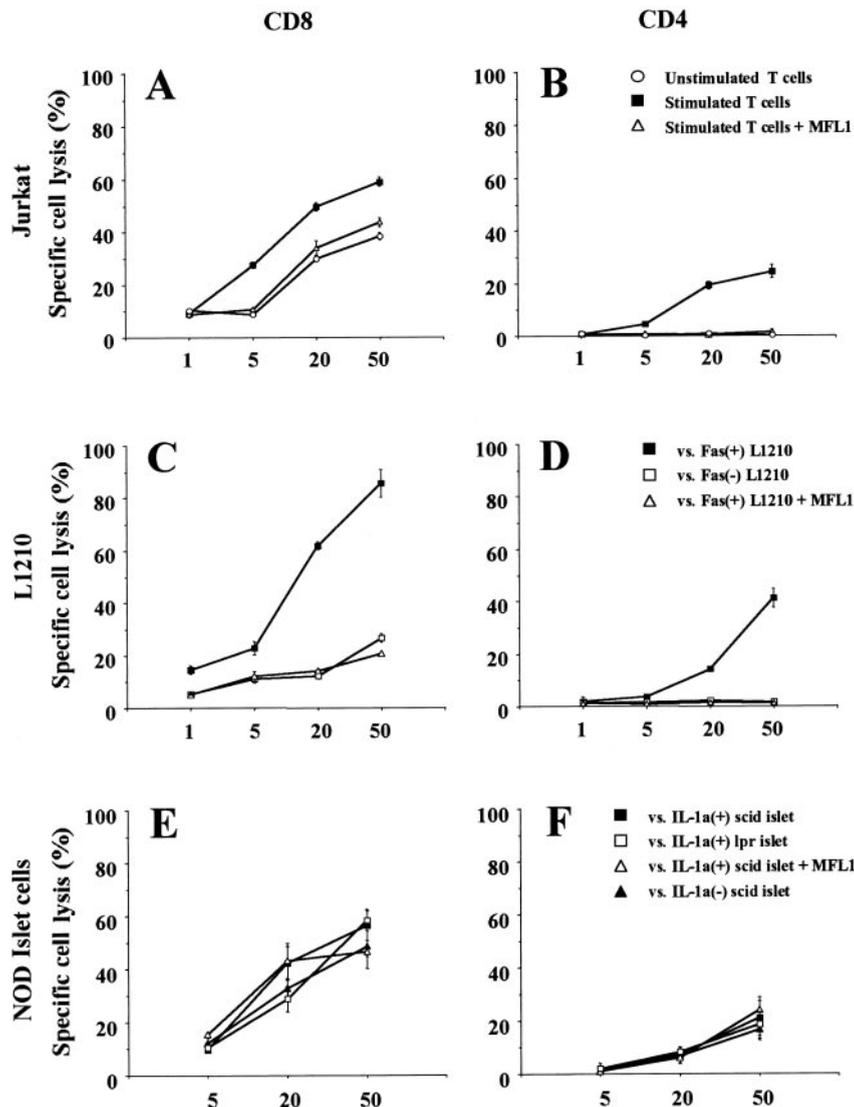
FIG. 2. Fas/FasL-mediated cytotoxic activity of islet-specific T-cells. Specific lysis by islet-derived CD8^{+} CTL (*A*, *C*, *E*) and the cloned CD4^{+} T-cell line (YNK7.3) (*B*, *D*, *F*) against Jurkat cells (*A*, *B*), Fas-positive or -negative L1210 cells (*C*, *D*), or NOD islet cells (*E*, *F*) was assessed by 8-h ^{51}Cr-release assay at the indicated effector:target ratios. *A* and *B*: unstimulated (○) or PMA/ionomycin-stimulated (■, △) CD8^{+} CTL and YNK7.3 cells were tested against [^{51}Cr]-labeled Jurkat cells in the presence of MFL1 antibody (△) or hamster IgG (○, ■). *C* and *D*: PMA/ionomycin-stimulated CD8^{+} CTL and YNK7.3 cells were tested against Fas-positive L1210 (■, △) and Fas-negative L1210 (□) cells in the presence of MFL1 antibody (△) or hamster IgG (■, □). *E* and *F*: PMA/ionomycin-stimulated CD8^{+} CTL and YNK7.3 cells were tested against intact NOD-scid/scid islet cells (▲), IL-1α–treated NOD-scid/scid islet cells (■, △), and NOD-lpr/lpr islet cells (□) in the presence of MFL1 antibody (△) or hamster IgG (■, □, ▲). One representative of three separate experiments is shown.

islets. The two exceptions, which showed severe insulitis similar to control NOD mice, may imply that inadequate blockade of Fas/FasL interactions in the initial phase of autoimmune diabetes can trigger full-blown autoimmune responses to pancreatic β-cells. These results suggest that the Fas/FasL interaction plays an essential role in triggering autoimmune diabetes in NOD mice.

DISCUSSION

This study demonstrated that islet-specific CD4^{+} and CD8^{+} T-cells, which can transfer diabetes, can express functional FasL on their surface when appropriately activated. When anti-FasL antibody was administered twice a week to NOD mice from 5 through 15 weeks of age, the onset of diabetes was delayed by ~4–5 weeks, suggesting a substantial but not essential contribution of the Fas/FasL interaction to β-cell destruction leading to overt diabetes.

In adoptive transfer models, anti-FasL antibody treatment or Fas deficiency in the recipients failed to block rapid induction of diabetes by islet-specific CD8^{+} T-cells. As we and others have previously reported, CD8^{+} effector T-cells induce overt diabetes without the help of CD4^{+} T-cells (17,21). These results indicated that Fas/FasL-

mediated cytolysis was not responsible for the CD8^{+} T-cell–mediated β-cell destruction. However, it has also been reported that a CD8^{+} T-cell clone (TGNF-H3), isolated from NOD mice, failed to transfer diabetes into NOD-lpr/lpr mice (10,21), and that perforin-deficient islet-specific 8.3–T-cell receptor (TCR) transgenic CD8^{+} T-cells (22) could induce diabetes in B7-transgenic NOD-scid/scid mice (23). These results suggested that Fas/FasL-mediated cytotoxicity could indeed represent a major effector mechanism for CD8^{+} T-cell–mediated β-cell destruction. Several explanations for these discrepancies can be suggested. First, different CD8^{+} T-cell stimulation may contribute. CD8^{+} T-cells used in the present study were derived from unmanipulated NOD islets, whereas in the others, CD8^{+} T-cell clones were activated by B7-transgenic NOD islets. Because the TGNF-H3 clone appeared to be optimally activated by B7 costimulation, FasL overexpression in adult lpr/lpr mice might kill the transferred CD8^{+} T-cells expressing Fas at high level (24). Second, 7-day-old NOD-lpr/lpr mice were used as the recipients in the present study, a time at which FasL overexpression was not yet apparent (25). Therefore, unmanipulated CD8^{+} T-cells used in our study might have survived and

TABLE 1
Effect of anti-FasL antibody on adoptive transferred diabetes

Donor	Control		Administration of MFL1		NOD-lpr/lpr	
	Diabetes	Insulitis	Diabetes	Insulitis	Diabetes	Insulitis
Diabetogenic splenocytes	14/14 (100%)	+++	8/12 (67%)*	+++	6/10 (60%)†	++/+++
Islet-derived CD8+ CTL	13/13 (100%)	+++	14/15 (93%)	+++	9/9 (100%)	+++
YNK7.3	6/10 (60%)	+++	2/11 (18%)*	++/+++	0/8 (0%)†	±/+

Spleen cells (6×10^7) from diabetic NOD mice or CD8+ CTL (1×10^7) were intraperitoneally injected into 7-day-old NOD or NOD-lpr/lpr mice. YNK7.3 (1×10^7) were intraperitoneally injected into the recipients on day 7 and 14. MFL1 (250 μg) or control hamster IgG (250 μg) was intraperitoneally injected three times a week from 1 day before to 3 weeks after transfer. Diabetic incidence was monitored until 30 days after the transfer of effector cells. Five pancreases from each group were removed at 3 weeks after transfer in the recipients with diabetic splenocytes, 36 hours after transfer in the recipients with CD8+ CTL, and 4 weeks after transfer in the recipients with YNK7.3. Paraffin-embedded sections were stained with hematoxylin and eosin and scored for insulitis in at least 20 islets in each pancreas using the following criteria: −, normal islet; ±, peripheral mononuclear cell infiltration; +, mononuclear cell infiltration in <25% of islet; ++, 25–50% of islet infiltrated; +++, >50% of islet infiltrated. Statistical significance of the data was evaluated by χ^2 test: *$P < 0.05$, †$P < 0.01$ vs. hamster IgG-treated NOD mice.

been able to destroy pancreatic β-cells even in NOD-lpr/lpr recipients. Third, because the TGNF-H3 and 8.3 clones were isolated from 5- to 7-week-old NOD mice, it remains possible that CD8+ T-cells might preferentially exert Fas/FasL-mediated cytolysis in the initial phase of autoimmune diabetes. On the other hand, the CD8+ T-cells in the present study were isolated from 15- to 20-week-old NOD islets. Although CD8+ CTL from adult NOD islets in this study exhibited both Fas/FasL-mediated cytotoxicity as well as that mediated by other mechanisms (possibly perforin), the former appeared not to be a major mechanism for killing pancreatic β-cells in vitro (Fig. 2). Consistent with our results, antigen-specific CD8+ T-cells in the RIP-HA transgenic model exerted both Fas/FasL-mediated and perforin-mediated cytotoxicity against pancreatic β-cells (26). These results suggest that the Fas/FasL interaction is not essential for the destruction of pancreatic islets by islet-specific CD8+ CTL derived from adult NOD islets.

In contrast to CD8+ T-cells, CD4+ T-cell (YNK7.3) transfer experiments showed that administration of anti-FasL antibody significantly suppressed the onset of diabetes, apparently without influencing insulitis. In contrast, when CD4+ YNK7.3 cells were transferred to NOD-lpr/lpr mice, neither destructive insulitis nor diabetes was observed in contrast to the significant insulitis in anti-FasL antibody–treated mice. It has been reported that transferred NOD spleen cells, especially CD4+ T-cells, are depleted in NOD-lpr/lpr mice, apparently owing to aberrant overexpression of FasL (24,27). Therefore, administration of anti-FasL antibody in normal NOD mice seems to reflect the physiological condition better than transfer into lpr/lpr mice. Taken together, the results indicate that diabetes transferred by CD4+ YNK7.3 cells was suppressed significantly, but not completely, by the blockade of Fas/FasL interactions, implying that the Fas/FasL interaction contributes significantly but not indispensably to CD4+ T-cell–mediated destruction of β-cells. However, CD4+ YNK7.3 cells seem not to show Fas/FasL-mediated cytotoxic activity against pancreatic islet cells in vitro. Nonetheless, they were weakly cytotoxic against Fas-deficient islet cells (Fig. 2), an effect possibly mediated by other mechanisms such as granule exocytosis or cytokines (28). In fact, CD4+ YNK7.3 cells could produce large amounts of IFN-γ and TNF-α. On the other hand, in vivo, these cells may affect macrophages (29), which in turn secrete cytokines, including IL-1, IFN-γ, and TNF-α, which have been shown to be cytotoxic against pancreatic β-cells via nitric oxide production and other mechanisms that remain to be clarified (28,30–33). In addition, these cytokines induce the expression of Fas on the target cells via transcription factors, which might facilitate Fas/FasL-mediated β-cell death (33–35). On the other hand, Amrani et al. (36) reported that islet-derived CD4+ T-cells from diabetic 4.1-TCR transgenic NOD mice could kill Fas-expressing NOD islet cells, and that this was abrogated by the absence of Fas expression on target NOD islet cells in vitro. Depending on types or conditions of islet-specific
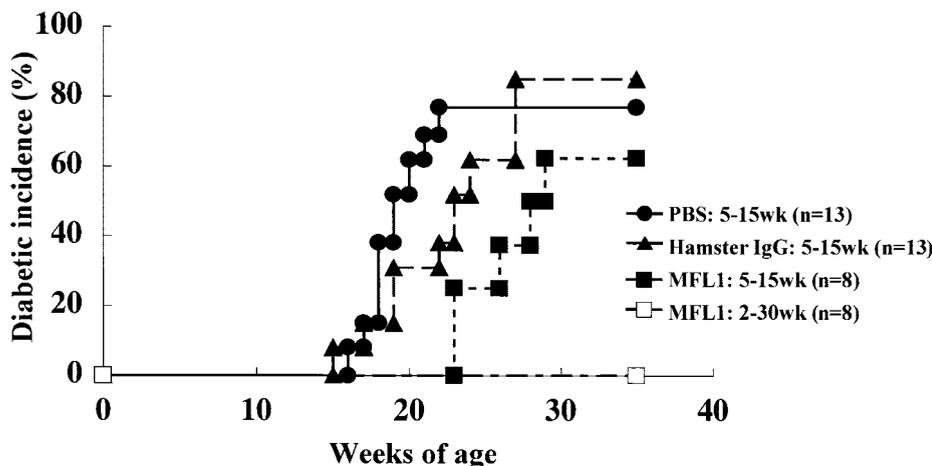

FIG. 3. Effect of anti-FasL antibody (MFL1) on spontaneous diabetes. MFL1 antibody (■, $n = 8$), PBS (●, $n = 13$), or control hamster IgG (▲, $n = 13$) was administered to female NOD mice from 5 through 15 weeks of age. In one group, MFL1 antibody was administered from 2 through 30 weeks of age (□, $n = 8$). Diabetic onset was monitored until 35 weeks of age.
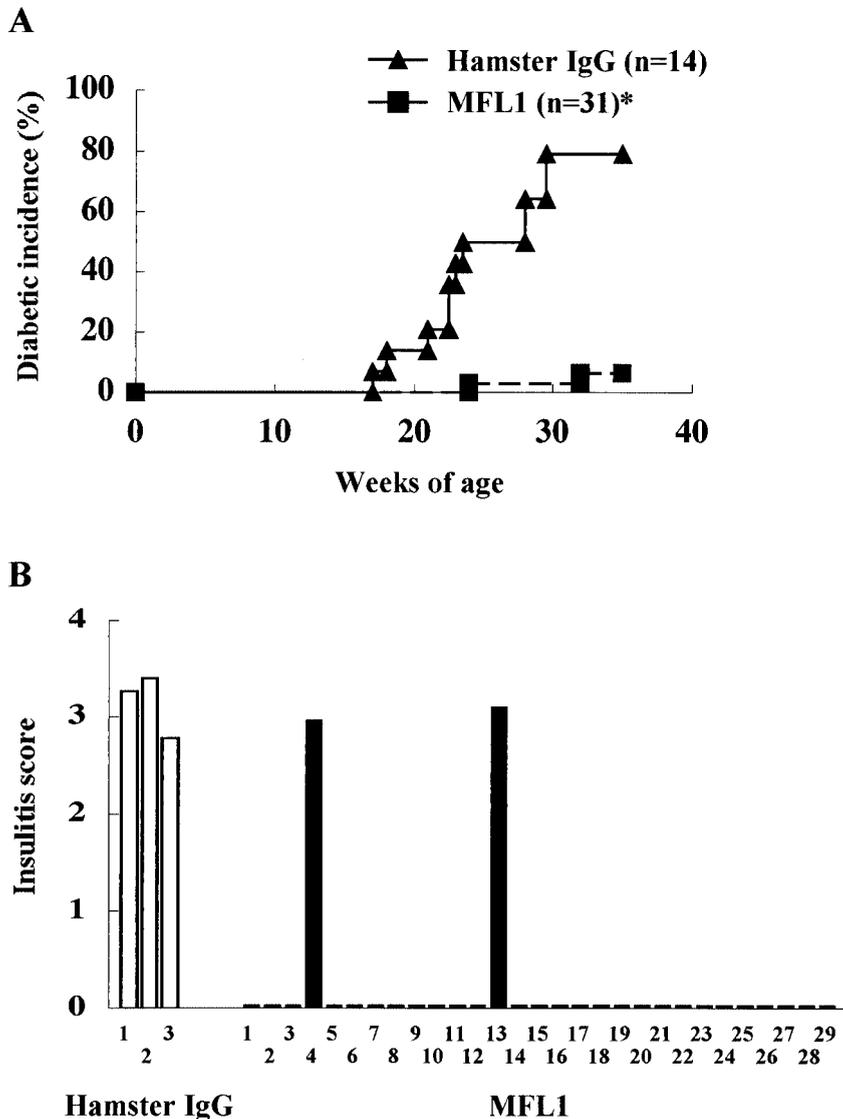
A

B

FIG. 4. Effect of anti-FasL antibody (MFL1) administration to young NOD mice. MFL1 antibody (■, $n = 31$) or control hamster IgG (▲, $n = 14$) was administered to female NOD mice from 2 through 4 weeks of age. *A*: Diabetes onset was monitored until 35 weeks of age. *$P < 0.0001$ vs. control (χ^2 test). *B*: Severity of insulitis in MFL1-treated (■) or hamster IgG–treated (□) normoglycemic NOD mice was examined at 35 weeks of age. Insulitis scores of individual mice are presented.

$CD4^+$ T-cells, each $CD4^+$ T-cell clone might show different degrees of Fas-mediated cytotoxic activity. However, although Fas-deficient *RAG-2$^{-/-}$* $CD4^+$ TCR (4.1)-transgenic NOD mice suffered much less overt diabetes, 20% still became diabetic (36). Taken together, these studies suggest that $CD4^+$ T-cell–mediated β-cell destruction may be caused not only by Fas/FasL-mediated direct cytotoxicity but also by other cytotoxic mechanisms, which may have the same final result even in the absence of Fas/FasL interactions.

The phenomenon that NOD-lpr/lpr mice showed neither spontaneous insulitis nor diabetes (10,11) appeared not to be explicable by the absence of $CD4^+$ T-cell–mediated killing via Fas/FasL interactions. Furthermore, FasL-deficient NOD-gld/gld mice also showed no insulitis or diabetes (12). The question why the absence of Fas/FasL interaction in NOD mice completely prevented the development of autoimmune diabetes remains unanswered. This study clearly revealed that the blockade of Fas/FasL interactions at a young age in NOD mice completely inhibited the development of insulitis and diabetes, suggesting an essential role of the Fas/FasL interaction in the initiation of autoimmune diabetes. The exact role of

Fas/FasL interactions in this induction phase of autoimmune diabetes remains to be determined.

In conclusion, our present results suggest that Fas/FasL-mediated cytolysis plays a substantial role in $CD4^+$ T-cell–mediated, but not $CD8^+$ T-cell–mediated, β-cell destruction in spontaneous autoimmune diabetes. However, a major role of Fas/FasL interactions was found in the induction phase rather than the effector phase of autoimmune diabetes, which explains the complete resistance of Fas- or FasL-deficient NOD mice to spontaneous diabetes. Further studies are underway to clarify the exact role of Fas/FasL interactions in the initiation of autoimmune diabetes.

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