Brief Definitive Report

Requirement of Fas for the Development of Autoimmune Diabetes in Nonobese Diabetic Mice

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Summary

Insulin-dependent diabetes mellitus (IDDM) is assumed to be a T cell–mediated autoimmune disease. To investigate the role of Fas-mediated cytotoxicity in pancreatic β cell destruction, we established nonobese diabetic (NOD)-lymphoproliferation (*lpr*)/*lpr* mice lacking Fas. Out of three genotypes, female NOD-+/+ and NOD-+/*lpr* developed spontaneous diabetes by the age of 10 mo with the incidence of 68 and 62%, respectively. In contrast, NOD-*lpr*/*lpr* did not develop diabetes or insulitis. To further explore the role of Fas, adoptive transfer experiments were performed. When splenocytes were transferred from diabetic NOD, male NOD-+/+ and NOD-+/*lpr* developed diabetes with the incidence of 89 and 83%, respectively, whereas NOD-*lpr*/*lpr* did not show glycosuria by 12 wk after transfer. Severe mononuclear cell infiltration was revealed in islets of NOD-+/+ and NOD-+/*lpr*, whereas islet morphology remained intact in NOD-*lpr*/*lpr*. These results suggest that Fas-mediated cytotoxicity is required to initiate β cell autoimmunity in NOD mice. Fas–Fas ligand system might be critical for autoimmune β cell destruction leading to IDDM.

Accumulating evidence has elucidated that insulin-dependent diabetes mellitus (IDDM) is a T cell–mediated autoimmune disease (1, 2). Histological examination of the IDDM pancreas revealed that mononuclear cells infiltrated into the islet (insulitis) and the infiltrate was mainly composed of T cells (3–5). The nonobese diabetic (NOD) mouse is an excellent animal model of human IDDM, and possesses a similar T cell predominance in the islet infiltrate (6). The significant role of T cells in IDDM has been extensively studied by using this mouse model. T cell manipulation by an administration of mAb decreased the incidence of diabetes and insulitis (7, 8). Insulitis and diabetes were adoptively transferred into neonatal or irradiated young NOD by splenic T cells, T cell lines, and T cell clones from diabetic NOD (9–12). Possible roles of these cells are to give an initial damage to β cells for launching inflammatory process, and/or to secrete cytokines for recruiting and activating other T cells, and/or to put the final damage to β cells for causing diabetes. Thus, T cells are essentially involved in the pathogenesis of IDDM, yet exact mechanisms of pancreatic β cell destruction remain obscure.

Recent studies have exposed that T cell–mediated cytotoxicity comprised two major pathways, perforin- and Fas-based mechanisms (13–15). Perforin is a protein present in the cytoplasmic granules of CTLs and secreted to form pores on target cell membranes. The presence of perforin in $CD8^+$ T cells in insulitis lesions of NOD mice suggested β cell lysis by a perforin-based mechanism (16). However, one study using transgenic mice expressing glycoprotein of lymphocytic choriomeningitis virus (LCMV) in β cells, transfer of perforin-deficient glycoprotein of LCMV–specific T cells failed to prevent insulitis (17). That study indicates that the perforin-independent pathway is required to initiate autoimmunity against pancreatic β cells.

Fas-dependent cytotoxicity is a possible molecular mechanism for triggering β cell destruction. To address this issue, we established Fas-lacking NOD mice by introducing lymphoproliferation (*lpr*) mutation from MRL-*lpr*/*lpr*. The MRL-*lpr*/*lpr* mouse carries an insertion of an early trans-

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posable element in intron 2 of the *Fas* gene leading to premature termination of the transcript and defective Fas expression on cell surface (18). Generated NOD-*lpr/lpr* did not develop diabetes or insulitis. In addition, splenocyte-transfer did not provoke insulitis or diabetes either. These results suggest that Fas-mediated cytotoxicity is critical to initiate β cell autoimmunity in NOD mice. Fas–Fas ligand (FasL) system might be required in an initial step of autoimmune β cell destruction leading to IDDM.

Materials and Methods

Mice. NOD/shi/osa mice were originated from the colony at Center for Experimental Animals Development (Shionogi, Koka, Japan) and bred under specific pathogen-free conditions at the Institute of Animal Research (Osaka University Medical School, Osaka, Japan). They were monitored for the development of diabetes with Tes-Tape (Eli Lilly, Indianapolis, IN) weekly. The incidence of spontaneous diabetes in our colony was 77% in females and 40% in males by 32 wk of age. MRL-*lpr/lpr* mice were purchased from Japan SLC (Hamamatsu, Japan). Experiments were approved by Osaka University Medical School Animal Care and Use Committee and performed according to Osaka University Medical School Guideline for the Care and Use of Laboratory Animals.

Breeding of NOD-lpr/lpr Mice. MRL-*lpr/lpr* mice (H-2^k; K^k, I-A^k, I-E^+, D^k) were outcrossed to NOD mice (H-2^{g7}; K^d, I-A^{g7}, I-E^-, D^b), and F_1 were backcrossed in NOD background. At the N_2 generation, H-2 was typed to select breeders homozygous for H-2^{g7} since this characteristic MHC haplotype was essential for insulitis and diabetes (19). Cervical lymphnode was biopsied and dispersed cells (10^6) were stained with the following monoclonal antibodies. SF1-1.1 for K^d and 28-8-6S for D^b were purchased from PharMingen (San Diego, CA) and 14-4-4S for I-E from Cedarlane (Hornby, Ontario, Canada). 11-4.1 for K^k, H116-32.R7 for I-A^k, and 10-2.16 for I-A^{g7} were provided by Dr. M. Hattori (Harvard Medical School, Boston, MA). FITC-conjugated anti–mouse IgG Fc (Cappel, Durham, NC) was used as a secondary antibody. Flow cytometric analysis was performed on a FACScan® (Becton Dickinson, Mountain View, CA). *lpr* mutation was also screened at each step of backcrossing. Tail was biopsied and extracted DNA was subjected to PCR with two different pairs of primers constructed according to the published sequence (20). The first pair is composed of NIL-1, 5′-CAG CAG GAA TCC TAT GAG GT-3′ and NIL-2, 5′-CTC GCA ACG TGA ACG GTT CG-3′, yielding a band of 381 bp for the mutated allele. The second pair is composed of NIL-3, 5′-CCT TCA TAA CTG GTG TCG CA-3′, and NIL-4, 5′-GCA GAG ATG CTA AGC AGC AG-3′, yielding a band of 346 bp for the mutated allele.

Genotyping and Phenotyping of Fas Allele. At the N_6 generation, heterozygotes for *lpr* were intercrossed and N_6F_1 was obtained. Genotype of the *Fas* allele was determined by PCR with Takara Ex Taq (Takara Shuzo, Otsu, Japan) and one set of primers, NIL-1, and NIL-4 covering an early transposable element. *lpr* type yielded a band of 5.7 kbp and wild type yielded a band of 265 bp. Phenotype was confirmed by flow cytometry and Northern blot analysis. Thymocytes and splenocytes were dispersed into single cells (10^6) and stained with Jo2 anti–mouse Fas antibody (PharMingen) followed by FITC-conjugated anti–hamster IgG (PharMingen), or a combination of PE-conjugated anti-CD4 (GK1.5; Becton Dickinson) and FITC-conjugated anti-CD8 (YTS 169.4; Cedarlane), or a combination of PE-conjugated anti-B220 (RA3-6B2; GIBCO BRL, Gaitherburg, MD) and FITC-conjugated anti-CD3 (145-2C11; PharMingen). Dead cells were excluded by 1 μg/ml of propidium-iodine (Sigma Chemical Co., St. Louis, MO) staining. For Northern blotting, total RNA was prepared from tissue using TRIzol (GIBCO BRL) and blotted onto a nylon membrane. EcoRI fragment of mouse cDNA was labeled with ^{32}P and used for hybridization as described (21).

Monitoring Spontaneous Development of Diabetes. After genotyping, female congenic mice were monitored for the development of diabetes with Tes-Tape weekly. Diabetes was diagnosed when Tes-Tape indicated $>2^+$ and hyperglycemia >300 mg/dl was confirmed by the measurement of blood glucose using Glutest E (Sanwa Kagaku Kenkyusho, Nagoya, Japan).

Adoptive Cell Transfer. Cell transfer was performed according to the method of Wicker et al. (22) with slight modification. Splenocytes from diabetic NOD were isolated in RPMI 1640 medium supplemented with 10% fetal bovine serum. RBCs were excluded by a lysis with NH_4Cl. After washing, numeration, and viability evaluation, splenocytes (5×10^7) were intravenously injected into sublethally irradiated male recipients. The dose of irradiation was 800 rad, and the age of recipients was between 9 and 13 wk when none of NOD males spontaneously developed diabetes. Development of diabetes was monitored three times a week by Tes-Tape after transfer.

Histological Examination. Pancreas was fixed on a filter paper in neutral-buffered formalin, and paraffin-embedded sections were prepared as maximum plane could be observed. Two nonconsecutive hematoxylin and eosin sections were examined. For transferred mice, sections were scored for insulitis, and the number of islets was counted by one investigator in a blind fashion. Score of insulitis was *0*, normal; *1*, peri-insulitis; *2*, mononuclear cell infiltration in $<50\%$ of the area of the islet; *3*, mononuclear cell infiltration in 50% or more of the area of the islet.

Analysis of Microsatellite Markers. To examine the extent of bringing in MRL genes surrounding *lpr* mutation to the NOD genetic background, genomes of the N_6 backcross used for the intercross were assessed with locus-specific microsatellite markers. IDDM susceptibility (*Idd*) genes with significant evidence of linkage to insulitis and diabetes (23, 24) were assessed similarly. Microsatellite markers were selected from the published data (25) and the database released by the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research (Cambridge, MA), and screened for polymorphisms between NOD and MRL strains. After MapPairs™ (Res. Genetics, Huntsville, AL) were subsequently used in this study: *Idd3, D3Mit270; Idd5, D1Mit122*, and *D1Mit318; Idd10, D3Mit10*, and *D3Mit140*; chromosome 19, *D19Mit11, D19Mit34, D19Mit43, D19Mit54*, and *D19Mit69.*

Statistical Analysis. The Kaplan-Meier method was used for the calculation of diabetes incidence. Insulitis scores and the number of islet were compared using the Kruskal-Wallis test.

Results

Genotyping and Phenotyping. Genotyping at the *Fas* locus determined NOD-+/+, NOD-+/*lpr*, and NOD-*lpr/lpr*. They were all maturated, but NOD-*lpr/lpr* showed marked lymphadenopathy at ~4 mo of age. This is due to impaired transcription of the *Fas* gene, defective expression of Fas antigen, and consequent accumulation of $CD4^-CD8^-CD3^{low}B220^+$ lymphocytes (Fig. 1). Although the pheno-

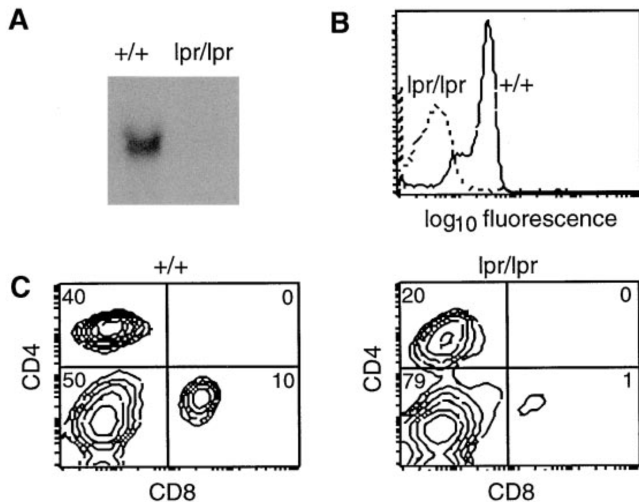

Figure 1. Fas mRNA expression in the thymus (*A*), Fas expression on thymocytes (*B*), and two-color flow cytometric analysis of cell surface markers on splenocytes (*C*) of NOD-+/+ and NOD-*lpr/lpr*.

type of NOD-*lpr/lpr* closely resembled a parental strain, MRL-*lpr/lpr*, NOD-*lpr/lpr* did not show high mortality as MRL-*lpr/lpr* making it possible to observe them until 10 mo of age. NOD-+/+ and NOD-+/*lpr* could hardly be discriminated by the phenotype.

Spontaneous Development of Diabetes. Female NOD-+/+ and NOD-+/*lpr* developed spontaneous diabetes after 3 mo of age. The incidence increased with age and reached 68% in NOD-+/+ and 62% in NOD-+/*lpr* by 10 mo of age (Fig. 2). This time course is comparable with that in NOD mice in our colony. In contrast, none of NOD-*lpr/lpr* developed diabetes during this observation period.

Diabetes after Adoptive Cell Transfer. Prevention of spontaneous diabetes might be due to the distorted cell proportion of immune cells since unusual $CD4^-CD8^-$ T cells accumulated in NOD-*lpr/lpr*. To avoid this influence, we performed transfer experiments from diabetic NOD to sublethally irradiated young congenic mice. In a total of four experiments, 8 of 9 NOD-+/+ (89%) and 10 of 12 NOD-+/*lpr* (83%) developed diabetes, whereas 0 of 7 NOD-*lpr/lpr* (0%) became diabetic during 12 wk of observation period (Fig. 3).

Histological Examination. Mild to severe insulitis existed in the pancreas of both genders of NOD-+/+ and NOD-+/*lpr*, irrespective of the development of diabetes (Fig. 4). In contrast, mononuclear cells were barely found in and around the islet of both genders of NOD-*lpr/lpr*. The severity of insulitis and the number of islets were evaluated in transferred mice. The average scores of insulitis (mean ± SE) were 2.47 ± 0.15, 2.36 ± 0.19, and 0.02 ± 0.02 in

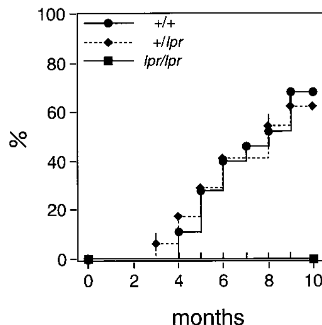

Figure 2. Cumulative incidence of spontaneous diabetes in female NOD-+/+ (n = 19), NOD-+/*lpr* (n = 18), and NOD-*lpr/lpr* (n = 17).

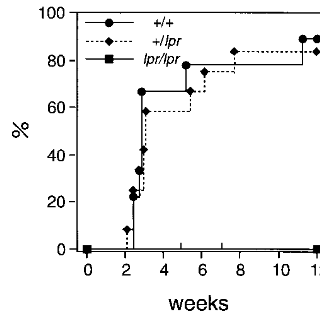

Figure 3. Cumulative incidence of adoptively transferred diabetes in male NOD-+/+ (n = 9), NOD-+/*lpr* (n = 12), and NOD-*lpr/lpr* (n = 7).

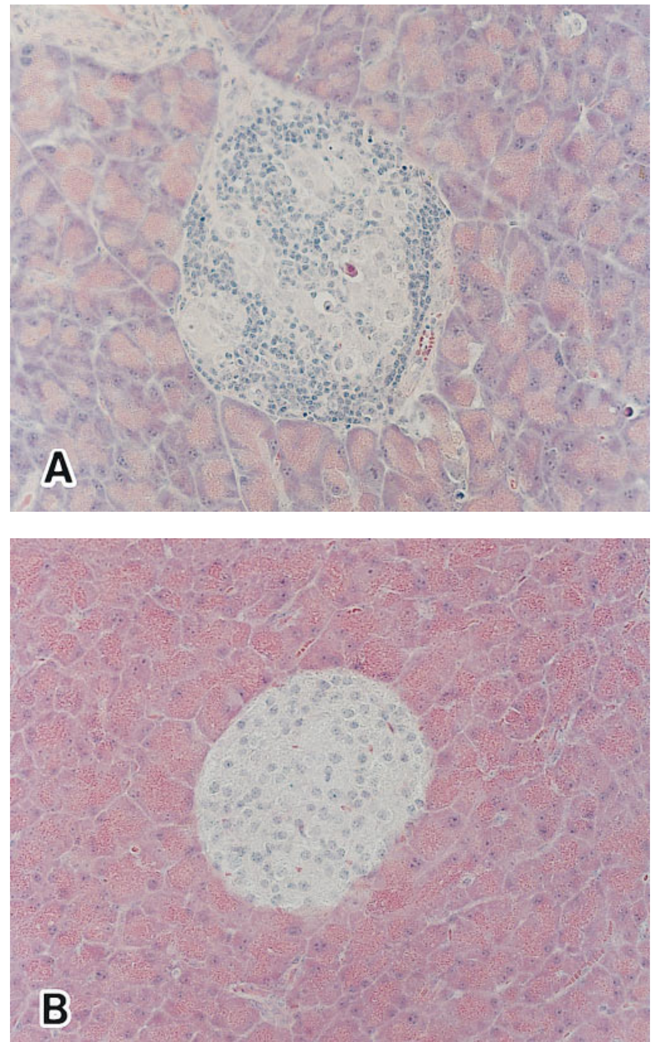

Figure 4. Hematoxylin and eosin staining of pancreatic islets of NOD-+/+ (*A*) and NOD-*lpr/lpr* (*B*) transferred with splenocytes from diabetic NOD. Original magnification was 200.

Table 1. *Analysis of Microsatellite Markers of the N_6 NOD-+/lpr Mice Used for the Intercross*

Pair No.	ID		*D19Mit43* (0 cM)	*D19Mit69* (9 cM)	*Fas* (24 cM)	*D19Mit11* (28 cM)	*D19Mit54* (39 cM)	*D19Mit34* (45 cM)
	Female	Male						
1	No. 272		D/D	D/D	+/*lpr*	D/M	D/M	D/M
		No. 277	D/D	D/D	+/*lpr*	D/M	D/D	D/D
2	No. 278	No. 283	D/D	D/D	+/*lpr*	D/M	D/M	D/M
3	No. 284	No. 286	D/D	D/D	+/*lpr*	D/D	D/D	D/D

D/D, NOD homozygote at the indicated locus; *D/M,* NOD/MRL heterozygote at the indicated locus; +/*lpr,* NOD/MRL heterozygote at the *Fas* locus.

NOD-+/+, NOD-+/*lpr,* and NOD-*lpr/lpr,* respectively (P <0.01). The number of islets per section was decreased in NOD-+/+ (9.89 ± 2.78), and NOD-+/*lpr* (12.3 ± 2.39), compared with that in NOD-*lpr/lpr* (52.1 ± 10.2) (P <0.01). Most islets of NOD-+/+ and NOD-+/*lpr* were inflamed and small in size, indicating the decrease in number resulted from destruction of the islet. Conversely, islets of NOD-*lpr/lpr* remained normal in morphology.

Analysis of Microsatellite Markers. Genetic regions encompassing *lpr* mutation were assessed in three pairs of the N_6 littermates used for producing the intercross. Although the position of recombination between NOD and MRL strains varied among the pairs (Table 1), either the incidence of diabetes in NOD-+/+ and NOD-+/*lpr* or the protection of insulitis and diabetes in NOD-*lpr/lpr* did not change among the F_1 intercross of these pairs. Markers linked to *Idd3*, *Idd5*, and *Idd10* loci were all homozygous for NOD alleles.

Discussion

The dominant role of T cells in the pathogenesis of IDDM has been strongly suggested by many investigators, but exact molecular mechanisms have remained elusive. Recent advances in the understanding of T cell–mediated cytotoxicity disclosed the possibility that β cells were destroyed by perforin- or Fas-based mechanisms. In fact, perforin-deficient LCMV-transgenic mice did not develop diabetes when infected with LCMV, suggesting that perforin-dependent cytotoxicity was crucial to cause diabetes by eliminating β cells (17). However, they did show insulitis, indicating that triggering islet inflammation required the perforin-independent pathway, which initially impaired β cells, exposed autoantigens to antigen-presenting cells, and launched an autoimmune cascade to diabetes. Our observation revealed that the Fas-dependent pathway might be precisely a complementary mechanism to initiate β cell destruction. NOD mice became completely free of insulitis by obtaining *Fas*-disrupting spontaneous mutation of *lpr.* Of course, there is a possibility that the lack of insulitis in NOD-*lpr/lpr* is due to the distorted T cell composition that contained unusual $CD4^-CD8^-$ cells. To avoid this influence, we performed transfer experiments from diabetic NOD to sublethally irradiated young congenic mice.

Even in this protocol, insulitis and diabetes were completely prevented in NOD-*lpr/lpr,* whereas they were rapidly induced in the littermates not homozygous for *lpr.* This finding strongly suggests that autoimmune cascade against pancreatic β cells is blocked at an early stage in Fas-lacking NOD mice. Transferred autoreactive T cells might initially damage β cells through Fas–FasL system, and then substantially destroy fragile target cells by other mediators such as perforin and cytokines. Two groups have reported that β cells can express Fas when stimulated with IL-1 (26, 27). IL-1 is a cytokine secreted by macrophages, the participation of which has already been proved essential for the onset of diabetes as well as T cells (28).

During the process of backcrossing, we screened H-2^{g7} (*Idd1*) homozygotes at the N_2 generation and *lpr* mutation at each generation. This implies that the prevention of insulitis and diabetes observed in NOD-*lpr/lpr* might be due to the absence of other *Idd* genes or the presence of unknown recessive genes linked to *lpr* mutation. However, microsatellite marker analysis revealed that other *Idd* loci significantly linked to insulitis and diabetes were replaced by the NOD alleles. In addition, either the incidence of diabetes in NOD-+/+ and NOD-+/*lpr,* or the protection of insulitis and diabetes in NOD-*lpr/lpr* did not change among the intercross with various recombinational positions from the *Fas* locus. This suggests that *lpr* mutation itself is responsible for the lack of disease in NOD-*lpr/lpr.*

To elaborate effective intervention therapies for IDDM, a better understanding of the pathogenesis is required. Given the fact that the Fas–FasL system is involved in the development of autoimmune diabetes, interference of this molecular mechanism would be a potent therapeutic strategy. Soluble form of Fas and anti-FasL Ab suppressed Fas-mediated cytotoxic activity (29, 30). Inhibitors of IL-1β–converting enzyme– or CPP32–like proteases blocked Fas-induced death signaling (31). If we devise a system to deliver one of these molecules at the site of inflammation, it might be a new beneficial treatment without systemic harmful effects for pre- and early stage of IDDM.

In conclusion, we have shown that autoimmune β cell

destruction is prevented by introduction of *Fas*-disrupting *lpr* mutation into NOD mice. Considering that islet inflammation was nearly completely abrogated, Fas-based cytotoxic mechanism is assumed to be critical in an initial step of β cell autoimmunity. Since there have been many β cell–specific T cell clones and lines, the analysis of cytotoxic pathways and abilities to induce diabetes of these cells would provide further evidence concerning the Fas–FasL system in IDDM.

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