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Protection of nonobese diabetic mice from autoimmune diabetes by reduction of islet mass before insulitis

(pancreatectomy/islet transplantation/mixed lymphocyte islet culture)

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ABSTRACT **Nonobese diabetic mice spontaneously develop diabetes that is caused by autoimmune cell-mediated destruction of pancreatic beta cells. Here we report that surgical removal of 90% of pancreatic tissue before onset of insulitis induced a long-term diabetes-free condition in nonobese diabetic mice. Pancreatectomy after development of moderate insulitis had no effect on the course of diabetes. The effect of pancreatectomy was abrogated with subsequent development of diabetes by infusion of islet-cell-specific T lymphocytes and by transplantation of pancreatic islets. Lymphocytes from pancreatectomized diabetes-free mice exhibited low response to islet cells but responded normally to alloantigens. These results suggest that the islet cell mass plays a critical role in development of autoimmune diabetes.**

Nonobese diabetic (NOD) mice spontaneously develop diabetes that has features similar to those of human insulin-dependent diabetes mellitus, a polygenic disease caused by autoimmune destruction of insulin-producing beta cells in the islets of Langerhans (1–3). The onset of this type of diabetes is preceded by a long period of insulitis that proceeds from lymphoid cell infiltration in the periphery of the islets (peri-insulitis) to cellular infiltration invading the islets (insulitis) (4–8). Although a T lymphocyte-mediated autoimmune etiology is suggested in this model (9–16), the antigen(s) responsible for triggering the activation of autoreactive T lymphocytes has not been fully elucidated. Molecules localized in the islets that have been characterized as the candidate antigens include insulin (17), glutamic acid decarboxylase (18), glycolipids (19, 20), carboxypeptidase H (21), and cellular proteins with molecular masses of 38 kDa (22), 52 kDa (23), and 69 kDa (24).

Regardless of the nature of islet autoantigen(s), initial activation of autoreactive T cells by the antigen(s) must take place sometime before the development of insulitis. Although it is likely that presentation of the autoantigen(s) to T cells occurs locally within the pancreas, there has been no study (to our knowledge) on whether the initiation of the diabetogenic process is a dose-dependent event in which a sufficient amount of islet antigen(s) in the pancreas must be presented to autoreactive T cells for activation. In this study, we examined whether reduction of overall islet mass by surgical removal of the pancreas has any influence in the subsequent development of diabetes in NOD mice.

MATERIALS AND METHODS

Mice. Female NOD/MrkTacfBR mice were purchased from Taconic. In this strain of NOD mice, hyperglycemia is first observed when the mice are 14 weeks old, and the cumulative incidence of diabetes is 80% in females at 27 weeks (data provided by Taconic). Male C3H/He (H-2^k) and DBA/1 (H-2^q) mice (7–8 weeks old) were obtained from The Jackson Laboratory. All mice were housed in accordance with U.S. Department of Agriculture Regulations Part III (Animal Welfare Act) at the animal facility of the Cancer Research Institute, Deaconess Hospital.

Pancreatectomy. Under sodium pentobarbital anesthesia (65 μg/kg i.p.), the pancreas and the spleen were surgically removed with careful conservation of the common bile duct and major vessels surrounding the duodenum. Approximately 10% (by weight and by insulin content) of the pancreas tissue was left intact adjacent to the lower duodenal loop. Pancreatectomy was performed when mice were either 7 or 13 weeks of age. As a control, sham operation splenectomy alone without pancreatectomy was performed when mice were 7 weeks of age. After 8 weeks of age, mice were tested weekly for urinary glucose. Once it became positive, mice were additionally tested for morning nonfasting blood glucose levels once a week by use of chemstrip tapes. Onset of spontaneous diabetes was defined when blood glucose levels became greater than 16.7 mmol/liter for two consecutive determinations. Blood glucose levels less than 11.1 mmol/liter were considered normal.

Adoptive Transfer of Diabetes. Nylon-wool nonadherent spleen cells (SPC) were prepared from spontaneously diabetic NOD mice 2 weeks after the disease onset and injected at 20×10^6 cells per mouse into either 8-week-old naive nondiabetic NOD mice or 30-week-old subtotally pancreatectomized nondiabetic NOD mice. Recipient mice were sublethally irradiated (750 rads) before cell injection.

Islet Transplantation. At the age of 4–5 weeks, NOD islet donors were treated with two doses of rabbit anti-mouse lymphocyte serum to suppress insulitis. Pancreases were removed from these mice at 8–9 weeks of age. Absence of lymphocytic infiltration in the islets was confirmed by histological examination. NOD islets as well as islets from diabetes-resistant C3H/He mice were isolated by a collagenase digestion and Ficoll gradient separation method and handpicked under a dissecting microscope as described (25). Varying numbers of NOD islets (500, 125, 60, and 30 islets) and 500 C3H/He islets were transplanted into the renal subcapsular space of pancreatectomized normoglycemic NOD recipients 1–2 weeks after pancreatectomy.

Mixed Lymphocyte–Islet Culture. Responder cells were prepared from lymph nodes of 13-week-old nondiabetic NOD mice that had received either the sham operation or the subtotal pancreatectomy at 7 weeks of age. Responder cells (5×10^5 cells) were mixed with irradiated (2500 rads) stimulator cells consisting of either 10^4 dispersed NOD islet cells, 5×10^5 fully allogeneic C3H/He (H-2^k) and DBA/1 (H-2^q) splenocytes, or 5×10^5 syngeneic NOD splenocytes. Single islet cells were prepared by incubating NOD islets at 37°C for

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Abbreviations: NOD, nonobese diabetic; SPC, spleen cells; LNC, lymph node cells.

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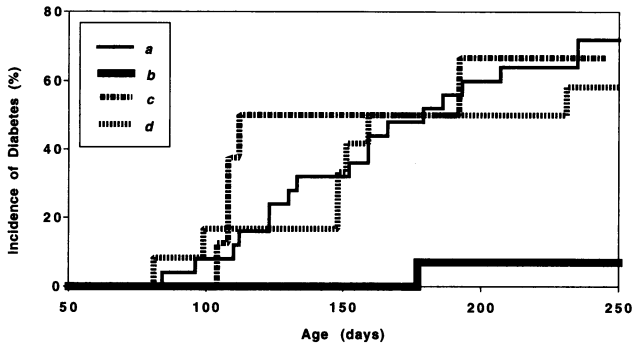

FIG. 1. Incidence of diabetes after pancreatectomy. a, Control untreated NOD mice (n = 25); b, pancreatectomy (90%) at 7 weeks of age (n = 15); c, pancreatectomy at 13 weeks of age (n = 8); d, sham operation (splenectomy alone) at 7 weeks of age (n = 12). P = 0.000 for a versus b, 0.618 for a versus c, 0.579 for a versus d, and 0.004 for b versus d.

3 min in 0.05% trypsin/EDTA solution with intermittent agitation. Mixtures of responder and stimulator cells were cultured in triplicates for 4–6 days in RPMI 1640 medium containing 5% fetal bovine serum and 2-mercaptoethanol (5×10^{-5} M) in 96-well U-bottom plates. All cultures were exposed to 0.5 μCi of [^{3}H]thymidine for 8 hr at the end of the culture period. [^{3}H]thymidine incorporation was expressed as mean counts per minute ($\times 10^{-3}$) for triplicate cultures ± SEM.

Histology. Tissues were fixed in Bouin's solution, processed, and parrafin-embedded. Sections 3–5 mm thick were cut and stained in hematoxylin and eosin.

Statistical Analyses. The analysis of diabetes incidence was calculated by the Kaplan–Meier estimate using the SYSTAT (version 5.1) and SURVIVAL programs (Systat, Evanston, IL). The significance level (P value) was obtained by Mantel's log rank test in the SURVIVAL program. Student's t test as well as χ^2 tests were also used to analyze statistical significance. A P value of <0.05 was considered significant.

RESULTS

Effect of Pancreatectomy on Development of Diabetes. The proportion of the remaining pancreas after pancreatectomy was determined immediately after surgery by measuring wet weight and insulin content of both the removed pancreas and remaining pancreatic tissue using randomly selected 7-week-old NOD mice (n = 5). Weight of the remaining pancreas was 29 ± 3 mg (or 11.3 ± 0.6%) compared with the whole pancreas weight of 255 ± 12 mg. Insulin content determined by radioimmunoassay was 2.1 ± 0.6 μg (or 9.2 ± 2.6%) in the remaining pancreas compared with 23.1 ± 1.7 μg in the whole pancreas.

After pancreatectomy, blood sugar levels in mice 7 weeks of age were maintained within a normal range (4.2–8.9 mmol/liter) without significant changes. All but one of 15 female mice remained diabetes-free for more than 250 days (Fig. 1). Whereas the spleen was removed at the time of pancreatectomy, sham-operated mice that underwent splenectomy alone developed diabetes in a manner similar to that of untreated control NOD mice. Pancreatectomy in mice at 13 weeks of age had no protective effect on the development of diabetes. Although all mice showed retarded body weight gain during

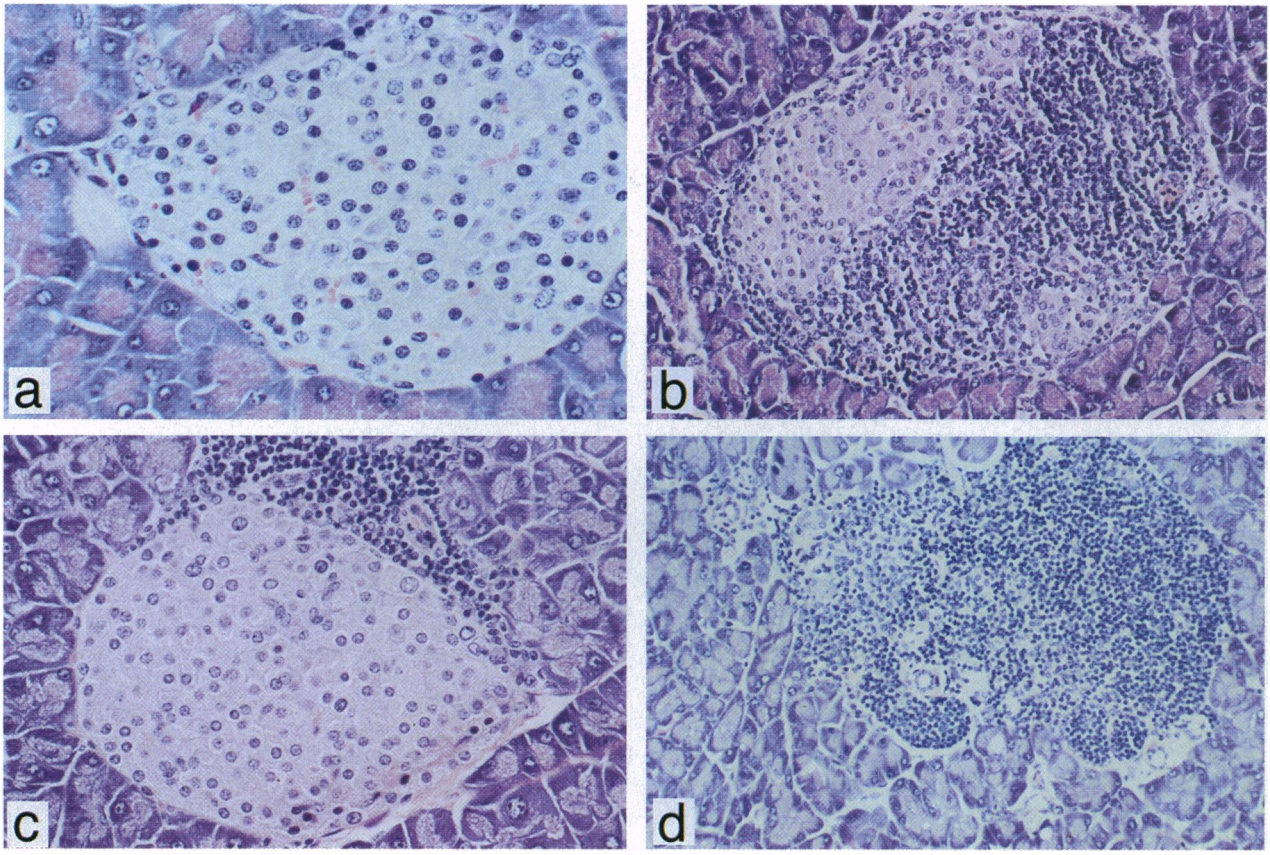

FIG. 2. Representative histology of islets (hematoxylin and eosin staining). (×400.) (*a*) Section of a 7-week-old NOD pancreas demonstrating a typical normal islet without lymphocytic infiltration. (*b*) Section of a pancreas from a 13-week-old mouse with massive peripheral lymphocytic infiltration. (*c*) Section of the remaining pancreas from a 34-week-old normoglycemic mouse that received a pancreatectomy; section shows minimum lymphocytic infiltration. (*d*) Section of a pancreas from a 16-week-old diabetic mouse that received a sham operation. The islet structure is obscured by infiltrating cells.

Table 1. Adoptive transfer of diabetes

Recipient mice	Onset of diabetes, days after transfer	Incidence of diabetes
Naive	12, 12, 12, 19, 27, >48	5/6
Pancreatectomized	12, 12, 12, 15, 15, 15, 15	7/7

Recipient mice were either 8-week-old naive nondiabetic NOD mice or 30-week-old pancreatectomized diabetes-free NOD mice. Incidence of diabetes was calculated at 30 days after adoptive transfer.

the first 2 weeks after pancreatectomy, they regained normal body weight in 3–5 weeks after surgery. Body weight changes were similar in mice that were pancreatectomized at 7 weeks of age and became diabetes-free and in mice that were pancreatectomized at 13 weeks of age and developed diabetes.

Histology. No lymphocytic infiltration was observed in the pancreases removed at 7 weeks of age (Fig. 2*a*). Because insulitis may be present at 3–5 weeks of age in other strains of NOD mice (1), absence of insulitis at 7 weeks of age in this strain of NOD mice (NOD/MrkTacfBR) was carefully confirmed by histological examination of multiple pancreases obtained from mice 3–7 weeks old. Moderate peripheral insulitis was seen in the pancreases removed at 13 weeks of age (Fig. 2*b*). At 34 weeks of age, the remaining pancreas tissue in subtotally pancreatectomized mice showed islets with no or minimum lymphocytic infiltration (Fig. 2*c*). The pancreas in sham-operated (splenectomy alone) mice revealed islets with severe lymphocytic infiltration involving the whole islet (Fig. 2*d*).

Adoptive Transfer of Diabetes. An adoptive transfer system (10, 13) was used to determine whether beta cells in the remaining pancreas were susceptible to destruction by activated autoimmune T lymphocytes. Injection of SPC obtained from overtly diabetic NOD mice into 30-week-old pancreatectomized nondiabetic NOD mice induced diabetes as rapidly as injection into 8-week-old naive NOD mice (Table 1).

Effect of Islet Transplantation After Pancreatectomy. Because reduction of beta cell mass by pancreatectomy created a diabetes-free condition, we tested whether the addition of beta cell mass in the form of islet transplantation could cause pancreatectomized NOD mice to develop spontaneous diabetes (Table 2). Islet transplantation with as few as 60 NOD islets abrogated the protection and caused eventual development of diabetes (Table 2, experiment B). It appears that 30 islets were insufficient to break the pancreatectomy-induced protection. Pancreatectomized NOD mice transplanted with 500 islets of diabetes-resistant, fully allogeneic C3H/He mice also developed diabetes. Allogeneic stimulation by renal subcapsular inoculation of 50×10^6 C3H/He SPC appeared to have no effect on the pancreatectomy-induced diabetes-free condition.

Mixed Lymphocyte–Islet Cultures. The capacity of lymph node cells (LNC) to respond *in vitro* to islet cells after pancreatectomy and sham operation was examined by mixed lymphocyte–islet cultures (Fig. 3). LNC prepared from pancreatectomized, nondiabetic mice failed to respond to isogeneic NOD islets, whereas LNC of sham-operated mice responded strongly to isogeneic islets. Responses of both groups of lymphocytes to alloantigens were comparable. Similar results were repeatedly obtained in multiple experiments.

DISCUSSION

In individuals genetically predisposed to develop autoimmune diabetes, autoreactive T cells are released into the periphery, where they subsequently become sensitized against islet antigen(s). It is postulated that initial nonautoimmune inflammatory responses in the islets recruit autoreactive T cells to the islets, where they are exposed to islet antigens released from damaged beta cells (26). Activated autoreactive T cells continue to expand and eventually destroy beta cells, leading to diabetes.

The results presented herein clearly illustrate the importance of early interaction within the pancreas between the islet antigen(s) and autoreactive T cells for initiation of the diabetogenic process. Removal of the pancreas before the development of insulitis (at 7 weeks of age in NOD/MrkTacfBR mice) induced a life-long protection of NOD mice from diabetes. However, progression of the diabetogenic process was not disturbed by pancreatectomy once the autoreactive T cells had been activated, as evidenced by moderate insulitis in 13-week-old pancreases. Although it is currently unknown whether the islet antigen(s) involved in the early T-cell activation is the conventional autoantigens (17–24) or other early beta cell-specific autoantigens (27), we propose that the amount of islet antigen(s) present in the remaining pancreas after pancreatectomy was insufficient to trigger activation of autoreactive T cells, thus resulting in protection of pancreatectomized mice from developing diabetes. Beta cells in these mice, however, continued to be susceptible to destruction by activated autoreactive T cells, as demonstrated by the adoptive transfer experiment, indicating that diabetes would have developed if the islet antigen(s) in the remaining pancreas was capable of activating autoreactive T cells.

Mice pancreatectomized after initiation of insulitis (i.e., 13 weeks of age) developed diabetes in a similar manner as untreated control mice. This was surprising to us for two reasons. First, we thought that 90% reduction of islet mass would shift the onset of disease to an earlier age because the fewer remaining beta cells would be more rapidly destroyed by the autoimmune process. Second, the remaining beta cells appeared to be more metabolically active because of loss of functional reserve, as evidenced by abnormal intravenous glucose tolerance tests (results not shown), and thus could be more susceptible to autoimmune destruction, leading to earlier disease onset. It has been suggested that metabolically active beta cells are more immunogenic and more preferentially

Table 2. Islet iso- and allografting in pancreatectomized NOD mice

Experiment	Islet donor	Islet nos.	Onset of diabetes, days of age	*P* value
A*	—	—	177, >250 × 14	—
B†	NOD	500	113, 150, 166, 172, 183, >250 ×3	0.002
	NOD	125	104, 104, 111, 138, 146, 205	<0.001
	NOD	60	86, 94, 122, 150, >230 × 2, >244	0.004
	NOD	30	>153 × 3, >154, 163, >196, >223	0.146
C‡	C3H/He	500	132, 134, 152, 167, >167, >218, >250	0.003
	(C3H/He SPC)		>153 ×5	NC

*Pancreatectomy was performed at 7 weeks of age.
†Varying numbers of NOD islets were transplanted 1–2 weeks after pancreatectomy.
‡C3H/He islet allotransplantation or C3H/He SPC injection into renal subcapsular space was done 1–2 weeks after pancreatectomy. The significance level (*P* value) was calculated against mice in experiment A.
NC, Not calculated.

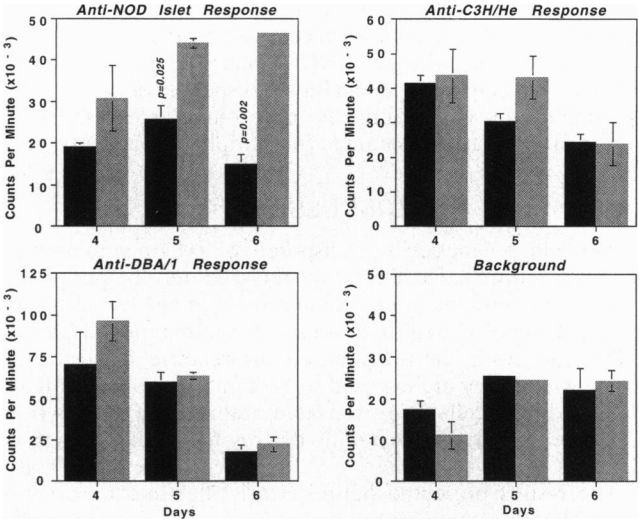

FIG. 3. Representative results of isogeneic mixed lymphocyte–islet cultures (anti-NOD islet response) and allogeneic mixed lymphocyte cultures (anti-C3H/He response and anti-DBA/1 response) by LNC of pancreatectomized mice (filled bars) and LNC of sham operated mice (shaded bars). Results of syngeneic mixed LNC–splenocyte cultures (background) are also shown.

destroyed by the autoimmune process (28). The mechanism(s) underlying slow development of autoimmune diabetes, regardless of the size of an islet mass, remains to be investigated.

The diabetogenic process that had been arrested by pancreatectomy was restored once a sufficient amount of islet antigen(s) was provided in the form of islet isografts. The interaction was dose-dependent in that transplantation of more than 60 NOD islets induced diabetes but transplantation of 30 NOD islets failed to cause diabetes. Since 30 islets contained 2.7 ± 0.4 μg (n = 3) insulin, which was equivalent to insulin content of the remaining pancreas after pancreatectomy and to approximately 10% of insulin in the whole pancreas, the minimal beta cell mass needed to initiate a diabetogenic autoimmune process was calculated to be approximately 20% of the total beta cells contained in the whole pancreas.

Diabetes also developed in pancreatectomized NOD mice after transplantation of allogeneic C3H/He islets. Thus, islets of diabetes-prone NOD mice and islets of diabetes-resistant C3H/He mice appeared to share diabetogenic autoantigen(s) that activated autoreactive T cells of NOD mice. Although a previous report (27) showed that T-cell clones derived from NOD mice proliferated *in vitro* in response to extracts of NOD islets as well as human islets, we have demonstrated for the first time that islets of allogeneic, diabetes-resistant mice were capable of initiating autoimmune response in NOD mice. This finding suggests that a diabetogenic antigen(s) is expressed on beta cells of all mice regardless of their genetic predisposition for autoimmune diabetes. Because pancreatectomized mice responded to alloantigens as strongly as control mice, allogeneic islets in pancreatectomized mice were probably rejected in 12–22 days as were those in streptozotocin-induced diabetic NOD mice (28). Thus, it appears that autoreactive T cells were activated in a relatively short period of time, and thereafter, the amount of beta cells (or the amounts of diabetogenic antigens) did not influence the course of diabetes. Consistent with this is the finding that pancreatectomy after the onset of insulitis failed to protect NOD mice from diabetes.

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