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

(pancreatectomy/islet transplantation/mixed lymphocyte islet culture)

AKIHKO ITOH AND TAKASHI MAKI*

Transplantation and Cellular Immunology Laboratory, Division of Organ Transplantation, Department of Surgery, Deaconess Hospital and Harvard Medical School, 1 Deaconess Road, Boston, MA 02215

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ABSTRACT Nonobese diabetic (NOD) 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 (perisinulitis) 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/MrTacFBR 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-2b) and DBA/1 (H-2k) 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 × 10⁶ cells per mouse into either 8-week-old naive nonobese diabetic 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 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 nonobese diabetic NOD mice that had received either the sham operation or the subtotal pancreatectomy at 7 weeks of age. Responder cells (5 × 10⁶ cells) were mixed with irradiated (2500 rads) stimulator cells consisting of either 10⁴ dispersed NOD islet cells, 5 × 10⁴ fully allogeneic C3H/He (H-2b) and DBA/1 (H-2k) splenocytes, or 5 × 10⁵ syngeneic NOD splenocytes. Single islet cells were prepared by incubating NOD islets at 37°C for 4 h. After digestion, the cells were washed and suspended in Hanks' balanced salt solution for 2 h. Cells were then counted in a Coulter Multisizer II cell counter.

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

*To whom reprint requests should be addressed.
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 [°H]thymidine for 8 hr at the end of the culture period. [°H]thymidine incorporation was expressed as mean counts per minute (×10^3) for triplicate cultures ± SEM.

Histology. Tissues were fixed in Bouin’s solution, processed, and paraffin-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 χ² 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.
cells

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on
Pancreatectomized NOD mice

were similar in mice that
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pancreatectomized diabetes-free NOD mice. Inci-
dence 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

Histology. No lymphocytic infiltration was observed in the
pancreases removed at 7 weeks of age (Fig. 2a). 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/MrkTacBR) was carefully con-
firmed 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. 2b). At 34 weeks of age, the remaining pancreas tissue in
subtotally pancreatectomized mice showed islets with no or
minimum lymphocytic infiltration (Fig. 2c). The pancreas in

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 acti-

Effect of Islet Transplantation After Pancreatectomy. Be-
cause 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 diabe-
tes (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 $50 \times 10^6$ C3H/He SPC appeared to have no

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

Discusssion

In individuals genetically predisposed to develop autoimmune
diabetes, autoreactive T cells are released into the periphery,
where they subsequently become sensitized against islet anti-
gens(s). It is postulated that initial nonautoimmune inflamma-
tory 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 con-
tinue to expand and eventually destroy beta cells, leading to
diabetes.

The results presented herein clearly illustrate the impor-
tance of early interaction within the pancreas between the islet
antigen(s) and autoreactive T cells for initiation of the diabe-
togenic process. Removal of the pancreas before the develop-
ment of insulitis (at 7 weeks of age in NOD/MrkTacBR 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 pancreatec-
tomy 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 auto-
reactive 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 1. Adoptive transfer of diabetes

<table>
<thead>
<tr>
<th>Recipient mice</th>
<th>Onset of diabetes, days after transfer</th>
<th>Incidence of diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>12, 12, 12, 19, 27, &gt;48</td>
<td>5/6</td>
</tr>
<tr>
<td>Pancreatectomized</td>
<td>12, 12, 15, 15, 15</td>
<td>7/7</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Islet donor</th>
<th>Islet nos.</th>
<th>Onset of diabetes, days of age</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td></td>
<td></td>
<td>177, &gt;250 x 14</td>
<td></td>
</tr>
<tr>
<td>B†</td>
<td>NOD</td>
<td>500</td>
<td>113, 150, 166, 172, 183, &gt;250 x3</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>NOD</td>
<td>125</td>
<td>104, 104, 111, 138, 146, 205</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NOD</td>
<td>60</td>
<td>86, 94, 122, 150, &gt;230 x 2, &gt;244</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>NOD</td>
<td>30</td>
<td>&gt;153 x 3, &gt;154, 163, &gt;196, &gt;223</td>
<td>0.146</td>
</tr>
<tr>
<td>C‡</td>
<td>C3H/He</td>
<td>500</td>
<td>132, 134, 152, 167, &gt;167, &gt;218, &gt;250</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>(C3H/He SPC)</td>
<td>&gt;153 x 5</td>
<td>NC</td>
<td></td>
</tr>
</tbody>
</table>

*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 pancreactectomized 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 pancreactectomy 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 $2.7 \pm 0.4 \mu g (n = 3)$ insulin, which was equivalent to insulin content of the remaining pancreas after pancreactectomy 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 pancreactectomized 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 autoantigen(s) is expressed on beta cells of all mice regardless of their genetic predisposition for autoimmune diabetes. Because pancreactectomized mice responded to alloantigens as strongly as control mice, allogeneic islets in pancreactectomized 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 pancreactectomy after the onset of insulitis failed to protect NOD mice from diabetes.

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