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Prevention of Autoimmune Diabetes in the BB Rat by Intrathymic Islet Transplantation at Birth

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Spontaneous diabetes in the BioBreeding (BB) rat, like human type I diabetes, results from the destruction of pancreatic islets by autoreactive T lymphocytes recognizing β cell–specific antigens. T cell tolerance is in part mediated by interactions of maturing thymocytes with antigens expressed in the thymic microenvironment; islets were therefore implanted into the thymus of neonatal diabetes-prone BB rats to determine whether exposure of T cell precursors to β cell antigens could influence the development of diabetes. This treatment completely prevented diabetes and insulitis in the native pancreas. The effect may be the result of specific modulation of diabetogenic T cells maturing in an islet-bearing thymus.

Rat pancreatic islet allografts transplanted into the thymus of allogeneic hosts survive indefinitely without the need for chronic immunosuppression. Recipients of these grafts are specifically unresponsive to extrathymic islets transplanted from the same strain, possibly as a result of the deletion or functional inactivation of donor-specific alloreactive clones (*1, 2*). This approach might also be used to alter T cell–mediated immunity to tissue-specific self antigens.

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We examined whether implantation of a small amount of islet tissue into the thymus of neonatal BB rats might prevent the development of autoimmune insulitis in the pancreas, a lesion that ordinarily causes 40 to 60% of these animals to become severely diabetic in young adulthood (*3–7*).

Litters of diabetes-prone BB rats [major histocompatibility complex (MHC) haplotype RT1^u] were separated at birth into randomly selected experimental and control groups. Each member of the experimental half-litters received an intrathymic inoculum of 60 to 80 islets (30 to 40 per thymic lobe) isolated from the pancreata of MHC-compatible Wistar Furth (WF,

RT1^u) adult male donors. Members of the other half-litters served as controls and received intrathymic injections of saline (5 to 10 μl per lobe). Blood glucose was monitored in all rats for at least 250 days, and no immunosuppression was administered to members of either group at any time (8).

None of the 18 BB rats that received intrathymic islets at birth developed diabetes during the experimental period (Fig. 1). In contrast, 7 of the 14 control animals that received intrathymic injections of saline developed spontaneous diabetes between 55 and 121 days of age (mean onset ± SD = 95 ± 22 days), a result not significantly different from the incidence observed in unmanipulated, diabetes-prone BB rats (51.9%, mean onset ± SD = 91 ± 20 days; n = 135).

Although the 60 to 80 islets transplanted intrathymically constituted a subtherapeutic dose of less than 10% of the amount required to reverse hyperglycemia in diabetic animals (approximately 1000 to 1500 islets per recipient), the possibility remained that persistent normoglycemia was the result of insulin production by the transplanted intrathymic islets rather than by islets in the native pancreas. Therefore, the thymus was removed from 13 rats that had been inoculated with islets at birth, 190 to 210 days previously. Eleven of these rats survived thymectomy and all remained normoglycemic for more than 70 days (Table 1), which indicated that the intrathymic graft was not responsible for maintenance of normoglycemia and suggested that the β cells of the native pancreas had escaped autoimmune destruction. Pancreat-

Table 1. Functional and histologic characterization of BB rats with intrathymic islet grafts. Baseline blood glucose levels of BB rats that had received intrathymic WF islets at birth (rats 1 to 13) and of saline-treated diabetic and nondiabetic littermate controls were determined at 190 to 210 days of age. The animals were thymectomized on that day (designated day 0) and monitored for changes in blood glucose until at least 280 days of age. Thymic specimens and pancreatic biopsies (obtained at the time of thymectomy) were examined for the presence of islets as described in Fig. 2. Blood glucose concentrations of animals that received intrathymic islets are depicted individually and as mean ± SD; concentrations in animals that received intrathymic saline are shown as mean ± SD. ND, not done.

Rats	Islets on day 0		Insulitis	Blood glucose (mg/dl)			
	Thymus	Pancreas		Day 0	Day 2	Day 7	>70 days
			Intrathymic islets				
1	+	+	−	114	104	97	80
2	+	+	−	114	102	94	89
3	+	+	−	97	124	111	79
4	+	+	−	118	97	112	103
5	+	+	−	81	111	97	121
6	+	+	−	120	120	94	91
7	−	+	−	99	124	67	95
8	+	+	−	128	69	81	80
9	+	+	−	112	104	97	86
10	+	+	−	130	127	90	87
11	+	+	−	99	76	67	109
12	+	+	−	120*	ND	ND	ND
13	+	+	−	90*	ND	ND	ND
				x ± SD: 109 ± 15	105 ± 19	92 ± 15	93 ± 13
			Intrathymic saline				
Diabetic		0/4	All end-stage	288 ± 86	390 ± 25	369 ± 68	ND
Nondiabetic		6/6	2/6	110 ± 28	104 ± 18	91 ± 12	86 ± 18

*Animal died during thymectomy.

Table 2. Surface phenotype of lymph node cells from islet-grafted and control BB rats. Superficial and deep cervical lymph nodes from 200-day-old BB and age-matched WF rats were teased into single-cell suspensions and incubated (5 × 10^5 to 6 × 10^5 cells per sample) with each of the following murine monoclonal antibodies: R7.3 [anti-αβ T cell receptor (TCRαβ)], W3/25 (anti-CD4; helper T cells and macrophages), OX-8 (anti-CD8; cytotoxic T cells and natural killer cells), and Mar 18.5 [anti-K light chain; membrane immunoglobulin (mIg)–positive B cells] (*25, 26*). Cells were then washed twice and exposed to fluorescein isothiocyanate–conjugated F(ab')$_2$ goat antibody to mouse IgG (Tago Inc., Burlingame, California). All incubations and washes were carried out at 4°C in Dulbecco's phosphate-buffered saline containing 1% bovine serum albumin and 0.1% sodium azide. Analysis was performed on freshly stained cells with the use of a FACScan flow cytometer (Becton-Dickinson, Sunnyvale, California). Background fluorescence was calculated with the use of cells incubated with the fluorescein conjugate alone. Data are presented as the mean percentage of positively staining cells ± SD.

Group (n)	Neonatal treatment	Cell number × 10^6	Positive cells (%)			
			TCRαβ	CD4	CD8	mIg
WF(4)	None	28.6 ± 5.9	78.7 ± 5.9	52.6 ± 3.1	27.9 ± 2.2	16.9 ± 6.7
BB(6)	None	11.8 ± 6.5	21.1 ± 6.0	24.3 ± 7.9	5.3 ± 3.9	58.8 ± 11.2
BB(5)	Intrathymic saline	12.7 ± 7.6	26.7 ± 2.9	24.2 ± 3.5	2.5 ± 0.6	59.8 ± 3.6
BB(8)	Intrathymic WF islets	13.2 ± 8.2	26.0 ± 4.5*	23.8 ± 4.1,	2.5 ± 0.7*	59.0 ± 8.6

*Not significantly different when compared with corresponding unmanipulated BB groups (Student's t test).

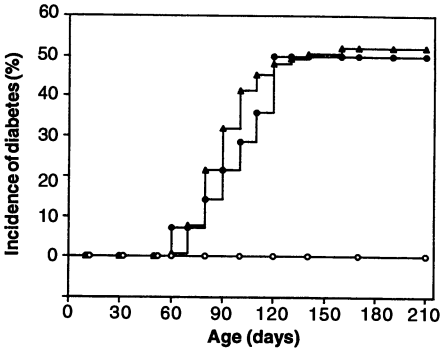

Fig. 1. Effect of intrathymic islet transplantation at birth on diabetes incidence in the BB rat. Pancreatic islets were obtained from adult male WF rats (Harlan Sprague-Dawley, Walkersville, Maryland) by collagenase digestion of the pancreas, centrifugation on a Ficoll density gradient, and manual removal of contaminating vascular, ductal, and exocrine components as described (*2*). Aliquots of 30 to 40 islets in 5 to 10 μl of phosphate-buffered saline were then inoculated directly into each thymic lobe of neonatal (<24 hours old), diabetes-prone BB rats (○; n = 18); control littermates (●; n = 14) received intrathymic injections of saline (5 to 10 μl per lobe). We monitored blood and urinary glucose concentrations in islet-grafted and saline-treated animals biweekly with the use of an Accuchek III blood glucose monitor (Boehringer Mannheim Corp., Indianapolis, Indiana) and Testape (Eli Lilly & Co., Indianapolis, Indiana), respectively. Rats were considered diabetic on the basis of persistent glycosuria and nonfasting blood glucose concentrations exceeding 250 mg/dl on three consecutive days. The incidence of spontaneous diabetes in age-matched, unmanipulated BB rats (▲; n = 135) is depicted for comparison. The difference in the incidence of diabetes in islet-grafted versus saline-treated rats was significant (P < 0.005; Fisher's exact test).

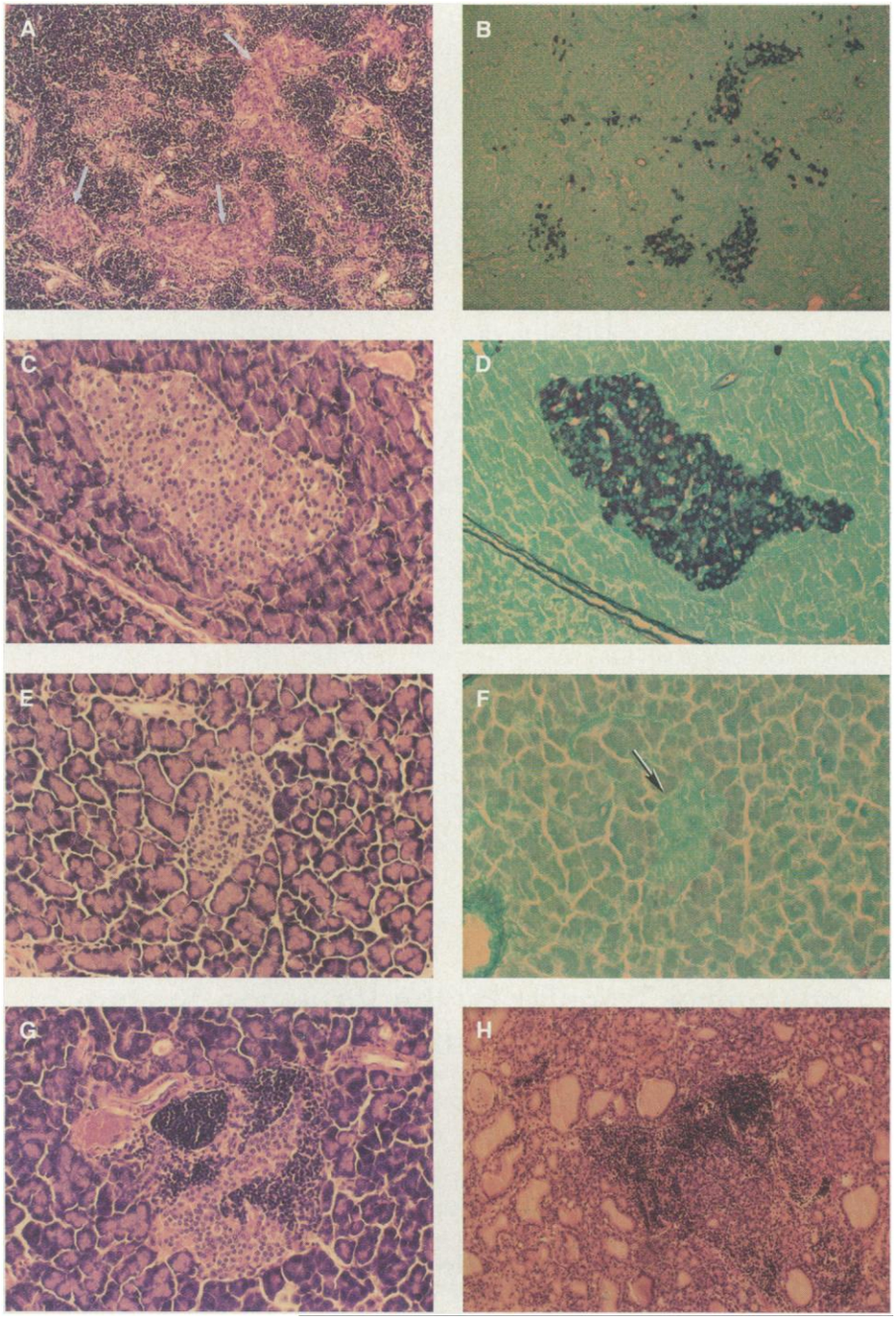

Fig. 2. Photomicrographs of thymus, pancreas, and thyroid from BB rats inoculated with WF islets or saline at birth. (**A**) Section of a thymus from a 200-day-old normoglycemic animal that had received an intrathymic islet graft at birth [Hematoxylin/Eosin (H & E), original magnification ×125]. Several clusters of islet cells are present (arrows). (**B**) Islet cells stain abundantly for insulin [Aldehyde-Fuchsin (AF), ×125]. (**C**) Pancreas from the same rat, demonstrating a healthy islet completely free of mononuclear cell infiltration (H & E, ×250). (**D**) The presence of β cells in this section was verified by insulin-specific staining (AF, ×250). (**E** and **F**) End-stage islet (arrow) in the pancreas of a chronically diabetic control animal that had received an intrathymic injection of saline at birth. No insulin-containing cells can be identified [(E) H & E, ×250; (F) AF, ×250). (**G**) Pancreas from an acutely diabetic BB rat with an islet heavily infiltrated by mononuclear cells (insulitis) (H & E, ×250). (**H**) Thyroid specimen from a 200-day-old animal that had been transplanted with intrathymic islets at birth. Dense cellular infiltration of the thyroid parenchyma is present (H & E, ×125). Thymic, pancreatic, and thyroid tissue were processed for histology by overnight fixation in Bouin's solution and embedment in paraffin. Serial sections (4 μm) from each sample were then stained with H & E to demonstrate general morphology or with AF to detect the presence of insulin.

ic biopsies performed at the time of thymectomy confirmed this interpretation (Fig. 2). All rats that had received intrathymic islets at birth possessed healthy islets in their native pancreas without any evidence of insulitis (Table 1 and Fig. 2, C and D); in contrast, the pancreata from diabetic, saline-injected controls contained only rare, end-stage islets devoid of insulin granules or islets heavily infiltrated by mononuclear cells (Fig. 2, E to G). Histologic examination of the excised thymus revealed small clusters of healthy, insulin-containing islet endocrine cells within the thymic parenchyma (Fig. 2, A and B), a finding consistent with the observation that islet allografts implanted in the thymus of spontaneously diabetic adult BB rats are not destroyed by rejection or autoimmunity (*2*).

To determine whether the prevention of autoimmune insulitis by neonatal islet tissue requires that the implant be situated in the thymus, we inoculated 16 neonatal BB rats with 60 to 80 WF islets beneath the renal capsule. This did not prevent diabetes, despite histologic evidence of survival of islets under the kidney capsule. Eight of the 16 rats that were inoculated with islets developed diabetes and insulitis in their native pancreas, as compared with 8 of 15 age-matched controls inoculated with saline beneath the renal capsule. The renal subcapsular islets in diabetic rats stained only weakly for insulin and were infiltrated by lymphocytes, whereas in rats that remained normoglycemic these islets appeared healthy and were not infiltrated.

To determine the specificity of the protection induced by intrathymic islet tissue, we examined animals with intrathymic islets for lymphocytic thyroiditis, another autoimmune endocrinopathy that ordinarily develops in 50 to 60% of BB rats (*7*, *9*–*11*). Despite the prevention of diabetes in rats that received intrathymic islets, the incidence of thyroiditis was not reduced in these animals when compared to the incidence in diabetic controls (6/12 versus 4/9, respectively; Fig. 2H).

Several nonspecific immunomodulatory methods decrease the incidence of both diabetes and thyroiditis in BB rats. These include systemic immunosuppression (*9*, *10*, *12*, *13*) and immunotherapeutic protocols such as induction of neonatal tolerance with MHC-compatible bone marrow and infusions of MHC-matched peripheral blood lymphocytes (*11*, *14*–*16*). These methods presumably act by nonspecifically suppressing cell-mediated immune responses or by correcting the abnormalities in T cell numbers and T cell function in the T lymphopenic BB rat. We obtained evidence

that the prevention of diabetes by intrathymic islet implantation was not due to such nonspecific alterations in T cell immunity by performing several in vivo and in vitro assays on recipients of intrathymic islets. Total cell numbers as well as phenotypic distributions of T cell subsets in the peripheral lymph nodes of islet-grafted animals were not significantly different from those of saline-treated and unmanipulated BB rats, and all groups exhibited the T lymphopenia characteristic of this strain (Table 2). Similarly, mixed lymphocyte culture and concanavalin A (Con A)–stimulated proliferative responses of lymph node cells from experimental and saline-treated BB rats were comparable, all being reduced as compared with responses of lymphoid cells that were not from the BB strain (*17, 18*). In vivo T cell–mediated responses were also present to a similar degree in experimental and control rats, as confirmed by the capacity of islet-grafted animals to reject allogeneic Lewis ($RT1^l$) skin grafts at a rate comparable to that observed in control diabetics (13 and 35 days versus 14, 22, 29, and 34 days, respectively).

These results indicate that the prevention of autoimmune diabetes by neonatal islet transplantation is β cell–specific, is not dependent on systemic alterations in immune function that have been associated with other methods of preventing diabetes in the BB rat, and requires that the islet implant be situated in the thymic microenvironment.

Prophylactic administration of high doses of insulin to normoglycemic, prediabetic BB rats reduces their incidence of diabetes, possibly by rendering the endogenous islets metabolically inactive and thus decreasing their expression of β cell–specific autoantigens (*19–21*). This mechanism seems an unlikely explanation for the prevention of insulitis and diabetes that we observed because of the small number of islets implanted in the thymus and the inability of renal subcapsular islet grafts to prevent disease. Furthermore, in the reports mentioned above, insulin therapy provided only partial protection from diabetes and did not prevent development of insulitis, whereas we observed complete prevention of both hyperglycemia and insulitis after neonatal intrathymic islet transplantation.

A more likely explanation for our findings is that intrathymic transplantation of islets into neonatal BB rats alters T cell development by promoting the deletion or functional inactivation of antigen-specific clones before their migration to the periphery (*22–24*). Such an effect could be mediated by interactions of maturing host T cells with islets expressing the β cell autoantigen–MHC complex or with host-derived thymic antigen presenting cells bearing processed β cell antigens that were shed from the islet graft. Alternatively, the long-term residence of islets in the thymus may stimulate the selection of specific regulatory cells capable of suppressing anti-islet autoimmune responses.

Our data show that introduction of a small number of MHC-compatible islets into the thymus of prediabetic BB rats prevents the development of spontaneous diabetes, an effect that appears to result from specific regulation of anti-islet autoimmunity. These findings could lead to the development of novel approaches for the prevention of diabetes and other organ-specific autoimmune diseases.

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Conjugative Transfer by the Virulence System of *Agrobacterium tumefaciens*

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Agrobacterium tumefaciens transfers part of its Ti plasmid, the transferred DNA (T-DNA), to plant cells during tumor induction. Expression of this T-DNA in plant cells results in their transformation into tumor cells. There are similarities between the process of T-DNA transfer to plants and the process of bacterial conjugation. Here, the T-DNA transfer machinery mediated conjugation between bacteria. Thus, products of the Vir region of the Ti plasmid of *Agrobacterium tumefaciens*, normally involved in transfer of DNA from bacteria to plants, can direct the conjugative transfer of an IncQ plasmid between agrobacteria.

The genes responsible for T-DNA transfer from *Agrobacterium tumefaciens* to plant cells are located in the Vir region of the Ti plasmid and in the bacterial chromosome (*1*). There are many similarities between T-DNA transfer and bacterial conjugation, including the introduction of single-stranded breaks in the DNA molecules that are transferred. In T-DNA transfer, the proteins VirD1 and VirD2, encoded by the *virD* operon of the Vir region, together act as an endonuclease on the bottom strands of the border sequences that surround the T region (*1*). In mobilizable plasmids, such as RSF1010, nicks are produced by Mob proteins at the origin of transfer (*oriT*), and there is sequence homology between border repeats and the *oriT* sequences of certain plasmids (*2*). Conjugative transfer involves

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