Induction of Tolerance to Autoimmune Diabetes with Islet Antigens

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Summary

The development of T cell tolerance to self-antigens is imparted principally through negative selection events during thymic ontogeny. However, this tolerance may be limited to antigens that are expressed in the thymus, and additional mechanisms are probably required to regulate autoimmune responses to tissue-specific antigens. Autoimmune diabetes can be induced experimentally by treating susceptible stains of mice with multiple low doses of streptozotocin (STZ). In this report we show that transplantation of isolated islets of Langerhans into the thymuses of adult C57BL/KsJ mice will induce tolerance to the subsequent induction of autoimmune diabetes. This tolerance is tissue specific and thymus dependent. It was not induced by thymic transfer of adrenal tissue or by kidney transfer of islets. Furthermore, depletion of mature T cells was required and the tolerant state was abrogated by the adoptive transfer of normal splenocytes. It is interesting that pretreatment of the islets with STZ enhanced their ability to induce tolerance, and suggests that antigen shedding induced by tissue damage may facilitate transfer of islet antigens to tolerizing cells in the thymus. These findings indicate that thymic tolerance specific for tissue can be stimulated to occur in the presence of atopical tissue-specific intrathymic antigens. Elimination of disease-related T cells in the absence of global immunosuppression represents a novel approach for the prevention of autoimmune disease.

Antigens encountered by T cell precursors during maturation in the thymus shape the repertoire of peripheral T cells. Initially, cells capable of reacting with antigens presented in the context of self MHC antigens develop through a process of thymic learning termed positive selection (1). Those T cells with the potential for autoreactivity are eliminated during passage into the thymic medulla. This event, termed negative selection or clonal deletion, is though to prevent the escape of autoreactive T cells into the peripheral tissues and represents the major mechanism for tolerance to self-antigens (2–4). Negative selection has been shown for T cells reactive with antigens expressed in the thymus such as I-E (5) and Mls (6), or H-Y antigen (7). Although it is an effective mechanism for deletion of cells reactive with antigens that are encountered in the thymus, clonal deletion may not always be complete, especially for antigens that are found only in the periphery (8–10). Thus, the repertoire of circulating T cells includes cells that are capable of responding to antigens expressed exclusively in the peripheral tissues (8, 9). These cells may be capable of causing autoimmune disease with appropriate activation signals and antigen presentation (9).

The multi-dose streptozotocin model (MDSDM)[1] of insulin-dependent diabetes mellitus (IDDM) is a T cell–mediated autoimmune disease (11). In this model, hyperglycemia and insulitis are induced about 10 d after administration of five subdiabetogenic doses of streptozotocin (STZ) in certain strains of male mice. Both insulitis and hyperglycemia can be prevented by T cell depletion using mAbs (12), and adoptively transferred with splenocytes (13). The mechanism of the disease and the effects of STZ on the islet cells are incompletely understood, but recent studies by Weide and Lacy (14) have shown that STZ treatment of islets is required to render them antigenic. These results suggest that a specific antigen(s), as yet unknown, is expressed by damaged islets which triggers autoreactive T cells.

Posselt et al. (15) have described a system whereby tolerance to alloantigen can be induced by surgical implantation of allogeneic islet cells into the thymus. Their study suggests that if T cells mature in the thymus in the presence of alloan-

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[1] Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; MDSM, multi-dose streptozotocin model; STZ, streptozotocin.

tigen, deletion or inactivation of alloreactive cells that normally populate the periphery can be induced. However, this study with alloantigen, as well as those previously reported involving Mls (6), IE (5), or H-Y (7), involve antigens that are not tissue specific and for which there is a relatively high frequency of antigen-reactive T cell precursors. It is unknown whether in adult animals, thymic tolerance can be induced towards tissue-specific antigens and whether it can be used to prevent organ-specific disease. We have, therefore, determined whether thymic transplantation of islet cells that express antigens that are recognized in autoimmune diabetes can induce tissue-specific tolerance in a manner that will protect against the development of disease.

Materials and Methods

Animals. Male C57BL/KsJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used between the ages of 5 and 9 wk. The mice were housed under pathogen-free conditions in the animal barrier facility at The University of Chicago. The animals were allowed access to standard laboratory chow and water ad libitum.

Preparation of Animals. Islets of Langerhans were isolated by standard collagenase technique (16). The islets were handpicked from the digest of pancreases with the aid of a stereomicroscope and placed in culture overnight with DMEM (containing 25 mM glucose) with 10% FCS, 10 mM morpholinopropane sulfonic acid (MOPS), penicillin (100 U/ml), streptomycin (100 μg/ml), and additional amino acids. The islets were again harvested by hand with a stereomicroscope and washed three times. They were treated for 30 min in Krebes-Hepes buffer (Hanks' buffered saline with 10 mM Hepes, 1% BSA, 14 mM sodium bicarbonate) with STZ (0.5 mg/ml), washed, and suspended in Krebs-Hepes buffer. Between 100 and 125 islets (in 25 μl) were injected into each of two lobes of the thymus of adult C57BL/KsJ mice. Some animals received an equivalent number of islets that were not treated with STZ, or minced fragments of adrenal tissue, that were (n = 2), or were not (n = 2) treated with STZ for 30 min as described for islets. The adrenal tissue was taken from islet donors. In other mice, 200–250 islets were transplanted under a kidney capsule. On the day after surgery, each of the mice was treated with anti-CD3 mAb (145-2C11, 300 μg intraperitoneally, purified with protein A–Sepharose from ascites). A fourth group of mice received intrathymic STZ-treated islets, but did not receive anti-CD3 mAb treatment.

Induction of MDSDM. 3.5 wk after surgery and treatment with anti-CD3 mAb, the mice were given STZ (40 mg/kg, in citrate buffer, intraperitoneally) daily for 5 d, to induce diabetes (11, 12). This protocol causes insulitis and hyperglycemia in ∼75–80% of normal C57BL/KsJ mice 1–2 wk after the final dose of STZ (12). Glucose levels were measured using a glucose analyzer (Beckman Instruments, Inc., Berkeley, CA) in plasma obtained from the retro-orbital sinus on days 12 and 16, corresponding to the 7th and 11th days after the fifth injection of STZ. In one experimental group, STZ-treated islets were placed into the thymus followed by anti-CD3 mAb as described, but the mice were administered 40 × 10⁶ splenocytes freshly isolated from another normal male C57BL/KsJ mouse 3 h before the first of five injections of STZ. The differences between the mean plasma glucose levels of each of the groups were compared by a Student's t test. Differences in the incidence of diabetes between the treatment and control groups were analyzed by Fisher's exact test.

Analysis of Splenocytes by Flow Cytometry. Single cell suspensions of splenocytes were prepared from mice receiving the indicated treatment and stained first with mAb 2.4G2 (17) (antimurine Fc receptor) followed by mAbs against CD3 (mAb 145-2C11; Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN), CD4 (Boehringer Mannheim Diagnostics, Inc.), CD8, and Thy1.2 (Becton Dickinson & Co., Mountainview, CA), which were directly conjugated to FITC. The stained cells were analyzed on a FACScan® (Becton Dickinson & Co.) with electronic gates placed around the peak corresponding to lymphocytes. Data from 10,000 cells were collected. The percent positive cells represent the percentage of cells with fluorescence intensity above staining with a FITC-conjugated irrelevant mAb (anti-Leu4, Becton Dickinson & Co.). The data presented in Table 1 were collected from five to seven mice in each group and represent mean ± SEM. The mean values in the experimental groups were compared with control values by a Student's t test.

Histologic Analysis of Insulitis. The pancreas from animals receiving the indicated treatments (14 from the group receiving intrathymic injection of buffer and anti-CD3 mAb and 7 from the group receiving intrathymic STZ-treated islets and anti-CD3 mAb) were harvested on days 16–18 and placed in formalin solution. Sections of the fixed pancreases, embedded in paraffin were prepared and stained with hematoxylin and eosin. Without knowledge of the source of the section, the presence of insulitis was evaluated by one of us (A. Montag), and graded as follows: 0, normal histology; 1, minimal cellular infiltrate into the islets, otherwise normal islet architecture; 2, extensive cellular infiltrate but preservation of islet architecture; 3, cellular infiltrate and loss of normal islet architecture. The differences in the scores between the two groups were compared by χ^2.

Immunohistochemical Staining for Insulin. At the time of killing, 12 d after the last dose of STZ, the thymuses and pancreases from animals were fixed in Bouin's fixative, and stained with guinea pig antiinsulin antibody using the avidin-biotin peroxidase method (18). Tissue sections were incubated with guinea pig antiinsulin antibody (Dako Corp., Carpenteria, CA) washed, and incubated with biotinylated goat anti–guinea pig antibody (Vector Laboratories, Inc., Burlingame, CA) followed by streptavidin complex (Dako Corp.). After washing, antibody complexes on the sections were localized with diaminobenzidene and counterstained with hematoxylin.

Results

Prevention of MDSDM after Intrathymic Placement of Islet Antigen, T Cell Depletion and Regeneration. Islets isolated from normal male (C57BL/KsJ) mice and treated with STZ in vitro, were placed into the thymuses of syngeneic adult C57BL/KsJ mice. Control mice received an intrathymic injection of Krebs-Hepes buffer. All mice then were given an injection of anti-TCR mAb. This treatment caused depletion of about 70% of T cells in the spleen by 72–96 h, modulation of the TCR on residual Thy1.2⁺ cells (Table 1), and elimination of virtually all T cells in the peripheral circulation. In previously reported studies, the residual T cells in spleen and lymph node have been found to have profound functional impairments (19, 20).

We rested the animals for 3.5 wk to allow for regeneration of T cells, and then analyzed whether the presence of islet antigens in the thymus modulated the course of the au-

Table 1. *Quantitative Analyses of Splenocytes by Flow Cytometry*

Group	CD3[‡]	CD4[‡]	CD8[‡]	Thy1.2[‡]
Intrathymic buffer/anti-CD3 mAb, studied 72–96 h after anti-CD3 mAb	0.8 ± 0.2* (3%)[§]	5.9 ± 1.7* (36%)	2.7 ± 0.6* (28%)	8.6 ± 2.5* (30%)
Animals studied on days 11–13 after STZ:				
Intrathymic buffer/anti-CD3 mAb	13.4 ± 1.8* (51%)	9.6 ± 1.6* (60%)	3.6 ± 0.5* (39%)	15.4 ± 2.3* (51%)
Intrathymic STZ-treated islets/anti-CD3 mAb	13.4 ± 1.1* (51%)	10.1 ± 0.8* (63%)	3.4 ± 0.3* (37%)	17.9 ± 1.1* (59%)
Untreated control mice	26.2 ± 1.0	16.0 ± 0.3	9.3 ± 0.6	30.2 ± 1.9

Splenocytes were harvested from groups of five to seven mice at the indicated times and stained with mAbs as described. The percent positive cells was calculated by comparing the staining to an irrelevant FITC-conjugated mAb (anti-human CD3).
* $p <0.001$ vs control
‡ Mean ± SEM % of 10,000 gated cells.
§ Percent control.

toimmune disease induced by STZ. During this time, splenic T cells had reached 51% of control levels, and TCR modulation was no longer seen (Table 1). Five doses of STZ were given to all of the animals, and the mice were studied for the presence of hyperglycemia and insulitis 7–13 d later. Overall, 76% of mice receiving an injection of Krebs-Hepes buffer and anti-CD3 mAb developed diabetes (plasma glucose >240 mg/deciliter (dl)) whereas only 14% of mice that had received intrathymic STZ-treated islets and anti-CD3 mAb became diabetic ($p <0.001$) (Table 2). The incidence of diabetes in the group receiving intrathymic Krebs-Hepes buffer and anti-CD3 mAb is similar to the frequency we have found previously in unmanipulated mice in this model (12, 21). Similarly, the plasma glucose levels on days 12 and 16 were reduced in mice receiving intrathymic STZ-treated islets and anti-CD3 mAb compared with mice with an intrathymic injection of Krebs-Hepes buffer and anti-CD3 mAb ($p = 0.011$ and $p <0.001$). Thus, exposure of developing T cells to islet

cells or antigen within the thymus protected the animals from subsequent induction of experimental diabetes.

The failure to develop diabetes was not due to insulin production by the intrathymic islet cells (15) that may have been sequestered from the effects of STZ on islets in the periphery. Animals that had received intrathymic STZ-treated islets and were protected from diabetes underwent thymectomy ($n = 6$), and the thymus tissue was studied for the presence of insulin-containing cells by immunohistochemistry. Insulin-containing cells were present in the thymuses of the protected mice, but after the thymectomy, the glucose levels did not increase (Figs. 1 and 2). In addition, the failure to induce MDSDM did not simply reflect general immunosuppression because of the residual effects of the anti-CD3 treatment. Full thickness skin grafts from C3H/HeN mice implanted on mice receiving intrathymic STZ-treated islets and anti-CD3 mAb were rejected at the same rate as grafts transferred to mice receiving an intrathymic injection of Krebs-

Table 2. *Prevention of Diabetes by Intrathymic Injection of STZ-treated Islets*

	Plasma glucose (mg/dl, mean ± SEM)		No. with diabetes*/Total	
Group	Day 12	Day 16	Day 12	Day 16
Intrathymic buffer/anti-CD3 mAb	269 ± 22	316 ± 25	13/21	16/21
Intrathymic STZ-treated islets/anti-CD3 mAb	191 ± 14[‡]	212 ± 15[‖]	2/14[§]	2/14[‖]

* Diabetes was considered present if plasma glucose >240 mg/dl.
‡ $p = 0.011$, § $p <0.01$, and ‖ $p <0.001$ vs. intrathymic saline and anti-CD3 mAb.
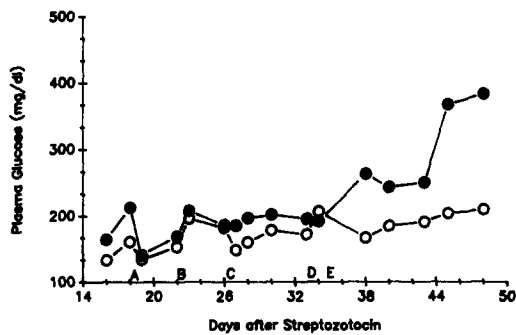
Figure 1. Effects of thymectomy on glucose levels, and adoptive transfer of diabetogenic cells with splenocytes from normal mice. Plasma glucose levels are shown from two mice that received intrathymic STZ-treated islets and anti-CD3 mAb that were studied further. These data are from a single experiment representative of four separate experiments. Both animals received five subdiabetogenic doses of STZ beginning on day 0, and both underwent thymectomy at day 16 (*A*). One animal received an injection (intraperitoneal) of 40 and 80 × 10⁶ splenocytes from normal male C57BL/KsJ mice (● — ●) or saline (O — O) on days 22 and 31 (*B* and *D*). Both animals received a single injection (intraperitoneal) of STZ (40 mg/kg in citrate buffer) on days 27 and 34 (*C* and *E*). Progressive hyperglycemia was seen in the recipient of normal spleen cells.

Hepes buffer and anti-CD3 mAb or to untreated control mice (data not shown).

The absence of hyperglycemia in mice receiving intrathymic STZ-treated islets and anti-CD3 mAb correlated with a decrease in the autoimmune response as indicated by a reduction in cellular infiltrates into the islets (Table 3, Fig. 3). Histologic sections of pancreases from mice given either intrathymic

STZ-treated islets and anti-CD3 mAb, or intrathymic Krebs-Hepes buffer and anti-CD3 mAb were evaluated in a blinded manner. Islets from normal C57BL/KsJ mice do not have insulitis. 61% of islets from animals receiving intrathymic injection of buffer showed evidence of insulitis, similar to that reported by ourselves and others in previous studies of MDSDM (11, 12, 21). Only half as many, 30%, of islets from mice receiving intrathymic injections of STZ-treated islets demonstrated cellular infiltrates (*p* <0.001). Furthermore, none of the islets from the animals injected intrathymically with STZ-treated islets showed severe degrees of insulitis which included loss of normal islet architecture and cellular infiltrates. Probably as a result of the repeated intraperitoneal injections, one animal from the group receiving STZ-treated islets developed exocrine pancreatitis, and 23% of the total islets that had insulitis in the group were from that one animal.

Immunohistochemical staining of the sections with an antiinsulin antibody indicated that a reduction in the number of insulin-containing cells accompanied more severe degrees of insulitis (Fig. 3). These data corroborate our evaluation of blood glucose levels, and indicate that placement of STZ-treated islets into the thymus during a period of T cell regeneration causes tolerance to MDSDM.

Specificity of Tolerance to MDSDM and Requirements for its Induction. Tolerance to diabetes was found to be tissue specific. Diabetes was not prevented when fragments of adrenal gland with or without STZ treatment were placed into the thymus instead of islets (Table 4). In addition, the antigen-bearing cells had to be placed into the thymus for tolerance to occur. Transfer of equal numbers of STZ-treated islets under the capsule of a kidney, followed by anti-CD3 mAb, did not protect
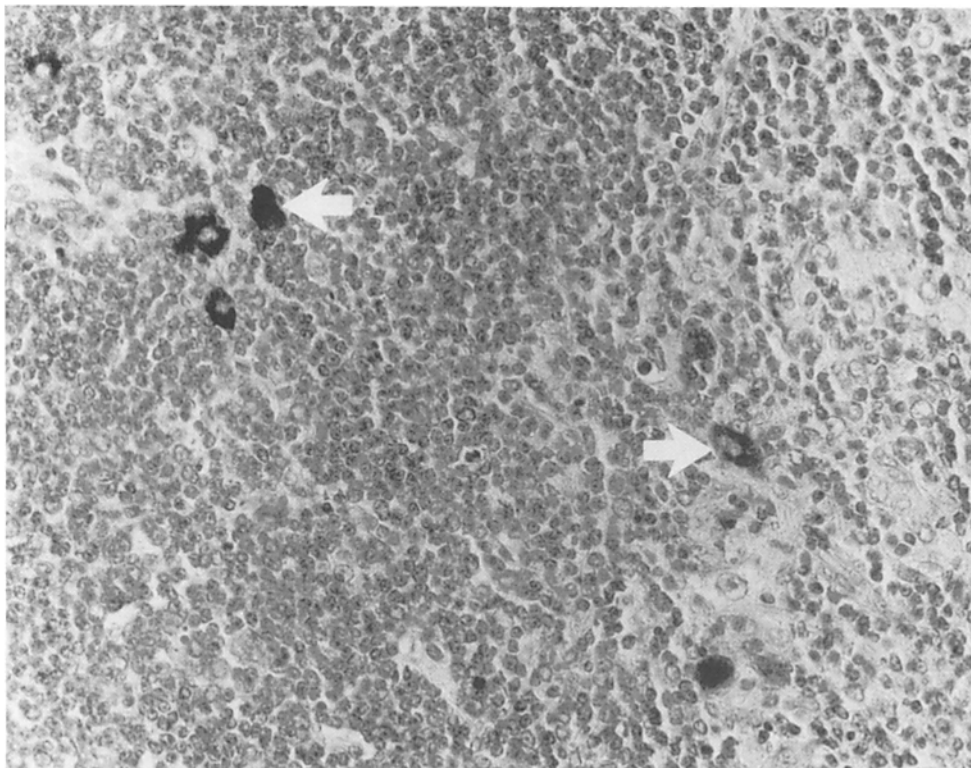

Figure 2. Identification of insulin containing cells in thymus removed from a tolerized mouse. A thymectomy was performed on mice that had received intrathymic STZ-treated islets and anti-CD3 mAb and had not developed diabetes after STZ. The thymus was studied for the presence of insulin-containing cells with immunohistochemical techniques. Single cells and small clumps of insulin containing cells could be identified at the cortical-medullary junction of the thymus (*arrows*). ×200.

Table 3. *Effects of Treatment on the Development of Insulitis*

| | Grade of insulitis* | | | | |
Group	0	1	2	3	Total no. islets
Intrathymic Krebs-Hepes buffer and anti-CD3 mAb (*n* = 14)	50 (39%)	42 (33%)	26 (20%)	10 (8%)	128
Intrathymic STZ-treated islets and anti-CD3 mAb‡ (n = 7)	46 (77%)	14 (23%)	4 (7%)	0	64

Pancreases were fixed in formalin, and sections of tissue (two from each mouse) were stained with hematoxylin and eosin and graded for the presence of insulitis as follows: 0, Normal histology; 1, minimal cellular infiltrate into the islets, otherwise normal islet architecture; 2, extensive cellular infiltrate but preservation of islet architecture; and 3, cellular infiltrate and loss of normal islet architecture. The differences in the scores between the two groups were compared by χ^2.

* Insulitis was absent from the islets of untreated normal male C57BL/KsJ mice.

‡ *p* <0.0001 vs intrathymic Krebs-Hepes buffer and anti-CD3 mAb. The data in this group includes one animal with exocrine pancreatitis. The distribution of lesions without that animal was: 0, 79%, 1, 21%, 2, 0%, and 3, 0%.

against the induction of MDSDM (Table 4). It is interesting that pretreatment of islets with STZ was necessary to render islets effective tolerogens. There was an initial reduction in the plasma glucose levels and incidence of diabetes (*p* <0.05) 12 d after STZ treatment in mice receiving intrathymic injection of islets that were not treated in vitro with STZ. How-

ever, by day 16, diabetes had developed in 80% of the animals, and the blood glucose levels were similar to mice that had received intrathymic injection of Krebs-Hepes buffer (Table 4).

In addition, T cell depletion with anti-CD3 mAb (Table 4), was needed for tolerance to occur. When mice received intrathymic STZ-treated islets but did not receive anti-CD3
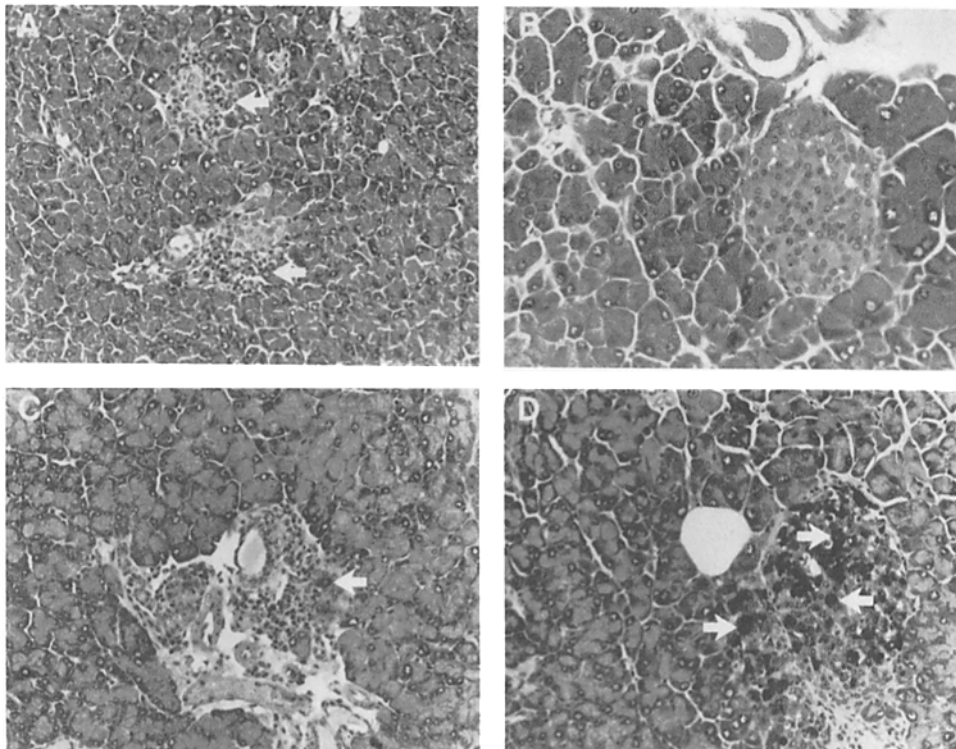

Figure 3. Prevention of insulitis with intrathymic injection of STZ-treated islets and anti-CD3 mAb therapy. 11–13 d after the last dose of STZ, mice were killed, and the pancreases were fixed in formaldehyde and embedded in paraffin. Some sections of the tissue blocks were cut and stained with hematoxylin and eosin (*A* and *B*) and others were studied for the presence of insulin-containing cells with the islets of Langerhans (*C* and *D*) as described. (*A* and *C*) Insulitis (*A*) and staining for insulin (*C*) in islets 12 d after the last dose of STZ in an animal pretreated with intrathymic injection of Krebs-Hepes buffer and anti-CD3 mAb. Lymphocytes (*A*, *arrows*) are found invading the parenchyma of the islet and there is loss of normal architecture. Only one insulin-containing cell can be identified (*C*, *arrow*). (*B* and *D*) Islet from a mouse 12 d after the last dose of STZ that was pretreated with intrathymic injection of STZ-treated islets and anti-CD3 mAb. The islet is free of insulitis (*B*), and many insulin-containing cells can be identified (*D*, *arrows*). (*A*) ×50; (*B*) ×100; (*C* and *D*) ×65.

Table 4. *Protection from MDSDM Requires that STZ-treated Islets be Present in the Thymus during T Cell Development*

Group	Plasma glucose (mg/dl, mean + SEM)		No. with diabetes*/Total	
	Day 12	Day 16	Day 12	Day 16
Intrathymic adrenal fragments/anti-CD3 mAb	275 ± 29	368 ± 11	4/5	5/5
Intrarenal STZ-treated islets/anti-CD3 mAb	280 ± 47	303 ± 41	4/5	4/5
Intrathymic non-STZ-treated islets/ anti-CD3 mAb	224 ± 25	314 ± 40	1/5‡	4/5
Intrathymic STZ-treated islets/ no anti-CD3 mAb	325 ± 66	278 ± 37	3/5	3/5
Intrathymic STZ-treated islets/anti-CD3 mAb, and syngeneic spleen cells before five doses of STZ	272 ± 46	349 ± 36	3/4	4/4

* Diabetes was considered present if plasma glucose >240 mg/dl.
‡ p <0.05 vs. intrathymic saline and anti-CD3 mAb.

mAb, 60% of animals developed diabetes, and their glucose levels were similar to animals receiving Krebs-Hepes buffer and anti-CD3 mAb.

Tolerance to MDSDM Can Be Abrogated by Splenocytes from Normal Mice. To verify that tolerance had resulted from deletion or inactivation of islet antigen-reactive T cells from the repertoire of the mice, we performed two additional experiments. First, mice that had received intrathymic, STZ-treated islets and anti-CD3 mAb received an adoptive transfer of 40 × 10⁶ splenocytes from normal C57BL/KsJ mice on the day of the first injection of STZ (Table 4). All of these mice developed diabetes when given the five doses of STZ, and the glucose levels were similar to those of mice treated with intrathymic Krebs-Hepes buffer and anti-CD3 mAb. Second, after thymectomy (Fig. 1), individual mice protected from diabetes were given syngeneic spleen cells (n = 4) or saline (n = 3), and one or two injections of STZ (40 mg/kg). Mice that received splenocytes and STZ became hyperglycemic, whereas mice that received saline and STZ did not. Thus, susceptibility to MDSDM could be adoptively transferred to tolerized mice with splenocytes from normal animals.

Discussion

We have shown that intrathymic transfer of islets of Langerhans and T cell regeneration can induce T cell tolerance to the development of autoimmune diabetes. Tolerance was specific for islets and was not due to general immunosuppression, since animals receiving the same anti-T cell mAb but without intrathymic islets developed diabetes similar to untreated mice, and animals with intrathymic islets rejected allogeneic skin grafts at a normal rate. Furthermore, the failure to develop hyperglycemia was not due to insulin production by the intrathymic islets that may have been protected from

the autoimmune attack that occurs in the periphery, since thymectomy of the tolerized mice did not result in hyperglycemia. The number of islets placed into the thymuses was relatively small (about 200/mouse), and in other experiments we have been unable to reverse hyperglycemia in mice with toxic diabetes using this number of syngeneic normal islets. Rather, our results indicate that tolerance to autoimmune diabetes was the result of clonal deletion or inactivation of islet-reactive T cells.

The development of diabetes in the MDSDM model has previously been shown to be dependent on T cell responses (11-14, 21). Pretreatment of mice with anti-CD4, anti-CD8 (12), or treatment with anti-CD3 mAbs (21) can prevent induction of disease. In addition, hyperglycemia can be adoptively transferred with splenocytes from a diabetic animal to a recipient treated with a single dose of STZ (13). The events that occur after STZ treatment leading to the induction of diabetes are not clearly understood, and may involve expression of novel antigens on the surfaces of β cells or presentation of constitutive proteins to T cells. Although in our experiments STZ treatment of the islets in vitro was necessary to completely prevent diabetes, there was a small effect of non-STZ treated islets on the natural history of disease as well. These findings raise the possibility that islet-specific T cell antigen may be present in low levels or sequestered on normal β cells. With injury and possibly shedding of antigen, as might occur to some degree during isolation of the islets, or to a greater extent after STZ treatment, these antigens may become more available to the immune system. This concept is consistent with the observation that expression of foreign antigens on the surfaces of β cells does not result in an inflammatory response against islets (8, 9) unless accessory signals are provided. These signals can be provided, for example, by viral infection or possibly cytokines (22).

In the thymus, antigens shed from islets may be presented to developing T cells by APCs that are either bone marrow or islet derived. Although bone marrow-derived cells are thought to be primarily responsible for mediating clonal deletion in the thymus (1, 3, 4), extrathymic APCs have also been shown to be capable of mediating clonal deletion of immature $CD4^+CD8^+$ thymocytes in suspension (23). Thus, the cells that present islet antigen to the developing T cells may be found within the islets themselves, or may be host derived and can process antigens that are shed by STZ-treated islets. If, in fact, shed antigen is presented by thymic cells, this approach for induction of tolerance may not require histocompatibility of the host and antigen-bearing cell, since transplantation of MHC incompatible tissues can be performed easily into the thymus with a single treatment with anti-T cell antibody (15).

Treatment with anti-CD3 mAb was required to induce tolerance, and tolerance did not develop when islets were placed outside of the thymus. Our findings suggest that induction of nonresponsiveness to autoimmune diabetes was due to T cell maturation in the presence of islet antigens which was stimulated by anti-CD3 mAb treatment. A single injection of anti-CD3 mAb has been shown previously to completely remove mature T cells from the peripheral blood (16). The residual ($\sim$30%) T cells in the peripheral organs show profound functional impairments even after TCR reexpression has occurred. The repopultion of the peripheral organs with T cells most likely reflects accelerated maturation of T cell precursors through the thymus and emigration into the peripheral sites. T cells on which the TCR has been modulated as a result of anti-CD3 mAb treatment may reexpress this complex, but evidence from thymectomized animals (16) indicates that T cell repopulation occurs in half the time if a thymus is present. It has previously been shown by Posselt et al. (25) that a reduction in the precursor frequency of alloreactive T cells occurs when T cell development takes place in the presence of allogeneic islets in the thymus (15). While our work was in progress, Koevary and Blomberg (24) and Posselt et al. (25) have reported that autoimmune diabetes may be prevented by intrathymic transplantation of islets into young or neonatal BB/W rats. Thus, our studies and those previously reported support the notion that islet antigen-reactive T cells have been either depleted from the repertoire or inactivated as a consequence of interaction with foreign antigens in the thymus during T cell development. Mature T cells that have developed in a thymus that lacks islet cells abrogate this protective effect. Thus, potentially autoreactive, islet-specific T cells escape thymic deletion in normal C57BL/KsJ mice. Failure to delete these T cells during normal development may reflect the inaccessibility of islet antigens for antigen presentation possibility because the antigens are not shed or are in low concentration.

Clonal deletion or clonal anergy may be the outcome of interaction of T cells with antigen present in the thymic medulla. For example, Hammerling et al. (25) have recently found that T cells reactive with the alloantigen K^b may be rendered nonresponsive after encounter with the alloantigen in the thymic medulla, but do not necessarily undergo apoptosis. Because there is no specific phenotypic marker (i.e., TCR-V region gene) known as yet for the autoreactive T cells in this model, we cannot determine whether islet-reactive T cells have actually been deleted, or whether they are still present but are incapable of causing diabetes. Either clonal anergy or deletion of antigen-reactive T cells is consistent with our observation that responsiveness to MDSDM may be restored with normal spleen cells. However, our findings indicate that induction of regulatory ("suppressor") cells in the periphery is not responsible for the effect we have observed.

When removed from mice 5 wk after surgery, the thymi of mice receiving intrathymic islets still contained insulin-producing cells. The small number of islet cells persisting in the thymus does not likely represent a significant source of insulin, but may be important in maintaining the tolerant state. We do not know how long the tolerant state will persist after thymectomy, but it is likely that continual maintenance of tolerance will require that antigen remain in the thymus to ensure removal of antigen-reactive cells will occur as new T cells develop.

The ability to induce antigen-specific tolerance, especially tolerance towards disease-related antigens, represents an optimal approach for prevention of autoimmune diseases. We have shown that this may be induced in animals with a mature T cell repertoire. With this technique, only T cells specific for disease-relevant antigens are eliminated as a consequence of the manipulation, and other cellular immune functions are left intact. Thus, the ability to eliminate disease-relevant T cells in the absence of global immunosuppression represents a novel approach to disease-specific immunotherapy.

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