BB Rat Thymocytes Cultured in the Presence of Islets Lose Their Ability to Transfer Autoimmune Diabetes

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Thymocytes from adult BB rats can adoptively transfer autoimmune diabetes to athymic recipients. It is also known that the development of BB rat T-cells is recapitulated in adult thymus organ cultures (ATOCs). Based on these observations, we tested the hypothesis that cells capable of the adoptive transfer of diabetes would be present in long-term ATOCs but could be rendered nondiabetogenic by co-culture with appropriate antigens. We observed that cells recovered from adult diabetes-resistant BB (BBDR) rat thymi cultured for up to 14 days can adoptively transfer disease to athymic WAG-*rnu/rnu* rats treated with polyinosinic: polycytidylic acid and a monoclonal antibody to preclude development of ART2a^{+} regulatory T-cells. Coculture of adult BBDR thymi in the presence of BBDR thyrocytes had no effect on the ability of recovered cells to induce diabetes in 70–80% of adoptive recipients. In contrast, co-culture in the presence of islets prevented transfer of diabetes, on average, in >90% of recipients. Fresh islets, frozen islets, and islets pretreated with streptozotocin to deplete insulin were equally effective in preventing diabetes, but none prevented insulitis in nondiabetic recipients. Co-culture in the presence of islets was not associated with detectable alterations in phenotype or in the secretion of γ-interferon or interleukin-4, either in cultures or in cells recovered from adoptive recipients. We conclude that islet antigens involved in the initiation of autoimmune diabetes in BB rats may be absent or deficient in BB rat thymi. Exposure of ATOCs to exogenous islets may lead to deletion or anergy of diabetogenic T-cells or to the positive selection of regulatory T-cells. *Diabetes* 50:972–979, 2001

Type 1 diabetes is a heritable T-cell–mediated disorder characterized by insulitis, an inflammatory infiltration of pancreatic islets, and by the selective destruction of islet β-cells (1). BB rats are used extensively to model this disease. Diabetes-prone BB (DPBB) rats develop spontaneous hyperglycemia that is clearly autoimmune in origin (2,3). They are lymphopenic and severely deficient in cells that express the ART2 (formerly RT6) marker of regulatory T-cells (4). Coisogenic diabetes-resistant BB (BBDR) rats were developed from diabetes-prone forebears selected for normoglycemia. The immune system of BBDR rats is phenotypically normal (2,3). These animals never become diabetic spontaneously but readily develop insulitis, hyperglycemia, and lymphocytic thyroiditis when treated in vivo with an activator of the immune system and depleted of regulatory T-cells that express the ART2 maturational alloantigen (2,3).

Both peripheral lymphoid cells (5) and thymocytes (6) from diabetes-resistant BBDR rats can adoptively transfer insulitis and thyroiditis to histocompatible athymic recipients. Successful transfer generally requires that recipients be treated with the nonspecific immune system activator polyinosinic:polycytidylic acid (poly I:C) and a monoclonal antibody to prevent the emergence of ART2^{+} regulatory T-cells. These and other data suggest that intrathymic events permit the generation of autoreactive effector T-cells in BBDR rats (3).

Attempts to modulate these intrathymic events in the BB rat have yielded conflicting results. “Prophylactic” intrathymic implantation of islet allografts reportedly reduced the frequency of subsequent diabetes in both young BBDP rats (7,8) and NOD mice (9). In contrast, intrathymic islets (both iso- and allografts) neither prevented nor reversed diabetes in ART2-depleted BBDR rats (10). To explain the latter finding in the BBDR rat, it was hypothesized that intrathymic islet grafts survive in BBDP rats because they are lymphopenic and immunocompromised, whereas immunocompetent diabetic BBDR rats successfully recapitulate the autoimmune disease process (10).

Developmental analyses show that diabetogenic T-cells are detectable in thymi from young adult but not neonatal BBDR rats (6). To enhance our ability to study the intrathymic events that are permissive to the development of autoreactive T-cells, we developed an adult thymus organ culture (ATOC) system (11). By day 7 of such a culture, the majority of cells present in BBDR rat thymi upregulate the αβ form of the T-cell receptor (αβTCR) and consist primarily of CD4 and CD8 single positive T-cells. The CD4:CD8 ratio is ∼2:1, approximating the ratio observed in peripheral lymphoid tissues in vivo. Over time, cells generated in BBDR rat ATOC also express de novo the maturational T-cell antigen ART2 and downregulate expression of Thy 1.1, which marks immature T-cells.

Building on these observations, we tested the hypothe-

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APC, antigen-presenting cell; ATOC, adult thymus organ culture; ELISA, enzyme-linked immunosorbent assay; γ-IFN, interferon-γ; IL, interleukin; mAb, monoclonal antibody; PE, R-phycoerythrin; poly I:C, polyinosinic: polycytidylic acid; STZ, streptozotocin.

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sis that cells capable of the adoptive transfer of diabetes would be present in BBDR ATOC and could be rendered nondiabetogenic by co-culture with appropriate antigens for an appropriate length of time. This hypothesis was confirmed, but the exact nature of the preventive antigen and the mechanism by which diabetogenicity was reduced remain to be identified.

RESEARCH DESIGN AND METHODS

Animals. BBDR/Wor/Brm (RT1^u, ART2a$^+$) and histocompatible athymic WAG-*rnu/rnu* (RT1^u) rats were purchased from Biomedical Research Models (Worcester, MA). All rats were certified by the vendor to be free of the Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus, H1 (Toolan's virus), GD7 (mouse poliovirus), Reo-3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, *Encephalitozöon cuniculi*, and pinworm. Rats were housed in a viral antibody–free facility, and monthly testing of sentinel rats was used to assure the absence of infection in experimental animals. All rats were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Preparation of islets and thyrocytes. Pancreatic islets for use in co-culture with thymocytes were isolated by collagenase digestion, handpicked, and counted as described (12). Islets were usually added to ATOC either immediately ("fresh islets") or after freezing at −80°C in the absence of cryopreservatives both to expose intracellular antigens and to destroy passenger antigen-presenting cells. In a preliminary experiment, the viability of a sample of frozen islets was tested by the method of trypan blue exclusion and was 0%. In one experiment, islets were exposed to streptozotocin (STZ) ("STZ islets") to deplete insulin stores before addition to ATOC. STZ treatment of islets was conducted in vivo. Because of disruption of islet architecture, native islets could not be isolated from STZ-treated animals by conventional collagenase digestion. To obtain STZ islets, islets were isolated from normal BBDR rats and placed into semipermeable hollow fibers with a molecular pore size of 80 kDa (Amicon, Bedford, MA). Islets were introduced through a 25-gauge needle into the fibers, which were then heat-sealed and implanted into the peritoneal cavity of athymic WAG-*rnu/rnu* rats. Recipients were then treated with STZ (85 mg/kg; Upjohn, Kalamazoo, MI) in phosphate citrate buffer, pH 4.7. Successful STZ treatment was defined as a plasma glucose concentration >17.8 mmol on 2 successive days (Beckman Glucose Analyzer II; Beckman, Fullerton, CA). Cells were recovered by flushing fibers three times with 10 ml complete ATOC medium (see below). Aliquots of these cells were either used immediately in ATOC or stored at −80°C until assayed for insulin content using a commercial enzyme-linked immunosorbent assay (ELISA) (ALPCO, Windham, NH). The amount of insulin present in STZ islets used in co-culture ranged from 3.9 to 4.7 μg per thymus. For comparison, the amount of insulin present in an identical number of otherwise untreated islets harvested after 3 days of culture ranged from 42.8 to 51.3 μg.

Thyroid tissue was recovered aseptically from BBDR donors by dissection, rinsed with sterile HEPES-RPMI, and digested in 100 PZ units of *Clostridium histolyticum* collagenase (Serva Biochemicals, Heidelberg, Germany) and 7.5 mg trypsin (Sigma, St. Louis, MO) in 10 ml Hanks' balanced salt solution supplemented with 0.1% fetal bovine serum. Thyroids were added in batches of 8–10 to the digestion solution and incubated for 20 min at 37°C and then triturated 10 times. The cycles of incubation and trituration were repeated a total of four times. The resulting cell suspension was filtered through a 70-μm nylon cell strainer to remove debris and washed three times in ATOC complete medium. The cells were counted, and cell pellets were stored at −80°C until use. The average yield of thyroid cells was 1.0×10^6 per donor.

ATOCs and co-culture procedures. ATOCs were established from 8- to 10-week-old BBDR rat thymi as described (11). Briefly, thymi were cut into 1-mm^3 fragments and cultured on filters placed on top of Gelfoam sponges (Upjohn, Kalamazoo, MI) in Dulbecco's modified Eagle's high-glucose medium supplemented with 20% fetal bovine serum (Hyclone, Logan, VT). Cultures were incubated at 37°C for 3–14 days in an atmosphere of 7% CO_2. Cultures maintained for longer than 7 days were fed by adding 5 ml media to the cultures on day 7. ATOCs were harvested using 10 ml collagenase solution per 6–8 ATOC filters (11).

In certain experiments, thymic fragments in the ATOC were co-cultured with syngeneic islets or thyrocytes from BBDR rat donors. Islet or thyroid cells were deposited directly on the surface of each individual thymus fragment. Cells were applied to each fragment twice at an interval of several minutes to optimize cell contact (0.25 μl per application). A total of 1,500–1,800 islets or 3×10^6 thyrocytes per thymus were applied. This number of islets is comparable to the number of intrathymic islets reported to prevent spontaneous BBDP rat diabetes in vivo (10). Typically, to generate cells for adoptive transfer, three thymi would be processed at one time for each treatment group, and the cells generated at the end of the culture were pooled. No attempt was made to remove islets or thyrocytes from ATOC at the conclusion of culture.

Adoptive transfer. Cells harvested from ATOC were washed three times in HEPES-buffered RPMI, counted, and transfused intravenously via the tail vein into WAG-*rnu/rnu* recipients in a final volume of 1 ml. The number of cells transferred to each recipient is given in the results for each experiment and in all cases represented the yield of 0.25–0.75 thymi. Recipient rats were injected intraperitoneally with anti-ART2a monoclonal antibody (mAb) (1 ml of 2× culture supernatant, five times weekly) and poly I:C. The rat anti-rat ART2a mAb was produced by culture of the DS4.23 hybridoma and was tested to document the absence of Mycoplasma contamination. Poly I:C was purchased from Sigma, dissolved in Dulbecco's phosphate-buffered saline (1 mg/ml), sterile filtered, and stored at −20°C until used. The dose of poly I:C in early experiments (Fig. 1) was 0.5 mg/100 g given three times weekly, but it was subsequently learned that lower doses of poly I:C could be used. In later experiments (Fig. 2), the dose of poly I:C was 0.025 mg/100 g three times weekly.

Flow cytometry. Flow microfluorometry was used to quantify the expression of surface markers on cells freshly harvested from ATOC and on recipient spleen and lymph node cells as described (6). Antibodies to the αβ T-cell receptor (αβ TcR, clone R73), interleukin (IL)-2 receptor α-chain (IL-2R, clone OX-39), CD4 (clone OX-35), CD8α chain (clone OX-8), transferrin receptor (clone OX-26), CD134 (clone OX-40), CD45RC (clone OX22), appropriate isotype control antibodies (mouse IgG1, mouse IgG2a, and rat IgG2b), and R-phycoerythrin (PE)- or CyChrome-conjugated streptavidin were purchased from Pharmingen (San Diego, CA). Anti-ART2a (clone DS4.23) was purified using Protein G Plus agarose (Calbiochem, La Jolla, CA). Anti-ART2a was biotin-conjugated using BIOTIN-X-NHS (Calbiochem). Antibodies were used either directly conjugated with fluorochromes (fluorescein isothyacyanate [FITC], PE, or CyChrome) or were used as biotin conjugates followed by PE- or CyChrome streptavidin. Samples were fixed in a final concentration of 1% paraformaldehyde in phosphate-buffered saline and analyzed using a FACScan. A minimum of 5,000 viable cells in each sample was analyzed. The lymphocyte fraction was gated on the basis of forward and side scatter.

Histopathology of pancreata from recipients of ATOC. Pancreas and thyroid samples for histology were fixed in Bouin's solution or in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Insulitis was scored as follows: 0, no infiltration; 1+, peri-islet infiltration only; 2+, infiltration of some but not all islets; and 3+, infiltration of most islets. Thyroiditis was scored as follows: 0, no infiltration; 1+, minimal focal infiltration; 2+, moderate infiltration; and 3+, widespread dense infiltration. Scoring was performed by a qualified pathologist (M.C.A.) unaware of the treatment status of the animals.

Elispot and ELISAs. Elispot assays to enumerate the frequency of cells secreting γ-interferon (IFN-γ) and IL-4 were performed as described (13). For IL-4, the capture antibody was OX81 (anti–IL-4; Pharmingen), and the detecting antibody was biotin-conjugated B11–3 (anti–IL-4). For IFN-γ, the capture antibody was DB.1 (anti–IFN-γ), and the detection reagent was biotin-conjugated polyclonal rabbit anti–IFN-γ (Torrey Pines, La Jolla, CA). Antibodies were used at a concentration of 1 μg/well. Serial dilutions of cells were activated for 2 days in 96-well plates with concanavalin A (5 μg/ml) in HEPES-RPMI supplemented with 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/l glutamine, 5×10^{-5} mol/l 2-mercaptoethanol (2-ME), and 10% fetal bovine serum (HyClone). Cells were then lysed with distilled water, incubated serially with the detecting antibody followed by streptavidin-alkaline phosphatase, overlaid with 0.6% agarose (Sigma), and incubated overnight at 37°C. The number of spots present in each well was counted using a Nikon E600 microscope, and results are expressed as the number of spots per 1 or 2×10^5 cells.

The concentrations of IFN-γ and IL-4 present in ATOC supernatants were measured using commercial kits (BioSource International, Camarillo, CA). In some experiments, suspensions of spleen and lymph node cells recovered from WAG-*rnu/rnu* recipients of cells recovered from ATOC were activated in vitro with concanavalin A (5 μg/ml) for 40 h, and the concentrations of IFN-γ and IL-4 present in the culture supernatants were measured in the same way.

Statistical analysis. Average latency to onset of diabetes is presented as the median. Statistical comparisons of diabetes-free survival in adoptive recipients were performed using the method of Kaplan and Meier (14). The equality of nondiabetic survival distributions for animals in different treatment groups was tested using the log-rank statistic (15). Parametric data are shown as arithmetic means ± 1 SD. Groups of three or more means were compared using either univariate general linear model analysis or one-way analysis of

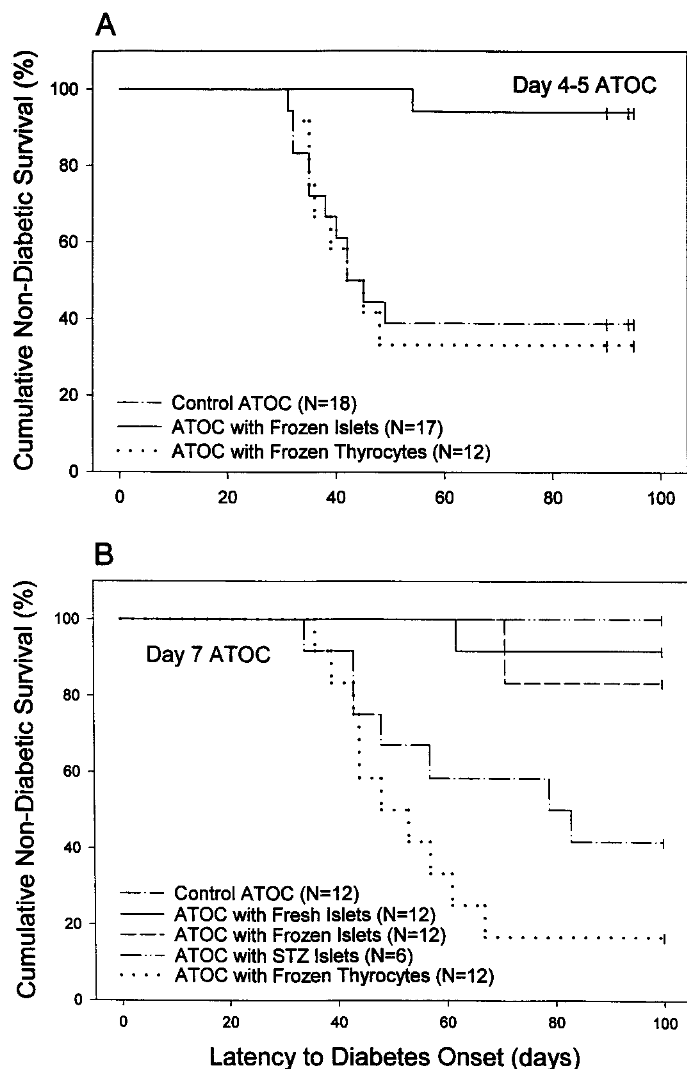

FIG. 1. Adoptive transfer of diabetes by fresh and cultured thymocytes. WAG-*rnu/rnu* rats were randomized, injected with either fresh or cultured BBDR thymocytes, and then treated with a combination of anti-ART2a mAb and poly I:C as described in RESEARCH DESIGN AND METHODS. *A*: Recipients in group A received the equivalent of one-fourth of a thymus, 2.0–2.5 × 10^8 cells. Recipients in group B received the equivalent of either one-fourth or one-half of a thymus (50–132 × 10^6 cells) cultured for 3–4 days. In a preliminary analysis, it was determined that there were no statistically significant differences between the outcomes obtained using one-fourth or one-half of a thymus, and the data for these two treatment conditions have been combined. Recipients in group C received the equivalent of either one-fourth or one-half of a thymus (10–52 × 10^6 cells) cultured for either 5–7 days. In a preliminary analysis, it was determined that there were no statistically significant differences between the outcomes obtained using one-fourth or one-half of a thymus, and the data for these two treatment conditions have been combined. Recipients in group D received cells from the equivalent of one-fourth of a thymus (5–7 × 10^6 cells) cultured for 9–10 days. Each curve represents the results of two independent trials. The cumulative frequency of diabetes was statistically similar in the recipients of fresh thymocytes (group A) and thymocytes after 3–4 days of culture (group B). The cumulative frequency of diabetes in the recipients of fresh thymocytes (group A) was significantly greater than that in recipients of cells from either 5- to 7-day ($P < 0.005$) or 9- to 10-day ($P < 0.01$) cultures. *B*: Recipients in groups E and F received the equivalent of either one-fourth (5 × 10^6 cells) or three-fourths of a thymus (15 × 10^6 cells) that had been cultured for 13–14 days. The curve for group E represents the pooled results of two independent trials; the curve for group F represents the results of a single trial. The cumulative frequency of diabetes in the two groups differs at the $P < 0.001$ level. Numbers of recipients are given in parentheses. Censored data (animals not diabetic at the time of death or termination of the experiment) are indicated by vertical bars.

variance and the Scheffe procedure for a posteriori contrasts (16). P values <0.05 were considered statistically significant.

RESULTS

Cultured BBDR thymocytes adoptively transfer diabetes. Thymocytes from adult BB rats can adoptively transfer autoimmune diabetes to WAG-*rnu/rnu* recipients (6). It has been also reported that the development of BB rat T-cells is recapitulated in adult thymus organ cultures (11). Based on these observations, we first tested the hypothesis that cells capable of the adoptive transfer of diabetes would be present in long-term ATOC. As shown in Fig. 1*A*, we confirmed that transfer of fresh thymocytes present in one-fourth of an adult BBDR rat thymus induces diabetes in ~80% of recipients with a median time to diabetes onset of 40 days. In addition, we observed that cells derived from the culture of one-fourth to one-half of an adult thymus are also capable of transferring diabetes, but the efficiency of diabetes transfer declined with extended culture (Fig. 1*A*). The reduced efficiency of transfer as a function of the duration of culture could be attributable to either the change in phenotype that occurs in ATOC over time or to the exponential decline in the number of viable cells present in ATOC (11). To investigate the effect of cell dose independent of changes in phenotype, we compared the ability of cells derived from the culture of one-fourth and three-fourths of an adult thymus to transfer diabetes. As shown in Fig. 1*B*, cells derived from one-fourth thymus cultured for 13–14 days were unable to transfer diabetes, whereas cells derived from three-fourths thymus cultured for 14 days transferred diabetes to ~70% of recipients with a median latency to onset of 72 days.

As expected (11), cells recovered from BBDR rat ATOC exhibited a mature phenotype by day 6 and maintained that phenotype thereafter. The phenotype of cells recovered from a representative 14-day culture was as follows: $CD4^+$, 53%; $CD8^+$, 31%; $CD4^+$ $CD8^+$, 11%; $\alpha\beta TCR^{hi}$, 95%; and TCR^+ IL-$2R\alpha^+$, 24%. In addition, 38% expressed de novo the $ART2a^+$ rat maturational T-cell alloantigen, which is normally absent in the thymus. The CD4:CD8 ratio of cells recovered after 6 or more days of culture approximated the 2:1 ratio characteristic of rat peripheral lymphoid tissues.

Culture of BBDR thymocytes in the presence of islet but not thyroid antigens reduces their ability to induce diabetes but not insulitis in adoptive recipients. Having determined that cells capable of the adoptive transfer of diabetes are present in cultured BBDR rat thymi, we next tested the hypothesis that exposure of cultured thymi to an appropriate antigen would reduce the diabetogenicity of recovered cell populations. We used ATOC carried out for 4–7 days based on our observation (Fig. 1) that cells cultured for 3–7 days transfer diabetes to about half of recipients.

As shown in Fig. 2*A*, we first observed that culture of BBDR rat thymocytes in the presence of frozen thyrocytes had no effect on the ability of the cultured cells to transfer diabetes. About two-thirds of recipients given thymocytes cultured in the presence or absence of thyroid tissue became diabetic with a median latency to disease onset of 42 days in both cases. In contrast, the frequency of diabetes in recipients of thymocytes cultured for 4–5 days

in the presence of frozen islet tissue was dramatically reduced. Only 1 of 17 recipients of such cells developed disease.

In a second experiment, we repeated this analysis using cells cultured for a slightly longer period of time (7 days). As in the previous experiment, the starting inoculum in the ATOC was one-half thymus, but because of the increased duration of culture, the actual number of cells surviving to the time of transfer was smaller. We also compared the efficacy of frozen, fresh, and STZ-exposed islets to alter the diabetogenicity of cultured BBDR rat thymocytes and performed histological analyses. As shown in Fig. 2*B*, we confirmed that the addition of thyrocytes to BBDR rat ATOC does not affect the ability of these cells to transfer diabetes. We also observed that the addition of any of the three forms of islet tissue to the cultures was associated with a reduction in the ability of cultured thymocytes to transfer diabetes. The three islet–co-culture groups were statistically similar to each other, and when considered as a group, they were significantly different from both the control and thyrocyte co-culture groups ($P < 0.001$ in both cases).

Histological analyses were performed on the pancreata of nondiabetic recipient rats at the conclusion of the experiment. The frequency of insulitis of any intensity (1+, 2+, or 3+) in nondiabetic recipients of thymocytes cultured in the presence of fresh (7 of 11), frozen (2 of 5), and STZ-treated (4 of 5) islets was statistically similar. Overall, some degree of insulitis was present in 62% of these 21 pancreata. In the case of pancreata from nondiabetic recipients of cells from control thymocyte cultures, four of five (80%) displayed insulitis. Very few recipients of cells from cultures performed in the presence of thyrocytes remained nondiabetic; the two pancreata that were available from such animals did not show insulitis. The average insulitis score for all animals in the experiment was 1.8 ± 0.9, and there were no statistically significant differences among groups with respect to the intensity of insulitis.

Histological analyses were also performed on 46 thyroid specimens obtained from both diabetic and nondiabetic recipients of cells recovered from ATOC. Overall, the frequency of thyroiditis was low (28%, 13 of 46). The frequency of thyroiditis in recipients of cells from control ATOC was 25% (3 of 12), and the mean intensity score was 0.6. The frequency of thyroiditis in recipients of cells from ATOC performed in the presence of any type of islet tissue was 33% (8 of 24), with a mean intensity score of 0.6. The frequency of thyroiditis in recipients of cells from ATOC performed in the presence of isolated thyrocytes was 20% (2 of 10), with a mean intensity score of 0.4. There were no statistically significant differences among these groups with respect to either the frequency or intensity of thyroiditis. Because nondiabetic recipients were on average observed for a longer period of time than diabetic recipients before recovery of thyroid specimens, this subgroup was reanalyzed separately, but again, there were no differences among groups with respect to the frequency or intensity of thyroiditis.

Addition of islet or thyroid tissue to BBDR rat ATOC does not produce detectable alterations in T-cell phenotype or in the number of cells secreting IFN-γ or IL-4 after 5 days of culture. In an effort to determine

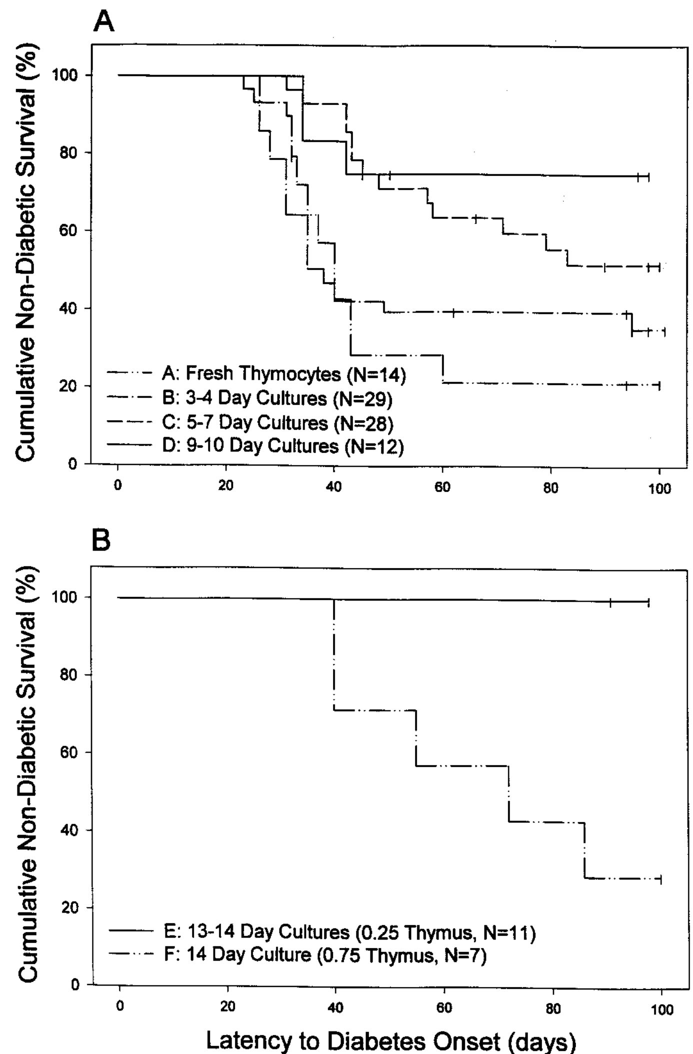

FIG. 2. Adoptive transfer of diabetes by thymocytes cultured in the presence or absence of antigen. WAG-*rnu/rnu* rats were randomized, injected with either fresh or cultured BBDR thymocytes, and then treated with a combination of anti-ART2a mAb and poly I:C as described in RESEARCH DESIGN AND METHODS. Recipients were injected with thymocytes cultured in the presence or absence of BBDR rat pancreatic islets or BBDR rat thyrocytes. Islets and thyroids were prepared as described in RESEARCH DESIGN AND METHODS. *A*: Cells were transfused into recipients after 4 or 5 days of culture. Each recipient received the equivalent of one-half of a cultured thymus. The number of cells transfused was 72–133 × 10^6 for day 4 cultures and 52–67 × 10^6 for day 5 cultures. In a preliminary analysis, it was determined that there were no statistically significant differences between the outcomes obtained using day 4 and day 5 cultures for any of the three experimental groups, and the data for day 4 and 5 cultures are combined. Each curve represents the results of three independent trials. The cumulative frequency of diabetes in recipients of thymocytes cultured in the presence of islets is significantly less than that in recipients of control thymocytes ($P <$ 0.005) or thyrocytes ($P < 0.001$). Frequency of diabetes in recipients of thymocytes cultured in the presence of thyrocytes is statistically similar to that in recipients of control thymocytes ($P = 0.8$). *B*: Cells were transfused into recipients after 7 days of culture. Each recipient received the equivalent of one-half of a cultured thymus. The number of cells transfused was 26–31 × 10^6. Each curve represents the results of two independent trials. The cumulative frequency of diabetes in recipients of cells cultured in the presence of either fresh, frozen, or STZ-treated islets is statistically similar. The cumulative frequency of diabetes in recipients of cells cultured in the presence of islets is significantly less than that in recipients of either control or thyrocyte-treated ATOC ($P < 0.001$ for both comparisons). Frequency of diabetes in recipients of thymocytes cultured in the presence of thyrocytes is statistically similar to that in recipients of control thymocytes ($P =$ 0.15). In addition, the frequency of diabetes in recipients of thymocytes cultured in the presence of thyrocytes in both *A* and *B* is statistically similar ($P = 0.98$). Censored data (animals not diabetic at the time of death or termination of the experiment) are indicated by vertical bars.

TABLE 1
Phenotype and cytokine production of BBDR thymocytes after 5 days of culture

	Cells added to ATOC		
	None	Frozen islets	Frozen thyrocytes
Phenotype (%)			
$CD4^+CD8^+$	11.4 ± 1.5%	10.9 ± 1.2%	9.5 ± 0.9%
$CD4^+$	57.5 ± 3.1%	60.1 ± 1.9%	59.8 ± 1.9%
$CD8^+$	27.5 ± 2.4%	24.7 ± 2.0%	26.5 ± 1.8%
$ART2a^+$	47.8 ± 2.6%	48.6 ± 1.7%	48.0 ± 2.4%
Transferrin receptor	21.0 ± 3.4%	23.0 ± 4.7%	19.7 ± 2.7%
$IL\text{-}2R^+$ ($CD25^+$)	18.9 ± 3.0%	20.4 ± 4.2%	18.0 ± 1.6%
$Elispot^+$ cell number/ 2×10^5 cells			
IFN-γ	49 ± 12	32 ± 7	36 ± 7
IL-4	50 ± 22	65 ± 22	37 ± 18

Data are means ± SD of six independent assays. Adult BBDR rat thymocytes were cultured in the presence or absence of islets and thyrocytes as indicated. Phenotyping data are expressed as the percentage of $\alpha\beta TCR^+$ cells present in the lymphocyte gate. Elispot data represent the number of cytokine-expressing cells per 2×10^5 cells in the cultures. There are no statistically significant differences among the three ATOC groups for any of the measured variables.

the mechanism underlying these observations, we measured the in vitro effect of co-culture with frozen islets or thyrocytes on the phenotype of BBDR rat thymocytes and their production of IL-4 and IFN-γ after 5 days of ATOC. As shown in Table 1, there were no statistically significant differences in the percentage of $\alpha\beta TcR^+$ cells expressing both CD4 and CD8, CD4 alone, CD8 alone, ART2a, transferrin receptor, or the IL-2 receptor α-chain (IL-2Rα, CD25). We also assessed the production of IFN-γ and IL-4 by these same cells. After 5 days of culture, we measured the frequency of cytokine-secreting cells using a sensitive elispot assay. We found no significant differences in the frequencies of IL-4– or IFN-γ–producing cells between the three treatment groups.

Addition of islet or thyroid tissue to BBDR rat ATOC does not produce detectable alterations in T-cell phenotype or in the number of cells secreting IFN-γ or IL-4 in adoptive recipients. We next analyzed WAG-*rnu/rnu* recipients of cultured BBDR rat thymocytes with respect to the in vivo phenotype of peripheral lymphoid cells and their production of IL-4 and IFN-γ. These animals had received thymocytes that had been cultured for 5 days in the presence or absence of either islets or thyrocytes. Lymph node and spleen cells were recovered either at the time of diabetes onset or at the conclusion of the experiment.

As shown in Table 2, there were no statistically significant differences in the percentage of CD4 single positive, CD8 single positive, or $IL\text{-}2R\alpha^+$ ($CD25^+$) cells present in nondiabetic recipients at the conclusion of the experiment. The percentage of $CD4^+CD8^+$ double positive cells in all recipients was comparably low in all groups, and the

TABLE 2
Phenotype and cytokine production of rat lymphoid cells recovered from WAG-*rnu/rnu* adoptive recipients of cultured BBDR rat thymocytes

	Cells added to ATOC		
	None	Frozen islets	Frozen thyrocytes
Phenotype (% of $\alpha\beta TCR^+$ cells)			
$CD4^+$			
Spleen cells	73, 85 (2)	81 ± 2 (5)	82 ± 2 (3)
Lymph node cells	—	84 ± 6 (5)	83 ± 4 (3)
$CD8^+$			
Spleen cells	10, 7 (2)	8 ± 2 (5)	7 ± 1 (3)
Lymph node cells	—	23 ± 2 (5)	27 ± 2 (3)
$IL\text{-}2R^+$ ($CD25^+$)			
Spleen cells	21, 31 (2)	24 ± 3 (5)	22 ± 2 (3)
Lymph node cells	—	23 ± 2 (5)	27 ± 2 (3)
Cytokine production (pg/ml)			
IFN-γ (lymph node cells)			
Nondiabetic	—	1,288 ± 514 (3)	795, 4,531 (2)
Diabetic	3,558 ± 871 (4)	—	2,935 ± 700 (3)
IFN-γ (spleen cells)			
Nondiabetic	866, 1,056 (2)	1,143 ± 502 (3)	679 ± 481 (3)
Diabetic	1,649 ± 1,221 (4)	—	1,714 ± 595 (3)
$Elispot^+$ cell number/ 1×10^5 spleen cells			
IFN-γ			
Nondiabetic	135, 175 (2)	186 ± 45 (5)	129 ± 75 (3)
Diabetic	189 ± 95 (4)	—	277 ± 58 (3)
IL-4			
Nondiabetic	63, 160 (2)	115 ± 47 (5)	67 ± 47 (3)
Diabetic	87 ± 32 (4)	—	97 ± 65 (3)

Data are means ± SD or raw data in cases where $n = 2$. Adult BBDR rat thymocytes were cultured for 5 days in the presence or absence of islets and thyrocytes as indicated, and recovered cells were then transfused into athymic WAG-*rnu/rnu* recipients. Lymph node and spleen cells were recovered from diabetic rats at the time of disease onset or from nondiabetic rats at the conclusion of the experiment. Phenotyping data are expressed as the percentage of $\alpha\beta TCR^+$ cells present in the lymphocyte gate. Elispot data represent the number of cytokine-expressing cells per 1×10^5 cells in the cultures. The number of recipients analyzed is given in parentheses. There are no statistically significant differences among the three ATOC groups for any of the measured variables.

percentages of $CD45RC^+$ and $OX40^+$ spleen and lymph node cells were statistically similar in all treatment groups (data not shown).

We also assessed the production of IFN-γ and IL-4 cells obtained from both diabetic and nondiabetic recipients in two ways. We measured the production of IFN-γ in spleen and lymph node cells by ELISA. We measured the frequency of IFN-γ– and IL-4–secreting spleen cells using a sensitive elispot assay. As shown in Table 2, there were no detectable differences in cytokine production or in the frequency of cytokine-secreting cells, either as a function of the type of cells transfused into the recipients or the presence or absence of diabetes.

DISCUSSION

These data document that, like fresh BBDR rat thymocytes (6), cells recovered from BBDR rat ATOC retain the ability to adoptively transfer diabetes and thyroiditis to histocompatible athymic recipients. In one respect, the finding is not surprising because the phenotype of T-cells generated in BBDR rat ATOC is similar to that of mature BBDR rat peripheral T-cells (11). In both the native BBDR rat and the athymic WAG-*rnu/rnu* recipient, the presence of these T-cells leads to diabetes, provided that the immune system is activated (e.g., by poly I:C) and populations of regulatory T-cells expressing the ART2 antigen are absent (3). In another respect, however, the observation is surprising in that the number of viable cells present in adult rat ATOC declines exponentially (11), and after 14 days of culture, only 1–2% of the cells originally present are viable (11). Nonetheless, diabetogenic cells remain recoverable throughout this time period.

These characteristics of BBDR ATOC—a stable but small population of diabetogenic cells that survive "intrathymically" in culture—make the ATOC a potentially powerful tool for the identification of autoreactive cell populations and analysis of the mechanisms underlying their development. In particular, over the interval between 3 and 7 days of culture, diabetogenic cells survived, were readily recoverable, and actually appeared to comprise a progressively larger percentage of surviving cells. As a first step toward exploiting this tool, we demonstrated that the diabetogenic potential of cells present in BBDR rat ATOC can be modified in vitro. The data show clearly that co-culture of thymic fragments in the presence of syngeneic islets, but not thyrocytes, almost completely prevents the adoptive transfer of diabetes. The observations are consistent with the hypothesis that thymocytes with diabetogenic potential differentiate in culture and that the presence of an appropriate antigen can modify the course of this process and reduce diabetogenicity.

A large number of islet constituents have been proposed as the candidate autoantigen responsible for the tissue-specific autoimmune process that leads to type 1 diabetes (1). Our observations suggest that defective intrathymic processing of one or more islet components may contribute to the development of autoimmunity (17). They are also consistent with previous observations that the prophylactic implantation of islet allografts intrathymically reduces the frequency of subsequent diabetes in young BBDP rats (7,8) and NOD mice (9).

We observed that fresh, frozen, and STZ-exposed islets were comparably effective in reducing the diabetogenicity of thymocytes present in BBDR rat ATOC. The effectiveness of fresh islets is consistent with a role for both insulin and islet surface antigens in this process. Fresh islets also supplied "passenger" antigen-presenting cells (APCs) to the co-cultures, and it could be argued that the presence of these cells was also important. The data generated using a frozen islet preparation devoid of viable cells argue against a role for metabolically active APCs in this process, but the possibility of indirect presentation of islet antigens by thymic APCs has not been excluded.

Our experiments using STZ-exposed islets were intended to investigate further the role of one of the principal candidate autoantigens, insulin. Extensive literature suggests that, despite its presence in the circulation and expression in the thymus, defects in expression, recognition, processing, or affinity intrathymically may, in susceptible animals, lead to loss of tolerance to insulin and the β-cells that produce it (1,18–23). Analyses of anti-insulin autoantibodies, insulin-reactive T-cells, and intrathymic insulin expression have implicated this protein as a potentially important autoantigen in both NOD mice and humans with type 1 diabetes (1,18–27). The potential importance of insulin as an autoantigen has also received support from studies demonstrating that insulin administration can prevent diabetes in NOD mice and possibly in humans (1,24,25). In our experiments, exposure to STZ reduced the insulin content of the islets used in co-culture by >90%. Nonetheless, STZ-exposed islets retained the ability to modify the cells generated in ATOC and to reduce their diabetogenicity. Because of the small residual amount of insulin remaining in these co-cultures, we cannot definitively exclude a role for insulin in reducing thymocyte diabetogenicity. The presence of even a small amount of insulin could in theory modulate the diabetogenicity of T-cells. We would point out, however, that other data do suggest that insulin may not be as strong a candidate autoantigen in the BB rat as in NOD mice or humans. It appears that parenteral insulin prevents diabetes in BBDR rats only at doses that produce persistent hypoglycemia and a state of "β-cell rest," and even in protected animals, autoreactive T-cells are still present (28). Similarly, neither oral insulin (29) nor intrathymic insulin B-chain peptide 9-23 (30) prevents diabetes in BB rats. In contrast, the intrathymic injection of 200 μg insulin β-chain ameliorates (31), and the systemic (including intrathymic) expression of proinsulin II (19) completely prevents, the expression of diabetes in the NOD mouse.

It has been reported that the in vivo implantation of intrathymic islets into young BBDR rats does not preclude the subsequent induction of diabetes in these animals by poly I:C and anti-ART2 mAb (10). This in vivo observation would seem to predict that the in vitro intrathymic modification of diabetogenicity should not be possible. The reason for the apparent discrepancy between the two data sets is not clear. Speculatively, it could be argued that the presentation of antigen in ATOC, dispersed widely across thymic fragments, might have been more efficient than that achieved by the method of bilateral injection into thymic lobes in vivo. Alternatively, it can be pointed out that the in vivo study was not performed in a viral antibody–free environment (10). Viral infection—e.g., with Kilham rat

virus (3)—could have prevented or circumvented the effect of the intrathymic islet antigen in the face of intercurrent regulatory cell depletion.

In the present study, we observed that co-culture in the presence of islets markedly reduced the ability of cultured BBDR thymocytes to transfer diabetes but eliminated insulitis in only ~40% of the animals that remained normoglycemic. Why ~60% of animals had nonprogressive insulitis cannot be determined from the available data, but the simplest explanation is that, because of the geometry of layering islets onto thymic fragments, a limited number of thymocytes in the ATOC was not adequately exposed to islet antigen. Speculatively, this limited cell population retained the ability to recognize and home to islets but was incapable of, or prevented from, completing the process of islet destruction. We have previously documented in studies of BB rat T-cells that the number of cells injected is a critical determinant of diabetogenicity (32). Alternatively, co-culture with islets may have affected the generation of a population of cells required to transform nonprogressive (or benign) insulitis to destructive (or malignant) insulitis (33). In the BDC2.5 TCR transgenic mouse, it was clearly shown that signals provided by IL-18, IL-12, and tumor necrosis factor-α are required for the development of diabetes in animals with otherwise nonprogressive insulitis (34).

We performed several analyses to attempt to define the mechanism by which islet co-culture altered the behavior of BBDR rat ATOC, examining both the cells generated in culture and the cells recovered from diabetic and nondiabetic recipients. One candidate mechanism studied was immune deviation, but we observed no differences in phenotype or the production of IFN-γ or IL-4 that were consistent with this mechanism. Other potential mechanisms include deletion and the generation of anergy. There was no detectable difference in the number of cells generated by BBDR rat ATOC in the presence or absence of islets, but the fact that islet co-culture prevented diabetes but not insulitis is compatible with either a partial reduction in the number of diabetogenic cells or the induction of anergy in some but not all of those cells. A final possible mechanism not addressed in the present study is positive selection of islet-reactive ART2^{-} regulatory cells. Such cells could in theory home to the pancreas and, in the form of "protective insulitis," prevent diabetes in the adoptive recipients of cells from ATOC.

The data on lymphocytic thyroiditis generated in these experiments are intriguing but inconclusive. Co-culture of BBDR thymocytes with thyroid cells did not reduce the frequency of thyroiditis in adoptive recipients, but overall the frequency of thyroiditis was low (~30%). Because lymphocytic thyroiditis in BB rats rarely, if ever, progresses to overt hypothyroidism (2,3), it is impossible to know from these limited data if anti-thyroid autoreactivity was modified.

In summary, we have demonstrated that it is possible to modify in vitro the generation of diabetogenic thymocytes that are genetically capable of inducing autoimmune diabetes. Given the expanding literature documenting the role of intrathymic self-antigen expression in the generation of self-tolerance (35,36), we propose that defective or deficient intrathymic expression of a relevant autoantigen present in pancreatic islets in BBDR rats is crucial to the development of autoimmune diabetes.

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REFERENCES

1. Abiru N, Eisenbarth GS: Multiple genes/multiple autoantigens role in type 1 diabetes. *Clin Rev Allergy Immunol* 18:27–40, 2000
2. Mordes JP, Bortell R, Doukas J, Rigby MR, Whalen BJ, Zipris D, Greiner DL, Rossini AA: The BB/Wor rat and the balance hypothesis of autoimmunity. *Diabetes Metab Rev* 2:103–109, 1996
3. Mordes JP, Bortell R, Groen H, Guberski DL, Rossini AA, Greiner DL: Autoimmune diabetes mellitus in the BB rat. In *Animal Models of Diabetes: A Primer*. Sima AAF, Shafrir E, Eds. Amsterdam, Harwood Academic, 2001, p. 1–41
4. Bortell R, Kanaitsuka T, Stevens LA, Moss J, Mordes JP, Rossini AA, Greiner DL: The RT6 (Art2) family of ADP-ribosyltransferases in rat and mouse. *Mol Cell Biochem* 193:61–68, 1999
5. McKeever U, Mordes JP, Greiner DL, Appel MC, Rozing J, Handler ES, Rossini AA: Adoptive transfer of autoimmune diabetes and thyroiditis to athymic rats. *Proc Natl Acad Sci U S A* 87:7718–7722, 1990
6. Whalen BJ, Rossini AA, Mordes JP, Greiner DL: DR-BB rat thymus contains thymocyte populations predisposed to autoreactivity. *Diabetes* 44:963–967, 1995
7. Posselt AM, Barker CF, Friedman AL, Naji A: Prevention of autoimmune diabetes in the BB rat by intrathymic islet transplantation at birth. *Science* 256:1321–1324, 1992
8. Koevary SB, Blomberg M: Prevention of diabetes in BB/Wor rats by intrathymic islet injection. *J Clin Invest* 89:512–516, 1992
9. Gerling IC, Serreze DV, Christianson SW, Leiter EH: Intrathymic islet cell transplantation reduces β-cell autoimmunity and prevents diabetes in NOD/Lt mice. *Diabetes* 41:1672–1676, 1992
10. Battan R, Mordes JP, Abreau S, Greiner DL, Handler ES, Rossini AA: Evidence that intrathymic islet transplantation does not prevent diabetes or subsequent islet graft destruction in RT6-depleted diabetes resistant BioBreeding/Worcester rats. *Transplantation* 57:731–736, 1994
11. Whalen BJ, Weiser P, Marounek J, Rossini AA, Mordes JP, Greiner DL: Recapitulation of normal and abnormal BB rat T cell development in adult thymus organ culture. *J Immunol* 162:4003–4012, 1999
12. Sutton R, Peters M, Mcshane P, Gray DWR, Morris PJ: Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation* 42:689–691, 1986
13. Hylkema MN, Van der Deen M, Pater JM, Kampinga J, Nieuwenhuis P, Groen H: Single expression of CD45RC and RT6 in correlation with T-helper 1 and T-helper 2 cytokine patterns in the rat. *Cell Immunol* 199:89–96, 2000
14. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. *J Am Statist Assn* 53:457–481, 1958
15. Matthews DE, Farewell VT: The log-rank or Mantel-Haenszel test for the comparison of survival curves. In *Using and Understanding Medical Statistics*. Matthews DE, Farewell VT, Eds. Basel, Karger, 1988, p. 79–87
16. Nie NH, Hull CH, Jenkins JG, Steinbrenner K, Bent DH: *Statistical Package for the Social Sciences*. New York, McGraw-Hill, 1975, p. 1–675
17. Ridgway WM, Fathman CG: The association of MHC with autoimmune diseases: understanding the pathogenesis of autoimmune diabetes. *Clin Immunol Immunopathol* 86:3–10, 1998
18. Jolicoeur C, Hanahan D, Smith KM: T-cell tolerance toward a transgenic β-cell antigen and transcription of endogenous pancreatic genes in thymus. *Proc Natl Acad Sci U S A* 91:6707–6711, 1994
19. French MB, Allison J, Cram DS, Thomas HE, Dempsey-Collier M, Silva A, Georgiou HM, Kay TW, Harrison LC, Lew AM: Transgenic expression of mouse proinsulin II prevents diabetes in nonobese diabetic mice. *Diabetes* 46:34–39, 1997
20. Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E, Polychronakos C: Insulin expression in human

thymus is modulated by *INS* VNTR alleles at the *IDDM2* locus. *Nat Genet* 15:289–292, 1997
21. Pugliese A, Zeller M, Fernandez A Jr, Zalcberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST, Patel DD: The insulin gene is transcribed in the human thymus and transcription levels correlate with allelic variation at the *INS* VNTR-*IDDM2* susceptibility locus for type 1 diabetes. *Nat Genet* 15:293–297, 1997
22. Sospedra M, Ferrer-Francesch X, Dominguez O, Juan M, Foz-Sala M, Pujol-Borrell R: Transcription of a broad range of self-antigens in human thymus suggests a role for central mechanisms in tolerance toward peripheral antigens. *J Immunol* 161:5918–5929, 1998
23. Heath VL, Moore NC, Parnell SM, Mason DW: Intrathymic expression of genes involved in organ specific autoimmune disease. *J Autoimmun* 11:309–318, 1998
24. Keller RJ, Eisenbarth GS, Jackson RA: Insulin prophylaxis in individuals at high risk of type I diabetes. *Lancet* 341:927–928, 1993
25. Fuchtenbusch M, Rabl W, Grassl B, Bachmann W, Standl E, Ziegler AG: Delay of type I diabetes in high risk, first degree relatives by parenteral antigen administration: the Schwabing Insulin Prophylaxis Pilot Trial. *Diabetologia* 41:536–541, 1998
26. Atkinson MA: NOD mice as a model for therapeutic interventions in human insulin dependent diabetes mellitus. In *NOD Mice and Related Strains: Research Applications in Diabetes*, *AIDS*, *Cancer and Other Diseases*. Leiter EH, Atkinson MA, Eds. Austin, TX, R.G. Landes, 1998, p. 145–172
27. Wong FS, Janeway CA Jr: Insulin-dependent diabetes mellitus and its animal models. *Curr Opin Immunol* 11:643–647, 1999
28. Gottlieb PA, Handler ES, Appel MC, Greiner DL, Mordes JP, Rossini AA: Insulin treatment prevents diabetes in RT6-depleted diabetes resistant BB/Wor rats. *Diabetologia* 34:296–300, 1991
29. Mordes JP, Schirf B, Roipko D, Greiner DL, Weiner H, Nelson P, Rossini AA: Oral insulin does not prevent insulin-dependent diabetes mellitus in BB rats. *Ann N Y Acad Sci* 778:418–421, 1996
30. Bieg S, Hanlon C, Hampe CS, Benjamin D, Mahoney CP: GAD^{65} and insulin B chain peptide (9–23) are not primary autoantigens in the type 1 diabetes syndrome of the BB rat. *Autoimmunity* 31:15–24, 1999
31. Cetkovic-Cvrlje M, Gerling IC, Muir A, Atkinson MA, Elliott JF, Leiter EH: Retardation or acceleration of diabetes in NOD/Lt mice mediated by intrathymic administration of candidate β-cell antigens. *Diabetes* 46:1975–1982, 1997
32. Whalen BJ, Greiner DL, Mordes JP, Rossini AA: Adoptive transfer of autoimmune diabetes mellitus to athymic rats: synergy of $CD4^+$ and $CD8^+$ T cells and prevention by $RT6^+$ T cells. *J Autoimmun* 7:819–831, 1994
33. Dilts SM, Lafferty KJ: Autoimmune diabetes: the involvement of benign and malignant autoimmunity. *J Autoimmun* 12:229–232, 1999
34. André-Schmutz I, Hindelang C, Benoist C, Mathis D: Cellular and molecular changes accompanying the progression from insulitis to diabetes. *Eur J Immunol* 29:245–255, 1999
35. Werdelin O, Cordes U, Jensen T: Aberrant expression of tissue-specific proteins in the thymus: a hypothesis for the development of central tolerance. *Scand J Immunol* 47:95–100, 1998
36. Hanahan D: Peripheral-antigen-expressing cells in thymic medulla: factors in self-tolerance and autoimmunity. *Curr Opin Immunol* 10:656–662, 1998