Rapid Publications

Prevention of Recurrent Autoimmune Diabetes in BB Rats by Anti-Asialo-GM1 Antibody

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BB rats exhibit a syndrome of spontaneous diabetes that has clinical and pathological characteristics analogous to those found in human insulin-dependent diabetes mellitus (IDDM). Islet tissue transplanted into spontaneously diabetic BB rats is uniformly destroyed by a recurrence of the autoimmune response that destroyed the diabetic subject's native islets. To examine recurrent autoimmune destruction of transplanted islets, it is necessary to exclude islet damage that might result from allograft rejection. We utilized neonatal tolerance induction to prevent rejection of Wistar-Furth (WF) (RT1^u) islet allografts by spontaneously diabetic BB recipients. We determined that islet-recipient treatment with anti-asialo-GM1 (anti-AGM1) antibody prevents recurrent autoimmune diabetes that would otherwise destroy transplanted WF islet grafts. Anti-AGM1 therapy significantly decreased peripheral blood natural killer (NK) cell activity. These data suggest a role for NK cells in the pathogenesis of recurrent diabetes in neonatally tolerant BB rats.
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There is increasing evidence that the destruction of pancreatic β-cells in insulin-dependent diabetes mellitus (IDDM) is the result of an autoimmune process. The possibility of recurrent autoimmune diabetes is of major concern in the transplantation of islets or pancreas for treatment of diabetes. The uniform recurrence of diabetes observed in BB rats after islet transplantation provided the first experimental indication that a recurrence of the diabetogenic autoimmune response could destroy transplanted β-cells (1). Evidence for a clinical counterpart of this phenomenon was subsequently provided by a report of recurrent diabetes in recipients of segmental pancreas isografts transplanted between identical twins discordant for IDDM (2).

The immune mechanism(s) that effects β-cell destruction is the focus of much investigation. Recent findings that islet cells are targets of natural killer (NK) cell lysis in vitro and that diabetic BB rats exhibit increased activity and numbers of NK cells has led to speculation that these cells may contribute to β-cell damage in autoimmune diabetes (3,4). To test this hypothesis, we administered anti-asialo-GM1 (anti-AGM1) antibody to BB rats and examined its effect on recurrent autoimmune islet destruction. Anti-AGM1 is a polyclonal antibody that binds the glycolipid AGM1 present on NK cells and reduces NK-cell activity when administered to rodents (5).

MATERIALS AND METHODS

Animals. Initial stocks of BB rats were obtained from P. Thiebert (Animal Resources Division, Health Protection Branch, Ottawa, Canada) and from A.A. Like (University of Massachusetts, Worcester, MA). The cumulative incidence of spontaneous diabetes (occurring between 60 and 180 days of age) in a Philadelphia colony of diabetes-prone BB rats (BBdp) is 50–60%. Diabetic rats (glucose >300 mg/dl) were inoculated subcutaneously with 2–5 U of protamine zinc insulin daily before islet transplantation. After islet transplantation, administration of insulin was withheld so that the level of blood glucose could be used as an index of islet-transplant function. Inbred Wistar-Furth (WF) rats were purchased from Harlan-Sprague-Dawley (Walkersville, MD) and used as islet donors. WF and BB rats are RT1 compatible, both possessing RT1^u haplotypes.

Neonatal tolerance induction and skin grafting. Immunologic tolerance was induced by neonatal (<24 h old) inoculation of BBdp rats with 50 × 10^6 T-lymphocyte–depleted WF bone marrow cells (BMCs). T-lymphocyte–depleted BMCs were harvested from T-lymphocyte–deficient WF rats. These rats were prepared by adult thymectomy, lethal irra-

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diation, and reconstitution of WF hosts with BMCs from another WF rat that had been drained from the thoracic duct for 5 days. Absence of T-lymphocytes in peripheral blood and bone marrow of the deficient rats was confirmed by immunofluorescent staining with monoclonal antibodies (MoAbs) specific for rat lymphocyte markers. Naive WF BMCs contain ~7% contaminating T-lymphocytes ($OX19^+$), whereas BMCs from WF T-lymphocyte–deficient donors contained <1% of $OX19^+$ cells. The status of immunologic tolerance of BBdp rats was confirmed in adulthood by their permanent acceptance of WF skin allografts as previously described (1).

Chemical induction of diabetes. BBdp rats of our colony that do not become spontaneously diabetic by 180 days of age are known to have <1% chance of becoming hyperglycemic. Thus, BBdp rats >180 days old were rendered diabetic with 65 mg/kg streptozocin i.v. and assumed to be hyperglycemic on the basis of chemically induced diabetes.

Islet isolation and transplantation. Islets were isolated from WF donors by collagenase digestion and centrifugation through a discontinuous Ficoll gradient as previously described (6). Spontaneously or chemically induced diabetic BB animals were transplanted intraportally with isolated islets (1000–1500) within 3 wk after the onset of hyperglycemia. Reversal of hyperglycemia (glucose <150 mg/dl) for at least 3 days was taken to indicate a successful islet transplant. Islet recipients were monitored by blood glucose measurements three times a week until a return of diabetes was confirmed. A group of prospective islet-transplant recipients were injected with 0.5 ml of anti-AGM1 twice a week beginning 1 wk before islet transplantation and continuing until experiment termination at 100 days posttransplantation.

Antibodies and fluorescence-activated cell sorter (FACS) analysis. The following mouse MoAbs were produced from hybridoma cell lines (obtained from A.F. Williams and D.W. Mason, Oxford University, Oxford, UK): W3/13 (reactive to rat T-lymphocytes, polymorphs, NK cells), OX19 (T-lymphocytes and thymocytes), W3/25 (helper-inducer T-lymphocytes, macrophages), and OX8 (cytotoxic-suppressor T-lymphocytes and NK cells). For immunofluorescent studies, lymph node cells (LNCs) were incubated sequentially with 0.05 ml of primary MoAb and FITC-goat $F(ab')_2$ anti-mouse IgG (Cappell, Cochranville, PA) that had been absorbed to eliminate cross-reactivity with rat IgG as described previously (7). Cells were fixed in 1% paraformaldehyde and analyzed with a FACS. Anti-AGM1 antibody was purchased from Wako (Dallas, TX) and diluted 1:25 with phosphate-buffered saline for in vivo administration. Lymphocytes for FACS analysis were harvested from four anti-AGM1–treated BB recipients of WF islets by cervical lymphadenectomy at 4, 35, 50, and 56 days after islet transplantation and 3 or 4 days after an injection of anti-AGM1.

NK-cell assay. Target YAC-1 cells ($2–3 \times 10^6$) were labeled with 200 μCi [^{51}Cr]sodium chromate (New England Nuclear, Boston, MA) in RPMI medium, washed three times, and seeded at 1×10^4 cells/well in 96 welled microtiter plates. Ficoll-gradient–separated peripheral blood lymphocytes (PBLs) were added to the wells (1.25×10^5 to 1×10^6 cells/well) in triplicate. The plates were centrifuged (100 g for 3 min) and incubated at 37°C for 4 h. At the end of incubation, plates were centrifuged, and an aliquot of the supernatant was collected from each well and counted in a Beckman γ-counter. Total ^{51}Cr release was determined by addition of 0.1 ml of 10% Triton X (Sigma, St. Louis, MO) to a set of target-cell wells. Specific cytotoxicity was determined by

$$\% \text{ lysis} = 100 \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}$$

where cpm is counts per minute. For all experiments, the ratio of spontaneous to maximum ^{51}Cr release for YAC-1 ranged from 7.1 to 13.2% in 4-h assays. NK-cell activity of individual animals treated with anti-AGM1 was tested 3 days after antibody administration.

RESULTS

We have previously demonstrated that BBdp rats inoculated at birth with unfractionated WF BMCs had a strikingly reduced incidence of diabetes in adulthood (1). Tolerance in these hosts was confirmed by their permanent acceptance of WF skin allografts. In our study, all BBdp rats were rendered neonatally tolerant by T-lymphocyte–depleted WF BMC transplantation. We and others have observed that elimination of contaminating mature T-lymphocytes from the bone marrow inocula abrogates the protective effect normally afforded by unfractionated BMC transplantation (8). At 6–8 wk, each BBdp rat received a WF skin graft to confirm a state of donor-specific immunological tolerance. To verify that tolerance also applied to islet tissue, BBdp rats that had not become diabetic by 180 days of age were rendered diabetic artificially with streptozocin and subsequently transplanted with WF islets. Because these animals had not become spontaneously diabetic, they were considered non-autoimmune. They were therefore not expected to destroy islets by recurrent autoimmunity. The finding that 7 of 7 WF islet grafts transplanted into streptozocin-induced diabetic BB rats were permanently accepted confirmed the presence

TABLE 1
Wistar-Furth islet allograft survival in BB diabetic subjects tolerant to WF antigens

Type of diabetes	Treatment of recipients	Graft survival (days)	Mean ± SD
Streptozocin induced		>100 × 7	>100
Spontaneous		3,6,6,9,10,10,10,11,75,85	22.5 ± 30.5
Spontaneous	Anti-AGM1	28, >100 × 4	82.0 ± 36*

AGM1, asialo GM1.
*$P < .01$ compared with untreated recipients.

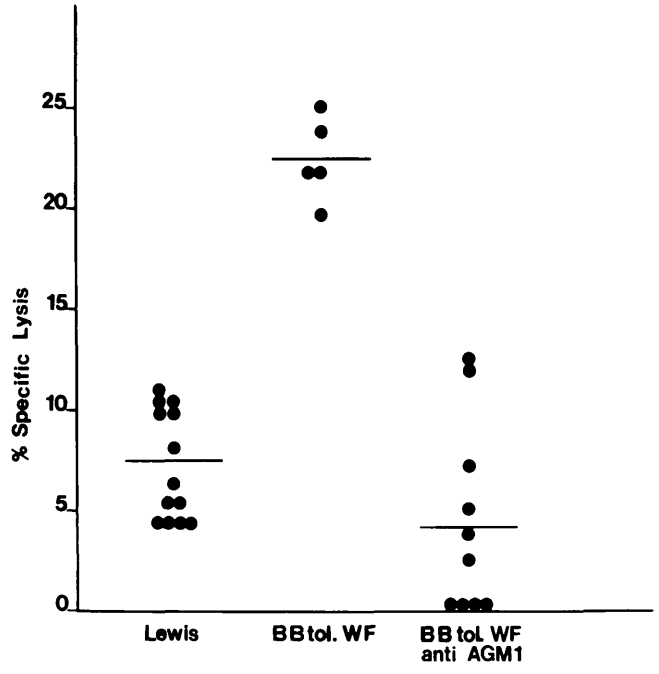

FIG. 1. Specific lysis of YAC-1 cells by peripheral blood lymphocytes (at 50:1 effector-to-target ratio) from normal Lewis and spontaneously diabetic BB tolerant [Wistar-Furth (WF)] rats. *Center panel* depicts specific lysis of PBLs from prospective WF islet recipients before initiation of anti-asialo-GM1 (AGM1)–antibody treatment. *Right panel* shows 10 data points from same 5 animals 2–10 wk after WF islet cell transplantation. PBLs from 1 animal that developed recurrence of diabetes 28 days after islet transplantation (during anti-AGM1 treatment) exhibited specific lysis of 12.5%.

of neonatal tolerance and the absence of islet autoimmunity (Table 1). In contrast, each of 11 islet grafts transplanted into spontaneously diabetic tolerant BB recipients was destroyed. Nine of them were destroyed within 2 wk of transplantation. Because rejection was precluded in these recipients by immunological tolerance, anti-islet autoimmunity had to be responsible for their demise.

In contrast to these results, spontaneously diabetic tolerant BB rats treated with anti-AGM1 accepted WF islets in four of five instances; one graft was destroyed after 28 days. Histological examination of accepted grafts at 100 days posttransplantation revealed morphologically intact islets without lymphocytic infiltration.

PBL NK-cell activity of tolerant BB recipients of WF islets before and after initiation of treatment with anti-AGM1 is shown in Fig. 1. PBLs from BB rats (before initiation of anti-AGM1 treatment) elicited a significantly higher specific lysis of YAC-1 cells than PBLs from normal Lewis rats (22.4 ± 2 vs. 7.5 ± 2.6%, $P < .01$). PBLs from anti-AGM1–treated BB rats exhibited a significantly decreased level of YAC-1 target-cell lysis compared with pretreatment levels (22.4 ± 2 vs. 4.2 ± 1.0%, $P < .01$). Of the 10 NK-cell activity determinations made in anti-AGM1–treated rats, 5 were made at 2–4 wk and 5 at 4–10 wk after initiation of treatment. Because no apparent difference was observed comparing NK-cell activity early and late in the course of treatment, the points were grouped together in Fig. 1.

FACS analysis of LNCs from four treated and seven control tolerant BB rats that had not been treated is shown in Table 2. No statistically significant difference was detected in the percentage of cells positive for the pan–T-lymphocyte markers W3/13 and OX19 or for the helper (W3/25) or cytotoxic-suppressor T-lymphocyte markers (OX8) in a comparison of treated and untreated rats. The nonsignificance of the decrease in the percentage of $OX8^+$ cells in treated rats was because only a few LNCs are NK cells. Thus, the remaining $OX8^+$ LNCs in treated rats are probably cytotoxic-suppressor T-lymphocytes.

DISCUSSION

Various immunologic effector mechanisms are purported to play a role in the diabetogenic autoimmune response in spontaneous diabetes. The in vitro lysis of islets can be accomplished by cytokines (9,10), cytotoxic T-lymphocytes (11), activated macrophages (12), antibody and complement (13), and NK cells (3). The BB rat is known to be deficient in many T-lymphocyte–dependent immune responses (7,14), but it displays elevated numbers and activity of NK cells (4). For this reason, we examined the effect of anti-AGM1 therapy on the recurrent autoimmune destruction of islets by BB rats. Anti-AGM1 treatment was effective in preventing recurrent autoimmune destruction of transplanted WF islets in diabetic rats. This effect was accompanied by a significant reduction in peripheral blood NK-cell activity in treated rats.

Several lines of evidence have suggested that T-lymphocytes play an important role in the destruction of islets in BB rats. Neonatal thymectomy, immunotherapy with polyclonal or monoclonal anti-lymphocyte antibody, and cyclosporin have all proved effective in preventing diabetes in this model (15,16). On the other hand, administration of an antimacrophage agent, silica, that apparently lacks specificity for T-lymphocytes has also been reported to prevent diabetes in BB rats (17). These findings suggest that multiple immune effector mechanisms may participate in autoimmune β-cell destruction. Like et al. (16) have postulated a role for both helper T-lymphocytes and NK cells in the diabetogenic immune response based on the finding that diabetes can be prevented by administration of OX19 or OX8 MoAbs to pre-

TABLE 2
FACS analysis of lymph node cells

Rats	Marker-positive cells (%)			
	W3/13	OX19	W3/25	OX8
Lewis (n = 5)	76.5 ± 3.4	75.4 ± 6.3	59.0 ± 3.9	27.1 ± 5.5
BB tolerant WF (n = 7)	40.0 ± 7.4	26.1 ± 5.5	26.3 ± 5.4	14.9 ± 10.7
BB tolerant WF anti-AGM1 (n = 4)	33.2 ± 6.6	23.0 ± 6.6	25.4 ± 8.3	6.4 ± 2.5

WF, Wistar-Furth; AGM1, asialo GM1.

diabetic BB rats (16). The efficacy of anti-AGM1 treatment in preventing recurrent autoimmune islet destruction directly implicates NK cells in β-cell damage. However, note that anti-AGM1 is not a specific marker for NK cells. Because AGM1 has been found to be expressed on activated peritoneal macrophages, thymocytes, BMCs, and alloimmune cytotoxic T-lymphocytes, a direct effect on other immune effector cells cannot be excluded (18,19). However, the markedly reduced NK-cell activity in anti-AGM1–treated recipients and an absence of significant alterations in the relative percentage of T-lymphocyte subsets argue that these results were achieved through the effect of anti-AGM1 on NK cells.

Our study was aimed at prevention of recurrence of diabetes after islet transplantation rather than prevention of disease initiation. Because diabetes in humans cannot yet be precisely predicted, protocols such as ours that are directed toward a reversal of the diabetic process may be of great clinical relevance.

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