DEPLETION OF RT6.1$^+$ T LYMPHOCYTES INDUCES DIABETES IN RESISTANT BIOBREEDING/WORCESTER (BB/W) RATS

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Diabetes-prone (DP)[1] BB rats develop spontaneous hyperglycemia and ketoacidosis that is fatal unless treated with insulin, and many lines of evidence indicate that the disorder has an autoimmune pathogenesis (1). Lymphocytic infiltration of the islets of Langerhans (insulitis), selective destruction of insulin-secreting β cells (2–5), and circulating islet autoantibodies (6–9) are observed. The disease is associated with the RT1^u allele of the rat MHC (10) and is prevented by immunosuppression (11, 12), thymectomy (13, 14), bone marrow allografts (15, 16), and lymphocyte transfusions (17–20). Con A–activated spleen cells from acutely diabetic BB rats adoptively transfer diabetes to naive recipients (21–23). DP-BB rats also have many abnormalities of cell-mediated immunity, including severe T cell lymphopenia (24–27).

Histocompatible diabetes-resistant (DR) BB rats have also been developed (28, 29). <3% of them develop spontaneous diabetes, compared with 40–70% of DP rats, and they appear to have an intact immune system with normal numbers of peripheral lymphocytes. The frequency of diabetes in DR-BB rats can be substantially increased by treating them with low-dose irradiation (30) or cyclophosphamide (23).

These observations on the DR-BB rat are of particular interest in light of the finding (31) that RT6$^+$ T cells are absent in the DP-BB rat but present in the DR in numbers similar to those found in normal Wistar Furth rats. The RT6 alloantigenic system (formerly termed ART2, Pta, AgF, and RTLy-2) consists of two allelic genotypes, RT6^a and RT6^b (32, 33). The surface antigen expressed by the RT6^a genotype is designated RT6.1; that expressed by RT6^b is designated RT6.2. RT6 is a nonglycosylated 21-kD surface membrane molecule (34) expressed on 60% of peripheral rat T cells but absent from thymocytes and bone marrow cells (35, 36). ~50% of helper/inducer (W3/25$^+$) and 70% of cytotoxic/suppressor (OX8$^+$) peripheral T cells express RT6 (36). RT6$^+$ lymphocytes participate in graft versus host responses (37), proliferate in response to Con A

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[1] *Abbreviations used in this paper:* DP, diabetes prone; DR, diabetes resistant.

and to allogeneic stimulation in MLRs (38), and secrete IL-2 (Lubaroff, D. M., personal communication). Cell fractionation studies suggest that $RT6^+/OX8^+$ T cells may be suppressors of in vitro MLRs (38) and antigen-specific proliferative responses (Mojcik, C. J., D. L. Greiner, I. Goldschneider, and E. S. Medlock, manuscript in preparation.

Previous studies have suggested that $RT6^+$ T cells may play a role in determining susceptibility to diabetes in DP-BB rats. Diabetes in RT6-deficient DP rats can be prevented by transfusions of DR-BB (17, 19) or Wistar Furth (18, 20) spleen and lymph node cells, and protection is associated with long-term persistence of donor-origin $RT6^+$ T cells in the transfused recipients.

To investigate further the role of $RT6^+$ T cells in BB rat diabetes, we selectively depleted DR rats of $RT6.1^+$ T cells by in vivo administration of an anti-RT6.1 lymphocytotoxic mAb. Our results show that depletion of $RT6^+$ T cells in young DR rats induces insulitis and diabetes and that RT6-depleted DR spleen cells induce rather than prevent diabetes.

Materials and Methods

Animals. DP and DR rats were obtained from the University of Massachusetts in Worcester (BB/W rats). DP-BB/W rats have been inbred for over 20 generations and have a cumulative incidence of diabetes between 40 and 70%. <0.5% become diabetic before 60 d of age, and >85% of diabetics appear between 60 and 120 d of age. $RT6^+$ T cells are not found in DP-BB/W rats at any age (31).

DR-BB/W rats were derived from fifth generation DP forebears and have subsequently been inbred for resistance to diabetes for over 20 generations. At the time of these experiments, the cumulative incidence of diabetes in the DR-BB/W rat was <3%, with most cases occurring before 60 d of age. Insulitis among nondiabetic DR-BB/W rats is rare. DR-BB/W rat T cells express the RT6.1 phenotype. The MHC haplotype of all BB rats is $RT1^u$.

Lewis (LEW, $RT1^l$, $RT6^a$) and Wistar Furth (WF, $RT1^u$, $RT6^b$) inbred rats were obtained from the Animal Genetics and Production Branch, National Cancer Institute, Frederick, MD. All rats were housed under standard laboratory conditions, provided with water and commercial chow ad libitum, and tested for diabetes twice weekly. In all experiments, diabetes was diagnosed on the basis of 4+ glycosuria and a plasma glucose >200 mg/dl.

Antibodies. Rat-mouse hybridoma cell lines secreting mAb to the RT6.1 (DS4.23) and RT6.2 (6A5) allelic forms of the RT6 peripheral T cell subset were prepared in our laboratory as previously described (35). The 6A5 cell line was the gift of Dr. D. M. Lubaroff, University of Iowa, Iowa City. W3/25 (helper/inducer T cell subset) and OX8 (cytotoxic/suppressor T cell subset) mouse mAbs were obtained from Accurate Chemical & Scientific Corp., Westbury, NY (39).

In Vivo Administration of mAb. DS4.23 anti-RT6.1 mAb was prepared for in vivo injection using hybridomas grown in tissue culture for 72 h in serum-free RPMI 1640 containing 1% Nutridoma-SP (Boehringer Mannheim Biochemicals, Indianapolis, IN). Cell-free tissue culture supernatant was concentrated 100-fold using a filtration system with a molecular weight cutoff of 50×10^3 (Amicon Corp., Danvers, MA). The protein content of concentrates was measured and the volume was adjusted to provide for the intravenous injection of 0.1–2.5 mg protein/kg body weight in 0.5 ml RPMI. Litters of male and female DP and DR rats were randomized to receive injections of mAb or medium. Experimental protocols are detailed below. In all experiments, representative animals from each injection group were killed for analysis of lymph node cell subsets by flow cytometry.

Immunofluorescence Analysis. Spleen and lymph node cell suspensions were prepared by extrusion of the tissues through a cell sieve followed by trituration with a Pasteur

pipette. Cells were washed with at least two changes of cold RPMI 1640 medium and viability was determined by exclusion of 0.1% trypan blue.

Cell suspensions were labeled by incubation with anti-RT6.1 or anti-RT6.2 mAb, followed by incubation with an $F(ab')_2$ fragment of FITC-conjugated goat anti-rat IgG antibody (heavy and light chain-specific; Cappel Laboratories, Malvern, PA) as previously described (40), or by incubation with anti-RT6.1 mAb that had been directly conjugated with FITC (36). Cells labeled with mouse mAb OX8 or W3/25 were developed for immunofluorescence analysis using an $F(ab')_2$ fragment of a FITC-conjugated goat anti-mouse IgG (heavy and light chain-specific) antibody that had been passed over a normal rat serum Sepharose 4B affinity column to remove crossreacting antibodies (31). Controls routinely included the FITC conjugate alone or irrelevant rat and mouse mAb substituted for the primary antibodies. All cell suspensions were fixed before analysis using saline-buffered 10% formalin.

Cell suspensions prepared for immunofluorescence were analyzed visually using a Zeiss fluorescence microscope or electronically on a FACS IV (Becton Dickinson & Co., Sunnyvale, CA), according to relative low-angle light scatter (1.5–15°) and relative fluorescence intensity as previously described (40). Dead cells and red blood cells were excluded from analysis by electronic gating. At least 5×10^4 nucleated cells were analyzed for relative fluorescence intensity.

Mitogenic Response of Lymphocytes to Con A. The proliferative response of RT6-depleted DR-BB/W spleen cells to various doses of Con A (0.125–1.0 μg/well) was measured by [^{3}H]thymidine uptake as previously described (41). Measurements at each dose of Con A were performed in triplicate and averaged.

Preparation of Lymphocytes for Adoptive Transfer of Diabetes. Spleen cells were prepared for adoptive transfer as previously described (21). Briefly, spleen cell suspensions were prepared aseptically, washed three times with cold RPMI 1640, and then cultured in RPMI supplemented with 10% FCS, 100 U/ml penicillin, and 5 μg/ml Con A (Miles-Yeda, Rehovot, Israel) for 72 h at 37°C in the presence of 5% CO_2. After incubation, the cells were washed, resuspended in RPMI and tested for viability by exclusion of 0.1% trypan blue. Each recipient rat received 40×10^6 viable cells intravenously via the tail vein. Adoptive transfer of BB rat diabetes without Con A activation has not been described (1). The phenotype of the cell(s) responsible for transfer remains unknown.

Histologic Procedures. Pancreata obtained at the end of an experiment were fixed in Bouin's solution and embedded in paraffin. Sections stained with hematoxylin and eosin were examined for insulitis by a pathologist (Dr. M. C. Appel, University of Massachusetts, Worcester, MA) who was unaware of the treatment status of the rats.

Statistical Procedures. Statistical analysis of the frequency of diabetes used the χ^2 and Fisher exact statistics (42). The Bonferroni adjustment for multiple comparisons (42) was made where appropriate. Only animals surviving either to the end of the experiment or to the onset of diabetes are included. Insulitis was scored as either present or absent, without regard to severity.

Experimental Protocols. Experiment 1 quantified the in vivo effect of anti-RT6.1 antibody injections on lymph node cell populations of DR-BB/W, LEW, and WF rats. 30-d-old DR-BB/W ($RT6^a$) animals were given one intravenous injection of DS4.23 (anti-RT6.1) at a dose of 0.1–2.5 mg mAb/kg body weight. 30-d-old WF ($RT6^b$) and LEW ($RT6^a$) rats, and 60-d-old DR-BB/W rats were also tested at a dose of 0.3 mg/kg. Controls were given injections of RPMI 1640. 24 h after mAb injection, the rats were killed and a sample of lymph nodes was removed. Aliquots of pooled lymph node cells were labeled by incubation with anti-RT6.1, anti-RT6.2, OX8, W3/25, or anti-Ig antibody and developed for immunofluorescence. The results of this study determined the dosage of anti-RT6.1 used in subsequent experiments.

Experiment 2 quantified the frequency of diabetes in 30-d-old DR-BB/W and LEW rats and in 60-d-old DR-BB/W rats given 0.3 mg/kg anti-RT6.1 antibody intravenously twice weekly for 4 wk. Control rats received RPMI 1640. All rats were tested for diabetes from the day of the first injection until 1 wk after the last one, at which time repopulation of $RT6^+$ T cells could be anticipated. The number of rats in each group is given in Table

TABLE I

Phenotypic Analysis of Rat Lymph Node Cells After In Vivo Administration of Anti-RT6.1 mAb

Rat strain	Treatment	Percentage of positive cells				
		RT6.1	RT6.2	OX8	W3/25	Ig
DR-BB/W	Anti-RT6.1	<1.0	<1.0	10.2	54.8	35.8
	Medium	30.3	<1.0	19.0	50.6	22.1
LEW	Anti-RT6.1	<1.0	<1.0	6.7	47.9	47.7
	Medium	42.9	<1.0	19.4	55.2	26.2
WF	Anti-RT6.1	<1.0	56.0	24.4	50.9	22.6
	Medium	<1.0	57.6	29.4	50.3	21.4

Rats were given a single intravenous injection of DS4.23 anti-RT6.1 mAb (0.3 mg/kg) or RPMI 1640 medium. 24 h after injection, lymph node cells were recovered and the mean percentage of total lymph node cells bearing each marker was determined by immunofluorescence analysis on the FACS using at least 50,000 nucleated cells. Results represent the mean of pools of three or four animals. Values of <1.0% indicate that no cells bearing this marker were detected.

II. During the final week of mAb injections, depletion (>95%) of $RT6^+$ lymph node cells in a representative sample of treated rats was confirmed by immunofluorescence analysis.

Experiment 3 was designed to determine if cells capable of the adoptive transfer of diabetes could induce the disease in nondiabetic DR-BB/W or LEW rats depleted of $RT6^+$ lymphocytes. Previous studies (23) had shown that intact rats of both strains are resistant to the induction of diabetes by this method (23). DR-BB/W rats are, however, susceptible to the adoptive transfer of diabetes by this method if they are first immunosuppressed (23). Treatment of DR-BB/W rats with mAb was begun at either 30 or 60 d of age; LEW rats were treated beginning at 30 d of age. Control rats were given injections of RPMI 1640 on the same schedule. After treatment with mAb for 3 wk (a total of six injections), DR rats were tested for diabetes and those that had remained nondiabetic were selected for further study. Both control and experimental animals were given a single intravenous injection of 40×10^6 Con A–activated spleen cells from acutely diabetic BB/W rats. Twice weekly injections of mAb or RPMI continued after injection of the Con A–activated cells until the conclusion of the experiment. Animals were tested for diabetes for 4 wk after the injection of activated spleen cells. The number of rats treated is given in Table III.

Experiment 4 was designed to test if cells capable of the adoptive transfer of diabetes were present in RT6-depleted nondiabetic DR-BB/W or LEW rats. Rats that had remained nondiabetic after 1–4 wk of twice-weekly injections of anti-RT6.1 antibody were used as spleen cell donors. Recipients were 30-d-old DP or DR-BB/W rats. A group of nondiabetic, $RT6^-$ DP-BB/W rats treated with anti-RT6.1 antibody or RPMI beginning at 30 d of age were also used as spleen cell donors according to the same protocol. The number of rats in each group is given in Table IV.

Results

Experiment 1: In Vivo Depletion of $RT6^+$ T Lymphocytes. Peripheral lymphoid tissues of intact DR-BB/W rats contained normal numbers of $RT6^+$ T cells (Table I). Doses of DS4.23 mAb ≥0.3 mg/kg body weight resulted in essentially complete (>95%) depletion of $RT6^+$ lymph node cells (Table I). The circulating $RT6^+$ T cell population remained depleted for at least 72 h after a single injection of mAb (data not shown). The effectiveness of anti-RT6.1 treatment was independent of age.

In vivo treatment of LEW ($RT6^a$) and DR-BB/W rats with anti-RT6.1 (0.3 mg/kg) slightly decreased the relative proportions of the helper/inducer ($W3/25^+$) and suppressor/cytotoxic ($OX8^+$) lymph node T cell subsets (Table I).

TABLE II

Frequency of Diabetes in Susceptible (DP) and Resistant (DR) BB/W and Lewis (LEW) Rats After Administration of DS4.23 Anti-RT6.1 Antibody at Different Ages

Strain	Age	Treatment	Number studied	Frequency of diabetes	
	d			*n*	%
DR-BB/W	30	Anti-RT6	38	19	50*
		Control	19	0	0
DR-BB/W	60	Anti-RT6	43	1	2
		Control	10	0	0
LEW	30	Anti-RT6	16	0	0
		Control	6	0	0
DP-BB/W	30	Anti-RT6	12	0	0
		Control	6	0	0

Rats were injected twice weekly for 4 wk with 0.3 mg/kg DS4.23 anti-RT6.1 mAb and tested for diabetes until 1 wk after the last injection. Data on the DP-BB/W rats are through 60 d of age, when spontaneous diabetes typically begins to occur. The DP-BB/W rats were tested for diabetes through 120 d of age. All diabetic rats had a plasma glucose ≥200 mg/dl. 4 of 12 (33%) RT6-depleted DP rats became diabetic as compared with 2 of 6 (33%) controls. These rates are comparable to those observed in the colony as a whole and suggest that diabetes in the DP rat is unaffected by anti-RT6-treatment. A sample of 10 RT6-depleted DR rats from the group treated beginning at 30 d of age was studied histologically at the end of the experiment. Four (40%) were found to have insulitis.

* $p < 0.001$ vs. control. No other comparisons are statistically significant.

Injection of WF ($RT6^b$) rats with anti-RT6.1 mAb had no effect on the level of $RT6^+$ peripheral T cells. On the basis of these findings, DS4.23 anti-RT6.1 mAb was given twice weekly at a dose of 0.3 mg/kg to deplete $RT6^+$ T cells in DR, DP, and LEW rats in subsequent experiments.

Experiment 2: Onset of Diabetes in RT6-depleted DR rats. When treated with anti-RT6.1 mAb beginning at 30 d of age, 50% (19/38) of RT6-depleted DR-BB/W rats, but none of 19 controls became diabetic ($p < 0.001$, Table II). The onset of diabetes was abrupt and the animals became ketotic. No rats became diabetic during the first 14 d of mAb treatment; 95% of all cases occurred during days 15–28 of mAb treatment. The mean age at onset of diabetes was 49 d. At the end of the experiment, a sample of 10 nondiabetic RT6-depleted DR rats was studied histologically; 4 of the 10 (40%) pancreata showed insulitis.

In contrast, diabetes could not be induced in DR-BB/W rats given anti-RT6.1 mAb beginning when they were 60 d of age despite comparable (>95%) depletion of $RT6^+$ lymph node cells (Table II). There was no difference between DR rats treated with mAb beginning at 60 d of age and untreated control DR rats with respect to diabetes. The 2.8% occurrence rate in the RT6-depleted, 60-d-old group corresponds closely with the rate of spontaneous diabetes in DR rats in our colony as a whole (<3%). Table II also shows that depletion of $RT6^+$ T cells in 30-d-old LEW rats did not induce diabetes.

Treatment of 30-d-old DP-BB/W rats with anti-RT6.1 mAb for 4 wk did not affect the frequency of diabetes, a result that was expected since DP-BB/W rats have no $RT6^+$ peripheral T cells (31). No diabetes occurred in either anti-RT6-

TABLE III

Induction of Diabetes in RT6-depleted Resistant BB/W (DR-BB/W) and Lewis (LEW) Rats After Injection of Con A–activated Spleen Cells from Acutely Diabetic BB/W Rats

Recipient strain	Age	Treatment	Number studied	Frequency of Diabetes	
	d			*n*	%
DR-BB/W	30	Anti-RT6	7	5	71*
		Control	8	0	0
DR-BB/W	60	Anti-RT6	11	1	9‡
		Control	8	0	0
LEW	30	Anti-RT6	10	0	0
		Control	6	0	0

All recipient rats were nondiabetic when tested. The anti-RT6-treated DR rats were selected from among those that failed to develop diabetes after 3 wk of antibody administration. All rats were given 40×10^6 Con A–activated spleen cells obtained from acutely diabetic BB/W rats 1 wk after the last injection of anti-RT6.1 antibody. Rats were tested for diabetes twice weekly for 4 wk after the injection of the spleen cells. All diabetic rats had a plasma glucose ≥200 mg/dl. Overall $\chi^2 = 27.86$, $p < 0.001$.

* $p < 0.02$ vs. control.

‡ $p < 0.05$ vs. group treated with anti-RT6.1 at 30 d. No other comparisons are statistically significant.

treated or control DP rats before 60 d of age. The frequency of diabetes between 60 and 120 d of age was also unaffected by anti-RT6 treatment, 4 of the 12 (33%) RT6-depleted rats and 2 of the 6 (33%) controls eventually becoming diabetic.

Experiment 3: Induction of Diabetes in RT6-depleted DR-BB/W Rats. Con A–activated spleen cells from acutely diabetic DP rats induced diabetes in 100% (3/3) of control 30-d-old DP recipients, verifying that the cells used were capable of the adoptive transfer of diabetes by established criteria (21). In the case of DP recipients, onset of diabetes before they become 60 d old is interpreted as evidence of the adoptive transfer of diabetes since <0.5% of animals in this age group become spontaneously diabetic (21). DR-BB/W rats are resistant to the adoptive transfer of diabetes unless immunosuppressed (23).

When Con A–activated spleen cells from acutely diabetic rats were given to 50–55-d-old RT6-depleted DR-BB/W recipients (treated with anti-RT6 antibody beginning at 30 d of age), diabetes occurred in 5 of 7 (71%) as compared with 0 of 8 medium-treated controls ($p < 0.01$, Table III). In contrast, when cells from the same incubation were given to eleven 80–85-d-old RT6-depleted DR-BB/W recipients (treated with anti-RT6 antibody beginning at 60 d of age), only one (9%) became diabetic ($p < 0.05$ vs. group treated at 30 d of age, Table 3). Similarly, injection of Con A spleen cells from acutely diabetic BB/W rats into untreated DR or LEW rats or into RT6-depleted LEW rats failed to induce diabetes (Table III).

Experiment 4: Adoptive Transfer of Diabetes Using Spleen Cells from RT6-depleted DR-BB/W Rats. In a preliminary experiment we compared the mitogen response of spleen cells from RT6-depleted DR-BB/W rats with the response of medium treated controls. After incubation in vitro with Con A (0.125–1.0 μg/well), we

TABLE IV

Frequency of Diabetes in 30-d-old DP and DR BB/W Rats After the Injection of Con A–activated Spleen Cells Obtained from Nondiabetic Rats After Treatment with Anti-RT6.1 Antibody

Spleen cell donors			Type and number of spleen cell recipients					
			DP-BB/W			DR-BB/W		
Strain	Age	Treatment	Tested	Diabetic		Tested	Diabetic	
	d			*n*	%		*n*	%
DR-BB/W	30	Anti-RT6	18	13	72*	6	5	83‡
		Control	17	1	6	9	0	0
DR-BB/W	60	Anti-RT6	10	1	10	5	0	0
		Control	8	0	0	5	0	0
LEW	30	Anti-RT6	8	0	0	ND	ND	
		Control	8	0	0	ND	ND	
DP-BB/W	30	Anti-RT6	12	0	0	ND	ND	
		Control	6	0	0	ND	ND	

Donor spleen cells were obtained from nondiabetic RT6-depleted DR-BB/W or LEW rats, or nondiabetic, anti-RT6.1-treated, $RT6^-$ DP-BB/W rats. Spleens were taken within 3 d after the final injection of anti-RT6 antibody. Splenocytes were incubated in the presence of Con A (5 μg/ml) for 72 h before injection (21). Recipient rats were 30 d old at the time of experimentation and were tested for diabetes for 4 wk after the injection of activated spleen cells. All diabetic rats had a plasma glucose ≥200 mg/dl.

* $p < 0.001$ vs. control.

‡ $p < 0.003$ vs. control.

found no significant difference in [^{3}H]thymidine incorporation between RT6-depleted and control spleen cells (data not shown). We then tested spleen cells obtained from intact and RT6-depleted rats and incubated with Con A for their ability to induce diabetes in both 30-d-old DP-BB/W and 30-d-old intact DR-BB/W rats.

We first tested Con A–activated spleen cells from RT6 depleted DR rats that had been treated with anti-RT6.1 mAb for 4 wk beginning at 30 d of age but did not become diabetic. When these cells were injected into 30-d-old DP-BB/W rats, 13 of 18 (72%) recipients become diabetic before 60 d of age ($p < 0.001$ vs. cells from non–RT6-depleted controls). When they were injected into intact DR-BB/W rats, 5 of 6 (83%) became diabetic within 3 wk ($p < 0.005$ vs. control cells, Table IV).

Con A–activated spleen cells from DR rats treated for only 1 wk with anti-RT6.1 antibody failed to induce diabetes in three DP recipients. Cells from rats treated for 2 and 3 wk, respectively induced diabetes in 1 of 2 and 2 of 2 of DP recipients before 60 d of age.

These results contrast strongly with those obtained using spleen cells obtained from nondiabetic, RT6-depleted DR rats that had been treated with anti-RT6.1 mAb for 4 wk beginning at 60 d of age. Only 1 of 10 (10%) DP recipients and 0 of 5 DR recipients became diabetic (Table IV). The cells obtained from these animals not only failed to transfer diabetes, but also failed to protect the DP recipients from diabetes during the 60–120 d of age high-risk period. By 120 d of age, 9 of the 10 (90%) DP recipients of RT6-depleted, Con A–activated DR spleen cells developed diabetes.

Con A–activated spleen cells from control (non–RT6-depleted) DR rats failed to induce diabetes in either DP or DR recipients. Injection of activated spleen cells from either RT6-depleted or control LEW rats into DP recipients neither induced nor prevented diabetes (Table IV). Lastly, activated spleen cells from nondiabetic anti-RT6-treated (0/12) or control (0/6) DP rats failed to transfer diabetes to 30-d-old DP recipients.

Discussion

These data support the hypothesis that diabetes in the BB/W rat is influenced by a regulatory T cell population that expresses the RT6.1 alloantigen. This hypothesis derives from previous studies (29) showing that DP-BB/W rats, 40–70% of which become spontaneously diabetic, lack $RT6^+$ T cells, while DR-BB/W rats, <3% of which become diabetic, have these cells. The present study now demonstrates that in vivo depletion of $RT6^+$ T cells is associated with the appearance of diabetes in DR-BB/W rats.

Treatment with anti-RT6.1 mAb at either 30 or 60 d of age depleted $RT6^+$ T cells in all DR rats tested. Depletion of RT6 T cells in 30-d-old DR rats was associated with an increase in the frequency of diabetes from 0% in 19 controls to 50% in 38 experimental animals. These was also an increase in insulitis, the pathologic substrate of diabetes, in treated rats, and even nondiabetic RT6-depleted DR rats were found to harbor spleen cells capable of the adoptive transfer of diabetes. When treatment of DR rats with anti-RT6.1 mAb was begun at 60 d of age, however, none of these effects could be observed.

Given these results, it is reasonable to speculate that during a critical developmental period a regulatory population of $RT6^+$ T cells may prevent spontaneous diabetes in DR rats. Once this temporal "window of susceptibility" has passed, however, the importance of this regulatory T cell population diminishes. In this regard it is useful to note that $RT6^+$ T cells normally appear late in ontogeny and do not reach adult levels until 40–50 days of age (36; Mojcik, C. J., D. L. Greiner, I. Goldschneider, and E. S. Medlock, manuscript in preparation). This corresponds closely to the age when RT6-deficient DP rats first show evidence of insulitis and islet cell autoantibodies (1, 8), both of which are indicators of autoimmunity and harbingers of impending diabetes mellitus. It is also of interest to note that an analogous temporal window has been observed by Like et al. (43) who found that a brief (10 d) course of immunosuppression with cyclosporine during the same age period conferred lasting protection from diabetes.

Our data suggest that the RT6-depleted DR-BB rat may provide a useful animal model of autoimmune diabetes that is complementary to the spontaneously diabetic DP-BB rat. In addition to diabetes itself, other similarities between these models include a lack of circulating $RT6^+$ T cells, the presence of insulitis in both diabetic and a substantial percentage of nondiabetic animals, abrupt onset of hyperglycemia, a requirement for exogenous insulin for survival, and the ability of spleen cells incubated in vitro with Con A to induce diabetes in adoptive recipients.

It must, of course, be cautioned that in vivo depletion procedures such as those used here can cause recipients to produce an immune response to injected

antibody. This could manifest itself as anaphylaxis (44) or in the form of antiidiotypic antibodies (45) that could negate the in vivo effect of the injected mAb. In this study, however, we used an alloantibody (i.e., rat anti-rat), not a xenotypic antibody, and it produced no detectable anaphylactic response. In addition, we have observed that continuous in vivo administration of DS4.23 anti-RT6.1 mAb to RT6.1$^+$ LEW rats for up to 4 mo is accompanied by persistent depletion of RT6$^+$ T cells without evidence of anaphylaxis (Greiner, D., and M. Angelillo, unpublished observations).

The slight reduction in the levels of circulating T cells after depletion of RT6$^+$ T cells in this rat model requires comment. It could be argued that the levels of T cells were in fact significantly reduced, but were not accurately quantified by our analyses. However, changes in lymphocyte subsets as small as 2–5% can be accurately quantified by FACS analysis (40). Alternatively, it could be argued that RT6$^+$ T cells are present but not detected by immunofluorescence analysis due to antibody-induced modulation (46) of the RT6 antigen from the cell surface. In related studies (Mojcik, C. J., D. L. Greiner, I. Goldschneider, and E. S. Medlock, manuscript in preparation), however, we have used thymectomized rats to show that RT6$^+$ T cells do not reappear after in vivo depletion with anti-RT6.1 mAb. This argues against a simple modulation mechanism to account for our depletion results. Lastly, it might be argued that depletion of RT6$^+$ peripheral T cells could induce an efflux of T lymphocytes from the thymus to peripheral lymphoid tissues. Thymocytes are RT6$^-$ (32), and require between 10 and 14 d to express RT6 antigen on their surface after emigration from the thymus (36). A rapid efflux of RT6$^-$ thymocytes could therefore account for the relatively slight reduction in the levels of peripheral W3/25$^+$ and OX8$^+$ T cells observed in RT6-depleted rats, but further experiments would be necessary to document this possibility conclusively.

The observation that spleen cells from RT6-depleted DR rats responded to Con A as well as cells from intact DR rats was not surprising because we have previously observed (Mojcik, C. J., et al., manuscript in preparation) that FACS fractionated populations of RT6$^+$ and RT6$^-$ LEW lymph node cells proliferate similarly in the presence of this mitogen. Furthermore, both RT6$^+$ and RT6$^-$ T cells produce IL-2, a potent stimulator of T cell proliferation in vitro (47). In contrast, DP rat lymphocytes proliferate poorly in response to Con A (48).

An important difference between RT6-depleted DR rats and DP rats is seen in the ability of their spleen cells to transfer diabetes to adoptive recipients. Previous studies (21–23), confirmed here, have shown that Con A–activated spleen cells from acutely diabetic DP rats transfer diabetes to 30-d-old DP recipients. Spleen cells from intact, nondiabetic DP or DR rats are incapable of transfer according to this protocol (21, 23). In the present study, spleen cells from nondiabetic RT6-depleted DR rats were found to be able to transfer diabetes to 30-d-old DP adoptive recipients. Interestingly, these spleen cells were also able to induce diabetes in untreated (nonimmunosuppressed) DR recipients. Previous reports (23, 30) had indicated that Con A–activated spleen cells from acutely diabetic rats could transfer diabetes only to DR rats that had first been immunosuppressed.

A final important difference between RT6-depleted DR rats and spontaneously

diabetic DP rats is the age at which diabetes typically appears. DR rats treated in vivo with anti-RT6.1 mAb beginning at 30 d of age became diabetic within 2–3 wk, on average when 49 d old. In contrast, the mean age at onset of spontaneous diabetes in DP rats is 89 d, with <0.5% of diabetes appearing before 60 d of age (1). Interestingly, in the small number of DR-BB/W rats (<3%) that do develop *spontaneous* diabetes, the mean age at onset is ~54 d.

There are at least three possible explanations for these observations. First, the delayed onset of diabetes in DP rats could involve regulatory mechanisms that depend on an $RT6^-$ cell population, for example the macrophage. DP-BB-adherent cells, possibly macrophages, have been shown (48, 49) to have a potent suppressive activity on T cell proliferation in vitro and may exert a regulatory influence on effector T lymphocytes in vivo. Second, the difference in time of onset of diabetes could be due to the selective expression of different effector mechanisms. Recent studies (50, 51) suggest that NK cell activity is elevated in DP rats and may be involved in the pathogenesis of diabetes. In contrast, NK activity is normal to decreased in DR rats and RT6-depleted DR rats (52). A third explanation for the delayed onset could be simply that fewer effector T cells capable of inducing diabetes are available in DP rats because of their lymphopenia (24–27). This concept of insufficient numbers of effector cells is supported by the observation that diabetes in DP rats is accelerated by the injection of Con A spleen cells from acutely diabetic rats (21–23) or RT6-depleted DR rats (Table II), both of which may be rich sources of effector cells.

Based on prior and present results, we propose that susceptibility of DR-BB/W rats to autoimmune diabetes depends on the delicate balance between its regulatory and effector cell populations (1). It has previously been observed that low-dose irradiation (30) or cyclophosphamide (23), both of which primarily affect suppressor T lymphocytes (53), induces diabetes in DR rats. On the other hand, a small percentage of DR rats spontaneously become diabetic (29), suggesting that DR rats do have an effector cell population. Our interpretation of the presence experiments is that in vivo depletion of $RT6^+$ cells in DR rats disrupts this delicate balance by removing the regulatory cell. According to our interpretation, DR rats contain at least two cell populations that determine the clinical expression of diabetes, a regulatory $RT6^+$ T cell subset that prevents it, and an $RT6^-$ effector population that produces it.

In vivo depletion of $RT6^+$ T cells in $RT1^l$ LEW rats does not induce insulitis or diabetes (Table II), suggesting that these normal animals lack the effector cells present in DP and DR rats. However, one would expect RT6-depleted LEW rats to become diabetic when injected with Con A–activated spleen cells from acutely diabetic DP rats, but this was not observed (Table II). Assuming that the injected DP spleen cells survived for a reasonable length of time in LEW recipients (16), at least two explanations for the negative outcome suggest themselves. First, Con A spleen cells from acutely diabetic rats may induce diabetes by an indirect mechanism in DP and DR rats, perhaps by activating a resident effector cell population. This explanation may not be tenable, however, because Con A spleen cells induce diabetes in WF rats (23) that would also be expected to lack effector cells. Alternatively, the effector mechanism important

in diabetes may be restricted at the MHC (16, 18) and unable to induce diabetes in histoincompatible LEW ($RT1^l$) recipients.

Since the RT6 antigen is known to occur only on a subset of peripheral T lymphocytes (31, 32), it is likely that the regulatory $RT6^+$ cell population important in the prevention of diabetes in DR rats belongs to the T cell lineage. In other experiments, we have shown that injection of lymphocytes from DR or WF rats to DP rats prevents diabetes (17, 18, 20). Cell fractionation has shown that the protective cell belongs to the helper/inducer (W3/25) T cell subset (19), and that the mechanism of protection is associated with the long-term persistence of donor-origin lymphocytes, including $RT6^+$ T cells (18). Taken together, these results suggest that the important regulatory cell that protects DR rats from diabetes belongs to the $RT6^+$, $W3/25^+$ T cell subset.

Although the mechanism by which $RT6^+$ T cells prevent diabetes remains uncertain, there is preliminary evidence that protection could be mediated by soluble factors (lymphokines) produced by $RT6^+$ T cells. When DR or WF lymphocytes sequestered in diffusion chambers are implanted into DP rats, diabetes does not occur (54), suggesting that lymphokines could be a component of the protective mechanism. IL-2, an important regulator of cell activation and proliferation (47), is known to be produced by $RT6^+$ T cells (Lubaroff, D. M., personal communication), and recent work by Prud'homme et al. (48) suggests that deficient IL-2 production by helper/inducer T cells in DP rats might contribute to their susceptibility to diabetes. We have recently observed (55), however, that pharmacologic doses of human IL-2 given chronically to 30-d-old DP rats do not alter either the frequency or time of onset of diabetes. While IL-2 may not be a protective lymphokine in the BB rat, it should be noted that the $RT6^+$ T cell population does not define a unique functionality and includes both helper/inducer ($W3/25^+$) and suppressor/cytotoxic ($OX8^+$) populations (36). Accordingly, many potential immunoregulatory lymphokines could be elaborated by the $RT6^+$ T cell population, and any one of these might be important in determining susceptibility to diabetes.

Finally, it is interesting to note that anti-RT6.1 treatment of DP rats begun at 30 d of age did not affect the time or rate at which diabetes occurred in these animals. This is consistent with our previous observation that DP-BB/W rats lack $RT6^+$ T cells (31). In related studies (16, 56) we have determined that the absence of $RT6^+$ T cells in DP rats can be traced to defects in the development of their prothymocytes. The potential importance of prothymocyte defects in BB rat diabetes is reinforced by the observation that transfer of DP bone marrow (an enriched source of prothymocytes) into DR adoptive recipients renders these irradiation chimeras susceptible to diabetes. Conversely, transfer of DR bone marrow into DP recipients confers resistance to diabetes (16). This is immunologically analogous to observations made in other animal models. Disease susceptibility in the New Zealand mouse model of SLE (57) and in the motheaten mouse model of severe combined immunodeficiency and autoimmunity (58) can also be traced to defects in prothymocytes, and predisposition to experimental allergic encephalomyelitis can be transferred from susceptible SJL/J mice to histocompatible resistant B10.S mice by bone marrow cells (59). Taken together, these results suggest that defects in the development of prothymocytes may be a

common predisposing factor for the T cell abnormalities important in the pathogenesis of insulin-dependent diabetes mellitus and other genetically determined autoimmune disorders.

Summary

To investigate the role of RT6+ T cells in the pathogenesis of diabetes in BB/W rats, we treated animals from the diabetes-resistant (DR) subline with anti-RT6.1 lymphocytotoxic mAb. This depleted >95% of peripheral RT6+ T cells but did not substantially reduce levels of circulating T cells or the in vitro response of spleen cells to mitogen.

Treatment of 30-d-old DR BB/W rats in this way: (*a*) induced insulitis and diabetes, (*b*) rendered nondiabetic RT6-depleted DR rats susceptible to the adoptive transfer of diabetes by spleen cells from acutely diabetic BB/W rats, and (*c*) yielded DR spleen cell populations capable of the adoptive transfer of diabetes to diabetes-prone (DP) or DR recipients. Treatment of DR rats beginning at 60 d of age failed to produce these effects.

These results suggest that both susceptibility and resistance to diabetes in the BB/W rat are in part regulated by the RT6+ T cell subset and provide evidence for the importance of regulatory T lymphocytes in the pathogenesis of autoimmunity and diabetes in BB/W rats.

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