Cyclophosphamide inhibits the development of diabetes in the diabetes-prone *BB* rat

D. O. Sobel¹, B. Ahvazi¹, H.-S. Jun², Y.-H. Chung², J.-W. Yoon²

¹ Georgetown University School of Medicine, Washington, District of Columbia, USA

² Julie McFarlane Diabetes Research Center, University of Calgary, Canada

Abstract

Aims/hypothesis. Cyclophosphamide has been shown to augment the diabetic process in NOD mouse and *BB* rat models of Type I (insulin-dependent) diabetes mellitus. Because cyclophosphamide has, however, been shown to increase immunoregulatory cell activity, we examined if cyclophosphamide treatment increases immunoregulatory cell activity and inhibits the diabetic process in *BB* rats.

Methods. The development of insulinitis and diabetes was explored in *BB* rats treated with saline and cyclophosphamide (60 to 175 mg/kg body weight). Subsets of spleen cells were assessed by flow cytometry and cytokine gene expression by RT-PCR. To determine if cyclophosphamide induces immunoregulatory cell activity, the development of diabetes was assessed in *BB* rats injected with spleen cells from rats treated with saline and cyclophosphamide.

Results. All dosages of cyclophosphamide decreased the development of diabetes. The degree of insulinitis was lower in pancreata from 55-day-old rats treated

with cyclophosphamide than those from controls. Cyclophosphamide caused no alterations in the numbers of NK cells, T-cell subsets, or RT6.1⁺ T cells. The adoptive transfer of spleen cells from cyclophosphamide-treated rats to *BB* rats inhibited the development of diabetes. Cyclophosphamide treatment decreased IL-12, IL-1 β , IL-2, IFN- γ and TNF- α gene expressions in mononuclear spleen cells but IL-4 gene expression increased.

Conclusion/interpretation. These findings show that cyclophosphamide treatment decreases the development of diabetes by inhibiting the development of insulinitis. This inhibitory action of cyclophosphamide on the diabetic process seems to be mediated by the induction of immunoregulatory cell activity. The suppression of cytokines that promote Th1 cell differentiation by cyclophosphamide treatment could also play a part in the diabetes sparing effect of cyclophosphamide. [Diabetologia (2000) 43: 986–994]

Keywords *BB* rat, Cytokines, Cyclophosphamide, Immunoregulatory cell

Biobreeding (*BB*) diabetes-prone rats have been extensively used as an animal model for human Type I

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Corresponding author: D.O. Sobel, MD, Georgetown University Children's Medical Center, Division of Pediatric Endocrinology and Metabolism, 3800 Reservoir Road, N.W., 2 PHC, Washington, D.C. 20007–2197, USA

Abbreviations: VAF: Viral antibody free, Con A: concanavalin A, *BB* rat: biobreeding rat, HPRT: hypoxanthine phosphoribosyltransferase, LPS: lipopolysaccharide, NOD: non-obese diabetic.

(insulin-dependent) diabetes mellitus. These rats spontaneously develop diabetes after autoimmune destruction of beta cells [1, 2]. As in human Type I diabetes, the pathogenesis of the disease in the diabetes-prone *BB* rat is through a cell-mediated autoimmune process characterized by lymphocytic infiltration of the islets and the presence of serum anti-islet cytotoxic antibodies [3, 4]. In addition, depressed suppressor cell activity is thought to play an important part in the development of diabetes in humans and the *BB* rat [5, 6, 7].

Cyclophosphamide is an alkylating agent with many immunological properties and has been used

in the treatment of human disease [8]. Cyclophosphamide has been shown to accelerate the diabetic process in non-obese diabetic (NOD) mice [9] and induce diabetes in diabetes-resistant *BB* rats [10]. Because other studies have shown that cyclophosphamide treatment can, however, augment the relative number of immunoregulatory cells with suppressor cell-like properties [11, 12], such treatment could potentially inhibit the diabetic process.

This study tests the hypothesis that cyclophosphamide treatment can induce immunoregulatory cells in *BB* rats and inhibit the development of insulinitis and diabetes.

Materials and methods

Animals. Diabetes-prone *BB* rats were obtained from the viral antibody-free (VAF) colony at the University of Massachusetts, USA. To preserve VAF conditions, animals were housed in sterilized cages which were covered with filter bonnets and placed in a laminar flow hood. Bedding and food were sterilized in an autoclave and the drinking water was acidified. Viral antibody-free conditions were monitored by performing viral serologic tests of sera from sentinel rats for the following viruses: respiratory (CAR) bacillus, H-1, Kilham rat (KRV), lymphocytic choriomeningitis (LCM), *Mycoplasma pulmonis*, parvovirus, pneumonia virus of mice (PVM), rat corona/sialodacryoadenitis (RCV/SDA), Reo, Sendai and Hantaan. Serologic testing was negative throughout. The principles of laboratory animal care were followed in compliance with the American Public Health Services Policy as outlined in the "Guide for the Care and Use of Laboratory Animals in Research and Teaching".

Experimental design. Male diabetes-prone *BB* rats from each litter were placed at random into saline and cyclophosphamide treatment groups. Cyclophosphamide at total doses from 60 to 175 mg · kg body weight or saline (0.9%) was i.p. injected. Except for the 175 mg/kg dose which was given as a single injection, all total doses were equally divided and given within 7 days of each other. Blood glucose concentrations were measured weekly by reflectometer. When blood glucoses reached 14 mmol/l on 2 consecutive days, the animals were diagnosed with diabetes and killed. Non-diabetic animals were killed at 130 days.

Analysis of glucose tolerance. After an overnight fast from food, rats were i.p. injected with 0.5 mg glucose · body weight⁻¹ using a 30 mg/ml glucose solution in water. Blood was obtained just before and at 30, 60, and 120 min after the glucose injection and assayed for glucose.

Pancreatic histology. Pancreata were fixed in buffered formalin and embedded in paraffin. Tissues were sectioned (5 µm), stained with haematoxylin and eosin and scored for islet inflammation under light microscopy as follows; 0 if no inflammation, 1 + if 1–10% of the islet was infiltrated, 2 + if 10–25%, 3 + if 25–75% and 4 + if more than 75% islet involvement or islet fibrosis. A minimum of ten islets a rat were analysed from at least two different sections. The average insulinitis score was calculated for each animal. The mean of the average insulinitis scores of animals from each treatment group was compared by Student's *t* test. The frequency of islets with

each insulinitis score was also calculated in each animal by dividing the number of islets with each score by the total number of scored islets. The mean frequency of each histopathologic score in *BB* rats treated with saline was compared with that of those treated with cyclophosphamide.

Analysis of cell surface phenotypes of mononuclear leucocytes. Mononuclear cells were isolated from spleen cells using Ficoll-Hypaque centrifugation. A minimum of 10,000 cells were analysed by flow cytometry (FacScan, Becton Dickinson, Mountain View, Calif., USA). We used OX19 (pan T-cell phenotype) FITC-conjugated antibody and OX8 (cytotoxic T-cell and natural killer (NK) cell phenotype) and PE-conjugated antibody (Serotec, Oxford, UK) to delineate the phenotypes. We identified OX19⁺OX8⁺ cells as cytotoxic/suppressor T-cell phenotype, OX19⁺OX8⁻ cells as helper/inducer cells and OX19⁻OX8⁺ cells as NK cells. We measured RT6.1⁺ T-cells, reported to have suppressor-like activity [13], using unconjugated DS4.23 (a rat monoclonal antibody from D. Lubaroff) and goat anti-rat IgG conjugated to PE (Serotec).

Spleen cell proliferation assay. Splenic mononuclear leucocytes were isolated by Ficoll-Hypaque gradient centrifugation. We incubated 100,000 cells per well (Costar 2779) in quadruplicate with concanavalin A (Con A) (Sigma, St. Louis, Mo., USA) or lipopolysaccharide (LPS, Sigma) in complete media (RPMI 1640 supplemented with 10% FCS, 2 mmol/l L-glutamine, 10 µmol/l 2-mercaptoethanol, 2 mmol/l non-essential amino acids, 25 µmol/l HEPES buffer, 100 µg/ml penicillin and 10 µg/ml streptomycin Gibco BRL Grand Island, N.Y., USA) or in complete media alone for 5 days in 5% CO₂ at 37°C. The cells were incubated with 18.5 kBq of ³H-thymidine (DuPont New England Nuclear, Boston, Mass., USA), for the last 16 h of the total incubation, harvested onto filter paper, and counted for radioactivity in a beta scintillation counter (Wallace 1205 Beta plate, Turku, Finland).

Adoptive transfer of spleen cells. To examine if the induction of non-specific immunoregulatory cell activity plays a part in the diabetes sparing effect of cyclophosphamide treatment, we determined if the adoptive transfer of splenocytes from *BB* rats treated with cyclophosphamide to young recipient rats inhibits the development of diabetes. Mononuclear splenocytes (60 × 10⁶ cells) were isolated from 55-day-old *BB* rats previously treated with cyclophosphamide (60 mg/kg total dose) or saline and then intravenously injected into untreated recipient *BB* rats (ten recipient rats for cyclophosphamide, nine recipients for saline) at 22–24 days of age and again 1 week later. The development of diabetes in rats receiving cells from animals treated with saline and from those treated with cyclophosphamide were then compared.

Cytokine gene expression. Total RNAs were extracted from spleen cells of cyclophosphamide-treated and saline-treated *BB* rats by the acid guanidine thiocyanate-phenol-chloroform method [14]. Splenic total RNAs (3 µg) were converted to cDNAs using Superscript II (Life Technologies, Gaithersburg, Md., USA) and oligo (dT)_{12–18} (Life Technologies) in 20 µl of the reaction mixture at 42°C for 1 h. After the synthesis of cDNA, PCR was run using specific primers for IL-1β, IL-2, IL-4, IL-10, IL-12, IFN-γ and TNF-α as described previously [15]. Hypoxanthine phosphoribosyltransferase (HPRT) was used as an internal standard. The PCR condition was optimized for each set of primers. We did PCRs using a different number of cycles to ensure that amplification occurred in a linear range. Cycle numbers for the different cytokines were as follows: 25 cycles for HPRT and IL-1β; 30 cycles for IL-12,

Fig. 1. Incidence of diabetes in *BB* rats given saline (□) ($n = 9$) or cyclophosphamide (◆) (175 mg/kg) ($n = 8$) i.p. at 44 days of age. The % cyclophosphamide-treated diabetic animals was calculated using the total number of animals not killed by drug toxicity as the denominator. Four rats died from the drug at ages 49–54 days

IFN- γ , IL-2 and IL-10; and 35 cycles for TNF- α and IL-4. The PCR mixture (50 μ l) contained 0.2 mmol/l concentration of each deoxynucleotide triphosphate, 1 μ mol/l concentration of each primer, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol-TRIS-Cl (pH 9.0) and 2.5 units of *Taq* polymerase (Pharmacia, Uppsala, Sweden). After amplification, the products were subjected to electrophoresis on 2% agarose gel and detected by ethidium bromide staining. Individual bands were image analysed with Image Master 1D (Pharmacia Biotech, Uppsala, Sweden). We normalized RT-PCR signals by calculating the ratio of each cytokine signal divided by the HPRT signal.

Statistical analysis. The product limit method of Kaplan and Meier was used to estimate survival from diabetes. Test group proportions were compared with chi-squared analysis. Group means were assessed by Student's *t* test. We considered *p* values less than 0.05 to be significant.

Results

Effect of cyclophosphamide on diabetes. The effect of three dosages of cyclophosphamide on the development of diabetes was studied. In all experiments, except the first, the total dose of cyclophosphamide was equally divided and given within 7 days of each other. In the first experiment, a single i.p. injection of 175 mg/kg cyclophosphamide ($n = 8$) or saline ($n = 9$) was given to 44-day-old *BB* rats ($n = 8$). Half of the rats injected with cyclophosphamide died of drug toxicity of the gastrointestinal system within 10 days of injection. The development of diabetes was significantly slower in rats treated with cyclophosphamide than in control animals by survival curve analysis ($p < 0.02$) (Fig. 1). The final incidence of diabetes was lower in the cyclophosphamide-treated

Fig. 2. Incidence of diabetes in *BB* rats treated with saline (□) ($n = 9$) or a total cyclophosphamide (◆) dosage of 120 mg/kg body weight ($n = 11$) equally divided and injected at 39 and 43 days of age

ed group by chi-squared analysis when assessing all animals not dying from drug toxicity (25% vs 89% $p = 0.05$).

Cyclophosphamide at a lower total dose (120 mg/kg) was divided into equal doses and given to *BB* rats ($n = 11$) at 39 and 43-days-of age (Fig. 2). None of the cyclophosphamide-treated rats died from drug toxicity. After 6 weeks of cyclophosphamide treatment, mean ($\pm$ SEM) leucocyte count of animals treated with cyclophosphamide and saline were 12.1 ± 0.9 and 8.5 ± 1.8 , respectively ($\times 10^3$ cells/ μ l blood) ($p > 0.05$). This dosage of cyclophosphamide also considerably decreased the development of diabetes compared with saline-treated control rats ($n = 9$) by survival curve analysis ($p < 0.015$). In addition, cyclophosphamide-treated rats had a lower final incidence of diabetes by chi-squared analysis by the end of the experiment (130 days) than saline-injected control animals (18% vs 78%, $p < 0.012$). Because animals at the end of this experiment could have potentially developed diabetes soon thereafter, the blood glucose tolerance of three non-diabetic rats treated with cyclophosphamide was assessed at the end of the experiment. Blood glucose concentrations after an i.p. injection of 0.5 mg/kg of glucose were entirely normal in these three animals (Fig. 3).

In younger animals, we next compared the effect of giving cyclophosphamide in a total dosage of 120 mg/kg with a lower total dosage of 60 mg/kg. We injected *BB* rats with either 30 mg/kg cyclophosphamide ($n = 18$), 60 mg/kg cyclophosphamide ($n = 8$) or saline ($n = 13$) at 30 and then again at 33 days of age. Of eight rats three died of gastrointestinal toxicity

Fig. 3. Glucose tolerance of non-diabetic 130-day-old BB rats ($n = 3$: $\square$ —, rat 1: $\blacklozenge$ — rat 2: $\blacksquare$ — rat 3) previously treated with a total cyclophosphamide dosage of 120 mg/kg body weight and a diabetic rat ($\diamond$ —). Blood glucose was determined at 0, 30, 60 and 120 min after giving i.p. glucose

ty after receiving the 120 mg/kg total dosage. We selected at random nine rats treated with saline, eight with 60 mg/kg and eight with 120 mg/kg cyclophosphamide to be weighed weekly. Due to deaths in the 120 mg/kg group, seven animals were weighed at 35 days and five at 42 and 49 days in this group. Animals in this group gained weight more slowly over the first 10 days compared with saline-treated rats ($p < 0.005$) (Fig. 4). After the first 10 days of cyclophosphamide treatment the mean rate of weight gain of surviving animals was, however, similar in this and the saline-treated groups. Rats injected with a total dosage of 60 mg/kg cyclophosphamide gained weight normally. None of these animals showed any behavioural side effects. The development of diabetes was lower in rats given a total dosage of 120 mg/kg cyclophosphamide than in control animals by survival curve analysis ($p < 0.005$) (Fig. 5). The final incidence of diabetes was less in the group treated with 120 mg/kg cyclophosphamide than controls at the end of the experiment by chi-squared analysis (40% vs 100%, $p < 0.03$). The overall development of diabetes was also lower in the group treated with a total dose of 60 mg/kg cyclophosphamide than controls by survival curve analysis ($p < 0.004$). The final incidence of diabetes in the first group (60 mg/kg) was not significantly altered.

Pancreatic histopathology. Pancreata from 55-day-old rats treated with cyclophosphamide (60 mg/kg total dose) ($n = 5$) or saline ($n = 4$) were compared. The total number of islets counted in saline-treated rats was 55 and that for cyclophosphamide-treated rats was 59 islets. Islet inflammation was present in both groups and consisted predominantly of mononuclear cells. There was very little exocrine involvement. The mean ($\pm$ SEM) insulinitis score was considerably lower in cyclophosphamide-treated rats (0.80 ± 0.34 vs

Fig. 4. Mean weights ($\pm$ SEM) of BB rats treated with saline ($\square$ —) ($n = 9$), cyclophosphamide at a total dose of 60 mg/kg body weight ($\blacklozenge$ —) ($n = 8$), or at a total dosage of 120 mg/kg body weight ($\blacksquare$ —) ($n = 8$). * $p < 0.01$; ** $p < 0.005$

Fig. 5. Incidence of diabetes in BB rats given saline ($\square$ —) ($n = 13$), cyclophosphamide at 60 mg/kg body weight ($\blacklozenge$ —) ($n = 18$) or at 120 mg/kg body weight ($\blacksquare$ —) ($n = 8$)

2.43 ± 0.33) ($p < 0.001$). The mean proportion of islets (% of total) with "0" insulinitis score was greater in cyclophosphamide-treated than control rats ($p < 0.006$). The mean proportion of islets with "2+" and "3+" scores was also lower ($p < 0.05$) in cyclophosphamide-treated rats [Fig. 6].

Con A and LPS induced blastogenesis. The proliferative response of mononuclear spleen cells to Con A and LPS was compared in 55-day-old BB rats treated with cyclophosphamide (60 mg/kg total dose) ($n = 4$) or saline ($n = 4$). A lower proliferative response to Con A concentrations of 1, 2, 4, and 8 μ g/ml was observed in spleen cells from cyclophosphamide-treated rats (Fig. 7). Spleen cell responses (Δ cpm $\pm$ SEM) to LPS (5 mcg/ml) of cyclophosphamide-treated rats were also diminished (1761 ± 185 cpm vs 2917 ± 185 cpm in controls, $p < 0.01$).

Mononuclear spleen cell count and subsets. The mean ($\pm$ SEM) total number of mononuclear spleen cells was higher in 55-day-old rats treated with cyclophosphamide (60 mg/kg total dosage) ($n = 4$) than in those treated with saline ($n = 4$) (161.2 ± 12.4 vs 132.0 ± 16 million cells, $p < 0.02$). Flow cytometric

Fig. 6. The frequency of islets with individual insulinitis scores from *BB* rats treated with saline (—) and cyclophosphamide (—). Islets from 55-day-old rats previously treated with saline ($n = 5$) or cyclophosphamide (60 mg/kg total dosage) ($n = 5$) were assigned histopathological scores as described in the Materials and methods. The mean frequency of each score was compared in rats treated with saline and cyclophosphamide. * $p < 0.05$

analysis of these mononuclear spleen cells was assessed. Similar proportions of OX8⁺OX19⁺ cells (NK cell phenotype), OX8⁺OX19⁺ (helper/inducer phenotype) and OX19⁺OX8⁺ was found in each group (Table 1). In addition, the proportion of RT6.1⁺ cells was less than 2% in each treatment group.

Adoptive transfer experiments assessing the induction of immunoregulatory cells. The induction of non-specific immunoregulatory cells by cyclophosphamide treatment was assessed by determining if the adoptive transfer of spleen cells from cyclophosphamide-treated rats into young recipient *BB* rats suppresses the development of diabetes (Fig. 8). The rate of development of diabetes was lower in rats receiving spleen cells from cyclophosphamide-treated animals than in rats receiving cells from saline-treated animals by survival curve analysis ($p < 0.0008$).

Cytokine gene expression after cyclophosphamide treatment. The effect of cyclophosphamide treatment (60 mg/kg total dosage) on cytokine gene expression in spleen cells of 55-day-old *BB* rats was examined. *BB* rats injected with cyclophosphamide had lower amounts of spleen cell IL-12, IL-1 β , IL-2, IFN- γ and

Fig. 7. The effect of cyclophosphamide treatment on Con A responses of spleen cells. Mononuclear spleen cells were obtained from 55-day-old *BB* rats ($n = 4$ per group) given saline (—) or cyclophosphamide (—) (60 mg/kg body weight total dosage) and incubated (10^5 cells/well) with Con A. The cells were incubated with ³H-thymidine (18.5 kBq/well) for the last 16 h of a 5-day total incubation time and then harvested and counted for radioactivity. ** $p < 0.001$

TNF- α mRNA than saline-treated controls (Fig. 9, 10) but IL-4 gene expression was increased.

Discussion

Cyclophosphamide treatment at all dosages used (60 to 175 mg/kg) inhibited the development of diabetes in *BB* rats. The diabetes sparing effect was overall related to dosages. The 120 mg/kg dosage resulted in a 77% decrease in final diabetes incidence. Although also effective, the higher cyclophosphamide dosage (175 mg/kg) was very toxic. Toxicity with the 120 mg/kg dosage occurred only when younger animals were treated.

The diabetes sparing effect seems to be mediated by the inhibition of the islet inflammatory process because the mean insulinitis score was considerably lower in cyclophosphamide-treated rats, a 67% reduction from the mean insulinitis score of control animals. In addition, the cyclophosphamide-treated rats had a greater proportion of islets with less inflammation and a lower proportion of islets with extensive inflammation.

The diabetes sparing effect of cyclophosphamide (120 mg/kg total dose) was also found to be long last-

Table 1. Cell surface phenotypes of mononuclear spleen cells from 55-day-old *BB* rats given saline or cyclophosphamide (60 mg/kg)

Treatment	Cell subset			
	OX19 ⁺ OX8 ⁺	OX19 ⁺ OX8 ⁻	OX19 ⁻ OX8 ⁻	OX19 ⁻ OX8 ⁺
Saline ($n = 4$)	12.3 $\pm$ 2.2	79.7 $\pm$ 2.3	6.9 $\pm$ 0.1	1.0 $\pm$ 0.1
Cyclophosphamide ($n = 4$)	11.7 $\pm$ 1.4	80.6 $\pm$ 1.8	6.8 $\pm$ 0.3	0.8 $\pm$ 0.1

Mean values $\pm$ SEM are depicted

Fig. 8. Inhibition of diabetes development after the adoptive transfer of spleen cells from cyclophosphamide-treated rats. Mononuclear spleen cells (60×10^6 cells) from *BB* rats previously given either saline or cyclophosphamide (60 mg/kg body weight total dosage) were injected i.v. into *BB* rats at 25 days of age and then again at 40 days of age. The development of diabetes was then compared in recipient animals; saline (□) ($n = 9$), cyclophosphamide (◆) ($n = 10$)

ing. Blood glucose tolerances of non-diabetic cyclophosphamide-treated rats at the end of the experiment were normal. Thus, even after 3 months of treatment, rats were protected and were not imminently about to develop diabetes.

Our experiments support the hypothesis that the inhibition of insulinitis and resultant decrease in diabetes by cyclophosphamide is mediated through the induction of immunoregulatory cells. We found the development of diabetes to be statistically significantly inhibited when *BB* rats are injected with spleen cells from cyclophosphamide-treated rats. This induction of immunoregulatory cells could account for the finding that treating *BB* rats with cyclophosphamide inhibited spleen cell responses to LPS and Con A in vitro. The decreased LPS response could also potentially be due to alterations in the number of B cells which was not evaluated. We cannot rule out the possibility that this decreased proliferative activity or inhibition of diabetes resulted in part from a direct inhibitory toxic effect of cyclophosphamide on T cells even though we found no decrease but an increase in the total number of splenic mononuclear cells.

The specific cell(s) responsible for the suppression of insulinitis and diabetes is not known. Phenotypic examination of spleen cells provided no evidence of its type. This analysis also showed that the proportion of RT6.1⁺ cells, putative suppressor cells [13], was not increased with cyclophosphamide. These data do not, however, rule out the possibility that any measured cell subset augments immunoregulatory activity which in turn plays a part in inhibiting the diabetic process.

Although cyclophosphamide can induce bone marrow suppression and in turn immunosuppression,

Fig. 9 A,B. RT-PCR analysis of cytokine gene expression in splenocytes. Total RNA was isolated from the splenocytes of saline ($n = 3$) and cyclophosphamide (60 mg/kg body weight) ($n = 3$) previously 55-day-old *BB* rats treated RT-PCR was done as described in Materials and methods. **A** Lanes 1–3: PBS-treated; Lanes 4–6: cyclophosphamide-treated. **B** Individual bands were image analysed. RT-PCR signals were normalized by calculating the ratio of each cytokine signal divided by the HPRT signal. The mean ratios are compared and depicted. ■ = saline, ▨ = cyclophosphamide. * $p < 0.05$, ** $p < 0.01$

bone marrow suppression probably does not account for the diabetes sparing effect of cyclophosphamide because peripheral leucocyte counts were never decreased 4 and 6 weeks after treatment with this agent, spleen mononuclear cell numbers were increased in treated animals even with dosages of 125 mg/kg, and the transfer of spleen cells from treated rats inhibited the development of diabetes.

Paradoxically, treatment with cyclophosphamide has previously been shown to decrease suppressor cell activity, increase cell-mediated cytotoxicity, reverse immunologic tolerance [16–18] and accelerate autoimmune processes such as experimental allergic encephalomyelitis [19]. In addition, injection with cyclophosphamide has been shown to accelerate the onset of diabetes in NOD mice which seems to be due to a decrease in suppressor-like cell activity [20]. Thus, cyclophosphamide could be expected to accelerate the onset of diabetes in our experimental rats. The reason for its dichotomous effects on the diabetic process in NOD mice is not known, but cyclophosphamide injections have previously elicited dichotomous immunologic actions, e.g. they can augment or depress B-cell and cell-mediated immunologic responses [21]. The dosage of cyclophosphamide could potentially be an important modulating factor. In other experimental models, low dosages (20 mg/kg) decreased suppressor cell activity [22] and high dosages (similar to those we found to inhibit diabetes) augmented suppressor cell activity [11, 23]. Further, our data supports the notion that cyclophosphamide has a positive dose-related diabetes sparing effect. Alternatively, differences in genetic factors and immune responses of the *BB* rat and NOD mouse could account for the discrepant biologic responses, acceleration versus inhibition of the diabetic process.

The augmentation of immunoregulatory cell activity by cyclophosphamide treatment and the resultant inhibition of insulinitis and diabetes could result from the correction of an existing immunologic dysfunction in the *BB* rat thought to be important in the pathogenesis of diabetes [24, 25]. We have, however, found no evidence that cyclophosphamide corrects any of the immunologic deficiencies examined in the diabetes-prone rat, such as suppressed Con A responsiveness of spleen cells or depressed numbers of RT6.1⁺ cells, putative suppressor cells.

Interleukin-12 has been thought to mediate autoimmune processes including the autoimmune diabetic process [26]. Increased IL-12 production is found in high-risk first-degree relatives of Type I diabetic people [27]. Gene expression of IL-12 in islets correlate with beta-cell destruction in NOD mice [28]. Further, treatment with IL-12 accelerates but with IL-12 antagonist inhibits the autoimmune process in NOD mice [29,30]. Thus, our finding that treatment with cyclophosphamide decreases IL-12 mRNA in spleen cells suggests this inhibitory ef-

fect plays an important part in mediating the diabetes sparing effect of cyclophosphamide.

There is evidence that IL-1 β is important for the pathogenesis of autoimmune diabetes. Treatment with IL-1 receptor antagonist and IL-1 β neutralizing antibody decreases diabetes development in NOD mice [31, 32]. Interleukin-1 β also accelerates the autoimmune process in *BB* rats [33]. Thus, our finding of decreased IL-1 β gene expression in cyclophosphamide-treated *BB* rats implies that inhibition of IL-1 β gene expression by this agent could help mediate its diabetes sparing effect.

Tumour necrosis factor- α could also play a part in mediating the autoimmune diabetic process because treatment with TNF accelerates the diabetic process in NOD mouse [34], TNF is hyperexpressed in *BB* rats and NOD mice [35, 36], and treatment with TNF antibodies suppresses the development of diabetes in NOD mice [34]. Depending upon experimental conditions, TNF- α can, however, inhibit autoimmune diabetes [37]. Nonetheless, the down-regulation of TNF- α mRNA in cyclophosphamide-treated *BB* rats could be blocking a diabetogenic effect of endogenous TNF- α .

As these IL-12, IL-1 β and TNF- α cytokines inhibited by cyclophosphamide are derived from macrophages, and macrophage activity is important in the pathogenesis of diabetes [38], Cyclophosphamide could be suppressing the diabetic process by inhibiting macrophage activity [39] and its production of cytokines thought to have a pathogenic role in diabetes.

The inhibition of IL-12, TNF- α and IL-1 by cyclophosphamide could collectively be suppressing the development of diabetes by inhibiting Th1 differentiation which is important in the diabetic process [40], IL-12 has been shown to induce Th1 differentiation [41] and TNF- α and IL-1 could be required for this induction [42]. This mechanism of diabetes sparing by cyclophosphamide is further suggested by our finding that IL-2 and IFN- γ mRNA seem decreased in spleen cells of cyclophosphamide-treated rats. As mRNA of IL-4, a Th 2 cytokine was increased in cyclophosphamide-treated animals, immune deviation could play a part in the diabetes sparing activity of this agent.

Other immune modulators have been shown to augment immunoregulatory cell activity and inhibit the development of diabetes in *BB* rats. Freund's adjuvant induces this activity in *BB* rats [43, 44] and decreases Th1 cytokines. Freund's adjuvant works, however, through a mechanism of direct immune stimulation and not by immunosuppression from preferential cell cytotoxicity by which cyclophosphamide is thought to act. Also Freund's adjuvant is thought to act in *BB* rats by up-regulating TNF- α [45, 46] and we found cyclophosphamide inhibits TNF- α gene expression.

Treatment with cyclophosphamide decreases the development of diabetes in the diabetes-prone *BB* rat by inhibiting the development of insulinitis. Further, this inhibition is associated with an increase in non-specific immunoregulatory cell activity. Experiments using cyclophosphamide treatment could be useful in explaining the pathogenesis of Type I diabetes.

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References

1. Nakhooda AF, Like AA, Chappel CL, Murray FT, Marlis EB (1976) The spontaneously diabetic Wistar rat: metabolic and morphologic studies. *Diabetes* 26: 100–112
2. Crisa L, Mordes JP, Rossini AA (1992) Autoimmune diabetes mellitus in the *BB* rat. *Diabetes Metab Rev* 8: 4–37
3. Dyrberg T, Nakhooda AF, Baekkeskov S, Lernmark A, Poussier P, Marlis EB (1982) Islet cell surface antibodies and lymphocyte antibodies in the spontaneous diabetic *BB* Wistar rat. *Diabetes* 31: 278–281
4. Castano L, Eisenbarth GS (1990) Type-1 diabetes: a chronic autoimmune disease of human, mouse, and rat. *Annu Rev Immunol* 8: 647–679
5. Fairchild RS, Kyner JL, Abdou NI (1982) Specific immunoregulation abnormality in insulin-dependent diabetes mellitus. *J Lab Clin Med* 99: 175–186
6. Crosti F, Secchi A, Falqui L et al. (1986) Impairment of lymphocyte-suppressive system in recent-onset insulin-dependent diabetes mellitus. *Diabetes* 35: 1053–1057
7. Mordes JP, Boretll R, Doukas J et al. (1996) The *BB*/Wor rat and the balance hypothesis of autoimmunity. *Diabetes Metab Rev* 12: 103–109
8. Fleming RA (1997) An overview of cyclophosphamide and ifosphamide pharmacology. *Pharmacotherapy* 17: 146S–154S
9. Harada M, Makino S (1984) Promotion of spontaneous diabetes in non-obese diabetes-prone mice by cyclophosphamide. *Diabetologia* 27: 604–606
10. Like AA, Weringer EJ, Holdash A, McGill P, Atkinson D, Rossini AA (1985) Adoptive transfer of autoimmune diabetes mellitus in Biobreeding/Worcester (*BB*/W) inbred and hybrid rats. *J Immunol* 134: 1583–1587
11. McIntosh KR, Segre M, Segre D (1982) Characterization of cyclophosphamide-induced suppressor cells. *Immunopharmacology* 4: 279–289
12. Brooks-Kaiser JC, Bourque LA, Hoskin DW (1993) Heterogeneity of splenic natural suppressor cells induced in mice by treatment with cyclophosphamide. *Immunopharmacology* 25: 117–129
13. Greiner DL, Mordes LP, Handler ES, Angelilo M, Nakamura N, Rossini AA (1987) Depletion of RT6.1⁺ T lymphocytes induces diabetes in resistant BioBreeding Worcester (*BB*/W) rats. *J Exp Med* 166: 461–475
14. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156–159
15. Chung YH, Jun HS, Kang Y et al. (1997) Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced autoimmune diabetes in diabetes-resistant biobreeding rats. *J Immunol* 159: 466–471
16. Mitsuoka A, Morikawa S, Baba M, Harada T (1979) Cyclophosphamide eliminates suppressor T-cells in age-associated central regulation of delayed hypersensitivity in mice. *J Exp Med* 149: 1018–1028
17. Rollinghoff M, Starzinski-Powitz A, Pfizenmayer K, Wagner H (1977) Cyclophosphamide-sensitive T lymphocytes suppress the in vitro generation of antigen specific T lymphocytes. *J Exp Med* 145: 455–459
18. Polak L, Turk JL (1974) Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. *Nature* 249: 654–656
19. Lando Z, Teitelbaum D, Arnon R (1979) Effect of cyclophosphamide on suppressor cell activity in mice unresponsive to EAE. *J Immunol* 123: 2156–2160
20. Yasunami R, Bach JF (1988) Anti-suppressor effect of cyclophosphamide in the development of spontaneous diabetes in NOD mice. *Eur J Immunol* 18: 481–484
21. Turke JL, Parker D (1982) Effect of cyclophosphamide on immunological control mechanisms. *Immunol Rev* 65: 99–113
22. Duclos M, Galanaud P, Devinsky O, Maillot MC, Dormont J (1977) Enhancing effect of low dose cyclophosphamide treatment on the in vitro antibody response. *Eur J Immunol* 7: 679–684
23. Nikcevic DA, Duffie GP, Young MR, Ellis NK, Kaufman GE, Wepsic HT (1987) Stimulation of suppressor cells in the bone marrow and spleens of high dose Cyclophosphamide-treated C57BL/6 mice. *Cell Immunol* 109: 349–359
24. Prud'homme G, Fuks A, Colle E, Seemayer TA, Guttman RD (1984) Immune dysfunction in diabetes prone *BB* rats. *J Exp Med* 159: 463–478
25. Greiner DL, Handler ES, Nakano K, Mordes JP, Rossini AA (1986) Absence of the RT-6 T cell subset in diabetes prone *BB*/W. *J Immunol* 136: 148–151
26. Adorini L, Gregori S, Magram J, Trembleau S (1996) The role of IL-12 in the pathogenesis of Th1 cell-mediated autoimmune disease. *Ann NY Acad Sci* 795: 208–215
27. Szelachowska M, Kretowski A, Kinalska I (1997) Increased in vitro interleukin-12 production by peripheral blood in high-risk IDDM first degree relatives. *Horm Metab Res* 29: 168–171
28. Rabinovich A, Suarez-Pinzon WL, Sorensen O (1996) Interleukin 12 mRNA expression in islets correlates with beta-cell destruction in NOD mice. *J Autoimmun* 9: 645–651
29. Trembleau S, Penna G, Bosi E, Mortara A, Gately MK, Adorini L (1995) IL-12 administration induces Th1 cells and accelerates autoimmune diabetes in NOD mice. *J Exp Med* 181: 817–821
30. Rothe H, O'Hara RM Jr, Martin S, Kolb H (1997) Suppression of cyclophosphamide induced diabetes development and pancreatic Th1 reactivity in NOD mice treated with the interleukin (IL)-12 antagonist IL-12. *Diabetologia* 40: 641–646
31. Dinarello CA (1992) Interleukin-1 and tumor necrosis factor: effector cytokines in autoimmune diseases. *Semin Immunol* 4: 133–145
32. Cailleau C, Diu-Hercend A, Ruuth E, Westwood R, Carnaud C (1997) Treatment with neutralizing antibodies specific for IL-1 beta prevents cyclophosphamide-induced diabetes in nonobese diabetic mice. *Diabetes* 46: 937–940
33. Vertrees S, Wilson DA, Ubungen R et al. (1991) Interleukin-1 beta regulation of islet and thyroid autoimmunity in the *BB* rat. *J Autoimmun* 4: 717–732

34. Yang X-D, Tisch R, Singer SM et al. (1994) Effect of tumor necrosis factor α on insulin-dependent diabetes mellitus in NOD mice. I. The early development of autoimmunity and the diabetogenic process. *J Exp Med* 180: 995–1004
35. Chosich N, Rockett E, Harrison LC (1994) Endogenous TNF production differs between high and low diabetes incidence non-obese diabetic (NOD) mice. *Autoimmunity* 18: 164–168
36. Rothe H, Ongoren C, Martin S, Rosen P, Kolb H (1994) Abnormal TNF-alpha production in diabetes-prone *BB* rats: enhanced TNF-alpha expression in defective PGE2 feedback inhibition. *Immunology* 81: 407–413
37. Satoh J, Seino H, Abo T et al. (1989) Recombinant human tumor necrosis factor alpha suppresses autoimmune diabetes in nonobese diabetic mice. *J Clin Invest* 84: 1345–1348
38. Lee KU, Pak CY, Amano K, Yoon J-W (1988) Prevention of lymphocytic thyroiditis and insulinitis in diabetes-prone *BB* rats by the depletion of macrophages. *Diabetologia* 31: 400–402
39. Pennington JE, Harris EA (1981) Influence of immunosuppression on alveolar macrophage chemotactic activities in guinea pigs. *Am Rev Respir Dis* 123: 299–304
40. Katz JD, Benoist C, Mathis D (1995) T helper cell subsets in insulin-dependent diabetes. *Science* 268: 1185–1188
41. Germann T, Gately MK, Schoenhaut DS et al. (1993) Interleukin-12/T cell stimulating factor, a cytokine with multiple effects on T helper type I (Th1) but not on Th2 cells. *Eur J Immunol* 23: 1762–1770
42. Shibuya K, Robinson D, Zonin F et al. (1998) IL-1 α and TNF- α are required for IL-12-induced development of Th1 cells producing high levels of IFN- γ in BALB/c but not C57BL/6 mice. *J Immunol* 160: 1708–1716
43. Sadelain MW, Quin HY, Sumoski W, Parfrey N, Singh B, Rabinovitch A (1990) Prevention of diabetes in the *BB* rat by early immunotherapy using Freund's adjuvant. *J Autoimmun* 3: 671–680
44. Qin HY, Suarez WL, Parfrey N, Power RF, Rabinovitch A (1992) Mechanism of complete Freund's adjuvant protection against diabetes in *BB* rats: induction of nonspecific suppressor cells. *Autoimmun* 12: 193–199
45. Rabinovitch A, Suarez-Pinzon WL, Lapchak PH, Meager A, Power RF (1995) Tumor necrosis factor mediates the protective effect of Freund's adjuvant against autoimmune diabetes in *BB* rats. *J Autoimmun* 8: 357–366
46. Lapchak PH, Guilbert LJ, Rabinovitch A (1992) Tumor necrosis factor production is deficient in diabetes-prone *BB* rats and can be corrected by complete Freund's adjuvant: a possible immunoregulatory role of tumor necrosis factor in the prevention of diabetes. *Clin Immunol Immunopathol* 65: 129–134