Prevention of Adoptive Transfer in BB Rats by Prophylactic Insulin Treatment

SYLVIE BERTRAND, MONIQUE DE PAEPE, CLAIRE VIGEANT, AND JEAN-FRANÇOIS YALE

Prophylactic insulin can prevent diabetes in the BB rat. We evaluated its use to prevent adoptive transfer of diabetes by activated splenocytes. ConA-activated spleen cells from acutely diabetic BB rats were divided into two equal aliquots and injected intravenously in paired diabetes-prone BB rat littermates. At the time of cell injection, subcutaneous insulin injections (15 $U \cdot kg^{-1} \cdot day^{-1}$) were started in one of each pair ($n = 21$) of littermates, and the control littermates ($n = 21$) were injected with saline. The incidence of diabetes, observed 35 days after cell injection, was 95% in control rats compared with 29% in insulin-treated rats ($P < 0.05$). To confirm the absence of diabetes, insulin was stopped in all nondiabetic insulin-treated rats at 63 days of age. An OGTT was performed at 65 days of age: 4 rats were glucose intolerant. All rats received comparable numbers of ConA-activated splenocytes. At the time the rats were killed, 3 insulin-treated rats had a completely normal morphology and a normal glucose tolerance. All control rats had insulitis whether they were diabetic or not. No significant difference in any mononuclear subset was observed in relation to insulin treatment. We conclude that prophylactic insulin can prevent adoptive transfer of diabetes in the BB rat without inducing changes in the mononuclear cell subsets. ***Diabetes*** **41:1273–77, 1992**

From the McGill Nutrition and Food Science Centre, Royal Victoria Hospital, Montreal, Quebec, Canada.

Address correspondence and reprint requests to Dr. Jean-François Yale, McGill Nutrition and Food Science Centre, 687 Pine Avenue West, Royal Victoria Hospital, Montreal, Quebec, Canada H3A 1A1.

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ConA, concanavalin A; OGTT, oral glucose tolerance test; IDDM, insulin-dependent diabetes mellitus; Cs, cyclosporine; CsA, cyclosporine A; IL-1, interleukin-1.

The diabetes-prone BB rat is an excellent animal model for spontaneous autoimmune IDDM. Both immunosuppression, such as with Cs, and immune reconstitution have prevented diabetes in BB rats (1). Prevention of spontaneous diabetes also has been achieved by prophylactic insulin, as documented by Gotfredsen et al. (2) and confirmed by others (3–5).

Adoptive transfer of diabetes has been reported by injection of ConA-activated spleen cells from acutely diabetic BB rats to young diabetes-prone BB rats (6,7). Cs administration to the recipient rats at the time of cell injection could not prevent adoptive transfer. Like has reported that long-term (>4 wk) insulin treatment of young diabetes-prone BB rats before adoptive-transfer experiments resulted in a marked decrease in the transfer success rate (3). Appel et al. (4) found a >75% incidence of diabetes transfer when they conducted adoptive-transfer studies on BB rats (at 170 days of age) 50 days after cessation of exogenous insulin that had been given since the age of 30 days and that had been successful in preventing spontaneous diabetes.

In this study, we assessed whether prophylactic insulin started at the time of cell injection could prevent the adoptive transfer of diabetes to young diabetes-prone BB rats.

RESEARCH DESIGN AND METHODS

Litters of young diabetes-prone BB rats and acutely diabetic BB rats (<3 days after diabetes onset) were obtained from Dr. Pierre Thibert (Animal Resources Division, Health and Welfare Canada, Ottawa, Ontario). The rats were kept in metabolic cages, in temperature- (20°C) and humidity- (70%) controlled rooms, in laminar flow hoods, with 12-h light-dark cycles. They were fed ad libitum (Purina Rat Chow, Ralston-Purina, St. Louis, MO).

The adoptive transfers were performed as described

previously (7). Briefly, splenocytes from acutely diabetic BB rats were stimulated with 5 μg/ml ConA for 72 h. At termination of the incubation period, 1.5 ml of the solution was taken for mononuclear cell counts. The cells from each spleen preparation then were separated into two equal aliquots, which were injected intravenously into two young (33 ± 1–day–old) diabetes-prone BB littermates. From the time of injection of the ConA-activated spleen cells, one of the littermates was treated with subcutaneous insulin (Ultralente, 15 U · kg^{-1} · day^{-1}), and the other was injected subcutaneously with saline daily. The recipient diabetes-prone BB rats were monitored for daily body weight and glycosuria. At 6 days after the cell injection, blood samples were taken (without anesthesia) from the tail vein of all rats for glucose measurements at 0, 4, and 8 h after the insulin or saline injection.

The onset of diabetes was diagnosed by the appearance of glycosuria and confirmed by the presence of fasting hyperglycemia (>11 mM). All diabetic rats were then treated according to a fixed protocol. Control rats received 1 U of insulin subcutaneously, whereas the rats from the insulin-treated group received their usual doses. Each day the insulin dose was adjusted according to glycosuria (Chemstrip uG 5000 K, Boehringer Mannheim, Montreal, Canada): it was decreased 0.5 U/day if negative and increased 0.5 U/day if >5 g/l; no change otherwise.

At 63 days of age, the subcutaneous insulin injections were stopped in all nondiabetic insulin-treated animals. An OGTT was performed at 65 days old. Then, 2.5 g glucose/kg (50 g/dl) were given by gavage. Plasma was sampled from the tail vein (without anesthesia) before and 60 min after the glucose load for plasma glucose measurements. Rats were considered glucose intolerant if their 60-min plasma glucose level was >11 mM.

Two days before the ConA-activated cell injections and on days 12 and 21 afterward, 2 ml of blood was sampled by cardiac puncture under light ether anesthesia for mononuclear cell counts and subset determination. All rats were killed between 66 and 68 days of age. The pancreas of each was carefully dissected, cut in half longitudinally, and each half weighed: one half was fixed in Bouin's solution for morphologic examination, the other half immediately frozen at −20°C for pancreatic insulin measurement.

Morphology. Paraffin-embedded sections of pancreas, 5 μm thick, were stained with hematoxylin-eosin and examined in coded fashion for insulitis, and periductular and acinar inflammation.

Assays. Plasma glucose was measured on a Beckman Glucose Analyzer II (Fullerton, CA). Each frozen pancreas sample was homogenized by ultrasonic disintegration at 4°C in 8 ml of an acid-ethanol solution (75% vol/vol ethanol, 1.5% vol/vol 12 M HCl, 23.5% vol/vol distilled water) and the extracts (supernatant after single centrifugation at 6000 *g* for 15 min) were kept at −20°C until assay. Insulin was measured on plasma and on appropriate dilutions of pancreatic extracts with guinea pig anti-rat insulin antibody (Linco Research, St. Louis, MO), ^{125}I-labelled porcine insulin, and rat insulin standards (24.5 IU/mg) (Novo Research Laboratory, Bagsvaerd, Denmark).

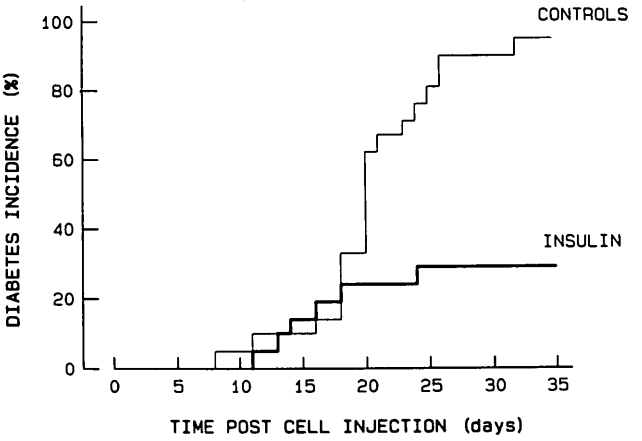

FIG. 1. Effects of prophylactic insulin treatment on diabetes incidence in young diabetes-prone BB rats after ConA-activated spleen cell injection from acutely diabetic BB rats, in relation to time. Incidence of diabetes was significantly lower in insulin-treated rats 35 days after cell injection ($P < 0.05$).

Mononuclear cell subsets were measured using a double-labeling direct immunofluorescence microassay. The combination of OX19-PE and W3/25-FITC monoclonal antibodies (both, Serotec, Bicester, UK) permitted measurement of the $CD4^+$ T-cells ($OX19^+W3/25^+$), $CD8^+$ T-cells ($OX19^+W3/25^-$) and monocytes ($OX19^-W3/25^+$). The double-labeling with OX19-PE and OX6-FITC (Serotec) allowed the measurements of B-cells ($OX19^-$ $OX6^{++}$) and Ia^+ T-cells ($OX19^+OX6^+$). Readings were performed using a FACScan Analyzer (Becton Dickinson, Sunnyvale, CA) and analyzed using a Consort 30 computer system (Becton Dickinson).

Statistical analysis. Analyses were performed using the SAS/STAT software (SAS Institute, Cary, NC) on a Hewlett-Packard Vectra computer. Data were analyzed using unpaired Student's *t* tests when the means of two groups were compared and Tukey's Studentized Range (HSD) test when the means of more than two groups were compared. Diabetes incidences were compared using Fisher's exact test. Results were considered significant for $P < 0.05$.

RESULTS

ConA-activated splenocyte injection was well tolerated by all BB rats. The incidence of diabetes in control rats 35 days after cell injection was 95%. Diabetes occurred, on average, 20.2 ± 1.2 days after cell injection. The prophylactic insulin treatment was well tolerated by all BB rats that received it, and it reduced the incidence of diabetes to 29% in the first 35 days after ConA-stimulated splenocyte injection (Fig. 1). Insulin treatment did not modify the early diabetes incidence, but almost completely prevented the occurrence of diabetes after 18 days. The OGTT, performed at 65 days of age, revealed that 4 of the insulin-treated rats (19%), but none of the control rats, were glucose intolerant.

The prophylactic insulin injections resulted in decreased blood glucose levels that lasted ~8 h (Fig. 2).

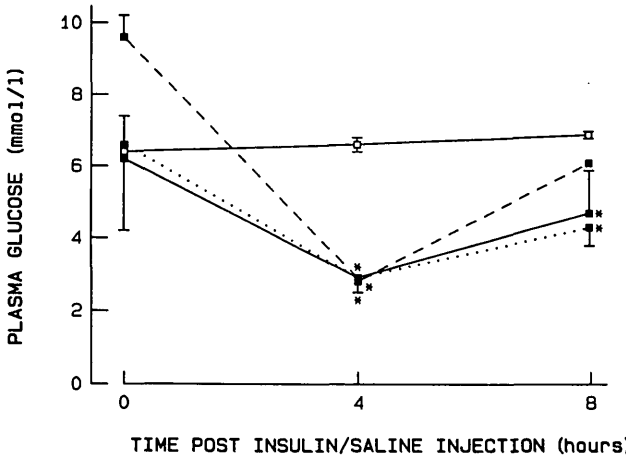

FIG. 2. Effects of prophylactic insulin treatment on the plasma glucose levels over the next 8 h, in relation to the subsequent metabolic outcome: (□—□), control rats; (■····■), insulin-treated rats with normal glucose tolerance; (■– – –■), insulin-treated rats with glucose intolerance; (■——■), insulin-treated rats with diabetes. *$P < 0.05$ vs. controls.

No significant difference was found between insulin-treated BB rats that later developed diabetes and those that did not in the level of glycemia obtained after the insulin injections. Neither were significant differences observed between control and insulin-treated BB rats, nor between rats that became diabetic and those that did not, in the number of ConA-activated spleen cells that were injected into the recipients, nor in the mononuclear cell subsets of the recipients before the cell injection—except for $CD8^+$ T-cells, which were lower in the insulin-treated rats that later became glucose intolerant (Table 1).

All diabetic BB rats had marked hyperglycemia at the time they were killed (control, 25 ± 1.7 vs. insulin-treated, 19 ± 2.5 mM; 12 h after insulin injection). Their pancreatic insulin content was markedly decreased (control, 0.77 ± 0.24 vs. insulin-treated, 1.6 ± 1.1 μg/g wet pancreas). Four insulin-treated BB rats were glucose intolerant at the time the rats were killed. At the OGTT, their mean blood glucose values were 9.1 ± 0.4 mM at 0 min and 16.8 ± 2.1 mM at 60 min. Their pancreatic insulin content was decreased (3.6 ± 2 μg/g wet pancreas). Also, 11 insulin-treated BB rats had a normal OGTT at the time they were killed (0 min, 7.9 ± 0.4; 60 min, 9.3 ± 0.2 mM); their pancreatic insulin content also was normal (37.6 ± 7.2 μg/g wet pancreas, $P < 0.05$).

Acinar inflammation was present in all BB rats examined except for 3 insulin-treated BB rats with normal glucose tolerance (Table 2). Periductular inflammation was present in all BB rats examined except for 3 control diabetic BB rats, 1 glucose-intolerant rat, and 5 insulin-treated BB rats with normal glucose tolerance.

Insulitis was found in all control BB rat samples examined, whether or not they were diabetic; 92% of control diabetic rats and 67% of insulin-treated diabetic rats revealed florid or end-stage insulitis at the examination. The nondiabetic control rat had florid insulitis. Three insulin-treated rats had a completely normal morphology at the examination and a normal glucose tolerance, and 2 of 4 glucose-intolerant rats showed insulitis. Of those remaining, one exhibited mild acinar inflammation without evidence of insulitis and the other had no islet on the section.

Independently of their metabolic outcome or insulin treatment, all rats showed a significant increase in $CD4^+$ T-cells 12 days after cell injection, with a partial decrease on day 21. As expected, all diabetes-prone BB rats had a profound T-lymphopenia, which was more severe in $CD8^+$ T-cells. $CD8^+$ T-cells did not change during the course of the study, remaining at very low levels (Fig. 3). The monocytes, B-cells, and Ia^+ T-cells were comparable during the whole study in the five groups (data not shown). No significant difference in any of these subsets was observed in relation to insulin treatment (Fig. 4).

DISCUSSION

We report, for the first time, a marked reduction in the incidence of adoptive transfer of diabetes through insulin treatment started at the time of injection of ConA-activated spleen cells of acutely diabetic rats to young diabetes-prone BB rats. This prevention was not related

TABLE 1
Comparison of immune parameters at time of ConA-activated cell injection in BB rats in relation to subsequent metabolic outcome and treatment group

		Insulin-treated rats		
	Control rats	Diabetes	Glucose intolerance	Normal glucose tolerance
n	21	6	4	11
Number of ConA-activated spleen cells injected (× 10^6)	45.5 ± 3.1	50.5 ± 2.0	41.5 ± 11.3	44.5 ± 4.5
Mononuclear cell subsets of recipients before cell injection (%)				
$CD4^+$ T-cells	17.6 ± 2.0	16.3 ± 8.6	9.7 ± 3.1	17.5 ± 2.0
$CD8^+$ T-cells	1.7 ± 0.2	1.7 ± 0.6	0.6 ± 0.2*	1.8 ± 0.15
Monocytes	24.6 ± 2.5	19.8 ± 10.4	27.3 ± 3.5	26.9 ± 3.3
NK-cells	30.6 ± 3.1	30.5 ± 1.0	36 ± 2.6	23.1 ± 1.3
B-cells	25.7 ± 2.6	31.8 ± 0.3	26.4 ± 2.5	30.7 ± 3.0
Ia^+ T-cells	1.6 ± 0.2	1.9 ± 1.4	1.3 ± 0.3	1.7 ± 0.2

Values are means ± SE. *n* = number of rats per group.
*$P < 0.05$ vs. other groups.

TABLE 2
Morphological data in relation to treatment group and metabolic outcome

	Control rats		Insulin-treated rats		
	Diabetes	Normal glucose tolerance	Diabetes	Glucose intolerance	Normal glucose tolerance
*n**	20	1	6	4	11
Insulitis					
Florid/end-stage (%)	92 (12)†‡	100	67 (3)§‖	67 (3)¶	45
Perl insulitis only (%)	8	0	33	0	27
No insulitis (%)	0	0	0	33	27
Acinar inflammation (%)	100 (17)†	100	100 (5)§	100	73
Periductular inflammation (%)	82 (17)†	100	100 (5)§	75	55
Pancreatic insulin content (μg/g)	0.77 ± 0.24	9.35	1.6 ± 1.1	3.6 ± 2.0	37.6 ± 7.2#

*n = number of rats in group. Data below are given as a percentage of this number, except where a different number of rats was used—as indicated in parenthesis with footnotes that explain the differences.
†Three pancreatic samples were inadequate for proper analysis.
‡In five samples, no islet could be identified.
§One pancreatic sample was inadequate for proper analysis.
‖In two samples, no islet could be identified.
¶In one sample, no islet could be identified.
#$P < 0.05$ vs. other four groups.

to changes in mononuclear cell subsets in the insulin-treated recipients.

Diabetes incidence was similar in control and insulin-treated rats up to 18 days after cell injection, suggesting that insulin treatment requires some time before its protective properties take effect. The mechanisms by which this effect occurs remain to be defined. Potential mechanisms include *1)* a protection from immune injury by inhibiting β-cell secretion and, potentially, the expression of a target antigen, *2)* an effect of either insulin or hypoglycemia on the immune effector cells, *3)* the induction of tolerance to insulin, and/or *4)* an increased secretion of adrenal cortical hormones as a result of hypoglycemia, with corticosteroid-induced immunosuppression.

Previous studies of spontaneous diabetes in the BB rat support the notion that the inhibition of β-cell activity is responsible for the prophylactic effects of exogenous insulin. We found that both exogenous insulin (with hypoglycemia) and diazoxide (with mild hyperglycemia) decreased the incidence of overt diabetes by 50% (5). In addition, numerous studies suggest that the expression of various autoantigens at the surface of the β-cells is highly dependent on the insulin secretory activity of these β-cells. Buschard et al. (8) reported a decrease in the binding of BB rat-derived islet-cell–specific monoclonal antibody IC2 by fasting—and an increase by glucose feeding. Appel et al. (9) revealed a decrease in the binding of three monoclonal antibodies (A2B5, R2D6, 3G5) to islet cells from NEDH rats in the presence of implanted insulinoma at the time of harvest of the target pancreas. Similarly, the binding of IC2 and A2B5 monoclonal antibodies to Lewis rat pancreatic islet cells has been shown to be glucose dependent in vitro (10). Finally, the expression of the 64,000-M_r autoantigen also

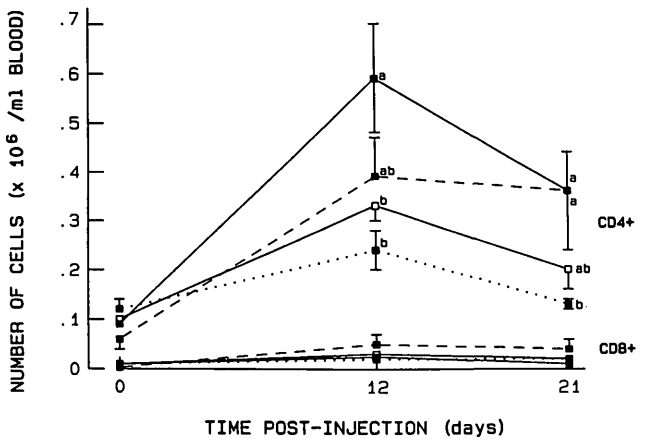

FIG. 3. Time-course of CD4⁺ and CD8⁺ T-cells in recipient BB rats after ConA-activated spleen cell injection, in relation to their treatment and subsequent metabolic outcome: (□—□), control rats; (■···■), insulin-treated rats with normal glucose tolerance; (■– – –■), insulin-treated rats with glucose intolerance; (■——■), insulin-treated rats with diabetes. Means sharing the same letter are not significantly different.

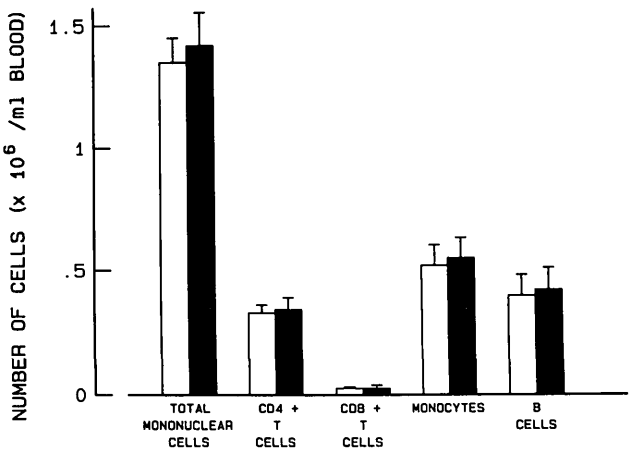

FIG. 4. Mononuclear cell subsets in recipient BB rats 12 days after the intravenous injection of ConA-activated spleen cells of the control BB rats (□) and insulin-treated BB rats (■). No significant difference was observed between the two groups.

was shown to be glucose dependent (11). These data suggest that protection by exogenous insulin could result from a decreased expression of autoantigens, "hiding" the resting β-cells from the activated immune system.

However, other mechanisms also may be operative. IL-1β–mediated β-cell impairment in vitro also was revealed to be modulated by the target β-cell secretory activity (12). These data suggest that β-cells are more resistant to some cytotoxic insults when they are at rest than when they are actively secreting insulin, by mechanisms presumably independent of the autoantigen density of their surface.

Although our adoptive-transfer study cannot address the mechanisms involved, it does provide further evidence that exogenous insulin can prevent not only initiation of the autoimmune process, but also the destruction of pancreatic β-cells by previously activated immune cells. This contrasts with our findings of unsuccessful prevention of adoptive transfer by CsA 10 $mg \cdot kg^{-1} \cdot day^{-1}$ in similar experiments (unpublished observations). Our data suggest that prophylactic insulin may be more potent than Cs in arresting the activated ongoing β-cell destruction.

The findings here may have important implications for human diabetes. Mirouze et al. (13) reported persistent remissions in subjects with newly diagnosed type I diabetes with the use of an external artificial pancreas. Suppression of endogenous insulin secretion by intensive insulin therapy for 2 wk at diagnosis of IDDM resulted in improved β-cell function and better metabolic control 1 yr post diagnosis compared with conventionally treated patients (14). In the Diabetes Control and Complications Trial, intensive insulin therapy resulted in improved β-cell function, as assessed by C-peptide, compared with standard treatment (15). In this context, our data suggest that intensive insulin treatment aimed at causing mild hypoglycemia might be more efficient than immunosuppressors in rapidly arresting the active ongoing β-cell destructive process at the onset of diabetes. Immunosuppressors might have a role in the more prolonged prevention of subsequent recurrence.

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