Spontaneous Diabetes Mellitus in the BB/W Rat

Effects of Glucocorticoids, Cyclosporin-A, and Antiserum to Rat Lymphocytes

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SUMMARY

Combination immunosuppression therapy with long-acting glucocorticoids, cyclosporin-A, and antiserum to rat lymphocytes (ALS) reduced the severity of spontaneous diabetes among BioBreeding/Worcester rats and decreased the frequency of diabetes in susceptible littermates. Combination therapy with glucocorticoids and three injections of ALS reversed hyperglycemia in a significant number of acutely diabetic animals. These results strengthen the hypothesis that autoimmunity plays a role in the pathogenesis of diabetes in these animals and increase our understanding of the requirements of treatment protocols for the prevention and cure of this spontaneous syndrome.

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Diabetes mellitus occurs spontaneously in approximately 40% of a partially inbred colony of BioBreeding/Worcester (BB/W) rats. The salient features of the syndrome include the genetic predisposition for the development of diabetes;[1] the abrupt onset of insulin-deficient, ketosis-prone diabetes between 60 and 120 days of age; lymphocytic insulitis with rapid and virtually complete destruction of pancreatic beta-cells;[2] lymphocytic thyroiditis without detectable abnormalities of thyroid function;[3] and the synthesis of autoantibodies to smooth muscle, thyroid colloid, and gastric parietal cells.[4,5] Other immunologic features of the syndrome include the reports that injections of antiserum to rat lymphocytes prevent and ameliorate the diabetic syndrome;[6] neonatal thymectomy prevents diabetes;[7] neonatal bone marrow transfusions reduce the frequency of diabetes;[8] susceptibility to develop diabetes is linked to the major histocompatibility complex;[9,10] and diabetic and prediabetic BB rats are profoundly lymphopenic.[11–13]

The observation that injections of antiserum to rat lymphocytes both prevent and ameliorate diabetes in the BB/W rat prompted us to test combinations of other clinically useful immunosuppressive agents for their ability to prevent and modify the diabetic syndrome.

In this communication, we present evidence that glucocorticoids and cyclosporin-A, in combination with antiserum to rat lymphocytes, significantly reduced the frequency of diabetes in susceptible animals. We also report that the combined administration of glucocorticoids and antiserum to rat lymphocytes normalized plasma glucose levels in a significant number of acutely diabetic BB/W rats.

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MATERIALS AND METHODS

ANIMALS

BB/W rats were obtained from the University of Massachusetts Medical School breeding facility. Animals between 60 and 90 days of age were tested for glycosuria three times weekly. Rats were defined as diabetic if their urine glucose indicated 2–4+ with Testape (Eli Lilly and Company, Indianapolis, Indiana) and if their plasma glucose (PG) concentrations exceeded 200 mg/dl. Animals were not included in the study if PG levels exceeded 400 mg/dl. This maximum level was chosen after a pilot study demonstrated that rats with an initial PG greater than 400 mg/dl on the day of detection virtually never responded favorably to the combination immune suppressive therapies used. Diabetic and sex-matched nondiabetic littermates (plasma glucose less than 180 mg/dl) were randomly assigned to five treatment groups.

TREATMENT PROTOCOLS

Group A. Animals assigned to this group received a single intramuscular injection (i.m.) of methylprednisolone acetate (Depomedrol, Upjohn Company, Kalamazoo, Michigan) 1 mg/100 g body wt. on the day diabetes was detected (day 0) and three intraperitoneal (i.p.) injections of rabbit anti-

serum to rat lymphocytes (ALS) (1.5 ml/rat) on days 0, 2, and 4.

Group B. Animals assigned to this group received one i.m. injection of Depomedrol, 1 mg/100 g body wt. and one i.p. injection of ALS (1.5 ml/rat), both on day 0 and cyclosporin-A (CSA), 1 mg/100 g body wt., for 30 consecutive days. CSA was dissolved in 0.2-ml olive oil and administered by stomach tube.

The quantities of Depomedrol and CSA given were the maximum tolerated without serious toxicity (weight loss and death).

Group C. Rats assigned to this group were the controls for group A and received one i.m. injection of Depomedrol vehicle [an aqueous solution of polyethyleneglycol (30 parts), sodium chloride (9 parts), and myristyl-gamma-picolinium chloride (0.2 parts)] and three i.p. injections of normal rabbit serum (NRS). The timing and quantities of these injections were the same as in group A.

Group D. These animals acted as controls for group B and received one injection of Depomedrol vehicle, one injection of NRS, and 30 doses of olive oil. The timing, routes of administration, and quantities of these agents were the same as in group B.

Group E. These rats received i.p. injections of ALS (1.5 ml/rat) on days 0, 2, and 4 of the experiment.

REAGENTS

Depomedrol and its vehicle were gifts of the Upjohn Company. Cyclosporin-A, the cyclic peptide fungal metabolite, was a gift of the Sandoz Ltd. (Basel, Switzerland). Rabbit antiserum to rat lymphocytes and normal rabbit serum were purchased from Microbiological Associates Inc. (Bethesda, Maryland). Olive oil was purchased from a local food market. Testape was a gift of Eli Lilly and Company.

ANIMAL CARE AND DISPOSITION

Diabetic animals entered into the study received daily subcutaneous insulin injections for the first 14 days, sufficient to prevent ketonuria and maintain normal body weight. The quantity of insulin administered was gradually reduced over the subsequent 5 days and stopped after day 19. Animals that became moribund (ketoacidosis and profound weight loss) after cessation of insulin injections were killed.

Nondiabetic animals that became diabetic after entering the study were not treated with insulin and were killed when moribund.

Diabetic rats that evidenced normalization of PG and nondiabetic animals that did not become diabetic were killed at 150 days of age. Diabetics that were free of ketonuria and maintained body weights after cessation of insulin therapy (stable diabetics) were also killed at 150 days of age.

POSTMORTEM PROCEDURES

At death, pancreatic tissue samples were fixed in Bouin's solution and paraffin-embedded sections stained with hematoxylin and eosin. Pancreata were examined (A.A.L.) without knowledge of the physiologic or treatment status of the animal and evaluated for the presence of insulitis.

RESULTS

Diabetic animals. Table 1 illustrates the response of the diabetic animals to the five treatment protocols. Only group A, the animals that received Depomedrol plus three injections of ALS, evidenced a significant cure rate (11/49, 22%). In comparison, none of the rats among the controls (group C) were cured. This difference is statistically significant ($X^2 = 4.9$, $P = 0.026$). The cure rate of group A was also significantly greater than that of group B ($X^2 = 7.5$, $P = 0.006$), which received Depomedrol, CSA, and one injection of ALS. Table 1 also illustrates the interesting fact that group A and B diabetic animals frequently became stable diabetics, i.e., maintained body weights and were free of ketonuria after cessation of insulin injections. Group E rats that received three injections of ALS did not evidence an improved cure rate but became stable diabetics with considerable frequency.

TABLE 1
Response of the diabetic rats to treatment protocols*

Group (N)	Cured	Remained diabetic	
		Stable	Ketotic
A (49)	11 (22%)	14 (29%)	24 (49%)
B (48)	1 (2%)	12 (25%)	35 (73%)
C (25)	0	1 (4%)	24 (96%)
D (23)	0	0	23 (100%)
E (50)	2 (4%)	23 (46%)	25 (50%)

*Chi square analyses: Cured: A versus B: $P = 0.006$; A versus C: $P = 0.026$; A versus E: $P = 0.0058$. Stable: A versus C: $P = 0.007$; B versus D: $P = 0.02$; B versus E: $P = 0.014$; A versus B: $P = 0.103$.

Figure 1 illustrates the plasma glucose response of diabetic group A animals. The upper portion of the figure records the pattern of unremitting hyperglycemia among the 38 animals that remained stable or ketotic diabetics. None of the ketotic animals survived after the fifth week of the study and relatively few of the stable animals survived beyond the tenth week. Most were killed when death appeared imminent in order to obtain tissues for morphologic study.

If grouped retrospectively according to the outcome (cured versus remained diabetic), the mean PG on the day diabetes was detected was somewhat lower among those group A rats that eventually evidenced normalization of PG (mean initial PG of cured diabetics = 285 ± 15 versus mean PG of ketotic diabetics = 317 ± 10). This difference, however, was not significant ($P = 0.085$).

There were no significant differences between the experimental groups when they were analyzed for age at entry into the study, sex distribution, body weights, and initial plasma glucose levels (data not illustrated).

Table 2 illustrates the response of the five groups of nondiabetic littermates to the treatment protocols. The frequency with which diabetes developed among susceptible littermates treated with Depomedrol plus three injections of ALS (group A) or Depomedrol, CSA, plus one injection of ALS (group B) was significantly less than that observed in their respective control groups (groups C and D). Three injections of ALS alone (group E) did not afford significant protection.

Transient diabetes was observed in 6 (12%) of the group B animals, but only in one member of group E. All seven of

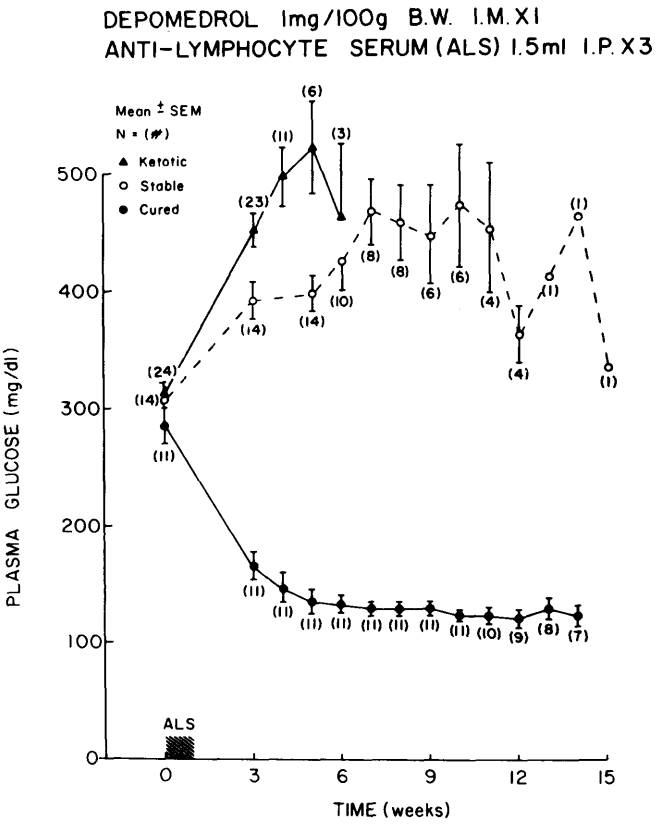

FIGURE 1. Plasma glucose (PG) concentrations (mean ± SEM) of diabetic animals treated with Depomedrol and three injections of ALS (group A), plotted retrospectively according to outcome. PG in cured animals returned to normal levels within 3 wk and remained normal for the duration of the experiment. PG concentrations in ketotic rats were generally higher than in stable animals. PG concentrations at day 0 were not significantly different in those animals destined to be cured.

these rats remained normoglycemic for the remainder of the experiment.

Although most of the permanently diabetic animals in these groups evidenced ketoacidosis and lethal syndromes, three rats of groups A and B, four of group C, six of group D, and five of group E were stable diabetics.

Figure 2 illustrates the PG values of nondiabetic group E animals (ALS × 3), and is, in general, also representative of the PG values of nondiabetic groups A and B. At the time of entry into the study, the PG values of those animals that would eventually become diabetic were not significantly different from the PG of the rats that remained normoglycemic throughout the experiment. It should be emphasized that the large SEMs recorded for the PG of the diabetic rats are a reflection of the fact that these animals first evidenced hyperglycemia at different times. Since this is a graph based on a retrospective grouping of animals according to the experimental outcome, data points at wk 7–10 included PG values of animals already diabetic as well as PG values of animals that were normoglycemic at that particular time but were to become hyperglycemic subsequently.

Morphologically, the pancreatic islets of diabetic rats that did not respond favorably to the various treatment regimens of groups A, B, and E could not be distinguished from the islets of the diabetic rats treated with the two vehicles and NRS (groups C and D), or the islets of the variously treated euglycemic littermates that eventually became diabetic. Most of these islets were small and revealed no evidence of surviving beta-cells. These "end-stage" islets were comprised essentially of non-beta-cells and a variable quantity of residual lymphocytes. The pancreata of cured diabetics and those rats with transient diabetes (see Table 2) evidenced variable degrees of islet injury, including active insulitis, focally scarred as well as normal and end-stage islets. The pancreatic islets of nondiabetic littermates that remained euglycemic during the entire period of observation were frequently but not always normal. Focal insulitis, fibrosis, and architectural disarray were observed.

TABLE 2
Response of nondiabetic littermates to treatment protocols*

Group (N)	Normal	Diabetic	
		Transient	Permanent
A (48)	38 (79%)	0	10 (21%)
B (48)	34 (71%)	6 (12%)	8 (17%)
C (50)	27 (54%)	0	23 (46%)
D (50)	27 (54%)	0	23 (46%)
E (49)	29 (59%)	1 (2%)	19 (39%)

*Chi square analyses: permanently diabetic: A versus C: P = 0.015; B versus D: P = 0.004; A versus E: P = 0.0245; B versus E: P = 0.0198; A versus B: P = 0.2037.

DISCUSSION

Although the precise pathogenesis and etiology of diabetes in the BB/W rat is not yet firmly established, the accumulated experimental data strongly suggest that these unique animals evidence abnormal immune regulatory mechanism(s). The initial abnormality that led to the animals' discovery, the spontaneous destruction of pancreatic beta-cells with resulting insulin-dependent diabetes, is preceded by lymphocytic insulitis.[2] It has since been reported[6,7] and confirmed[14,15] that antiserum to rat lymphocytes and neonatal thymectomy will ameliorate and prevent the diabetic syndrome. The results of both experimental interventions strengthen the autoimmune pathogenesis of the syndrome and implicate the key role of thymus-derived effector cells.

The data presented in this manuscript strengthen further the hypothesis that the BB/W rats evidence abnormal immune regulatory mechanisms. The successful normalization of plasma glucose levels in 22% of diabetic animals treated with one injection of glucocorticoids and three injections of ALS and the significantly reduced frequency of diabetes among susceptible euglycemic littermates treated with glucocorticoids and ALS, or glucocorticoids, ALS, and CSA are consistent with the concept that beta-cell destruction is mediated by an immunologic inflammatory attack.

The data presented above suggest that three injections of ALS *alone* were insufficient to cure or prevent diabetes but did result in a reduction in the severity of the diabetic syndrome as evidenced by the increased fraction of diabetic animals that were stable, i.e., survived for prolonged periods without insulin administration. The combination of one injec-

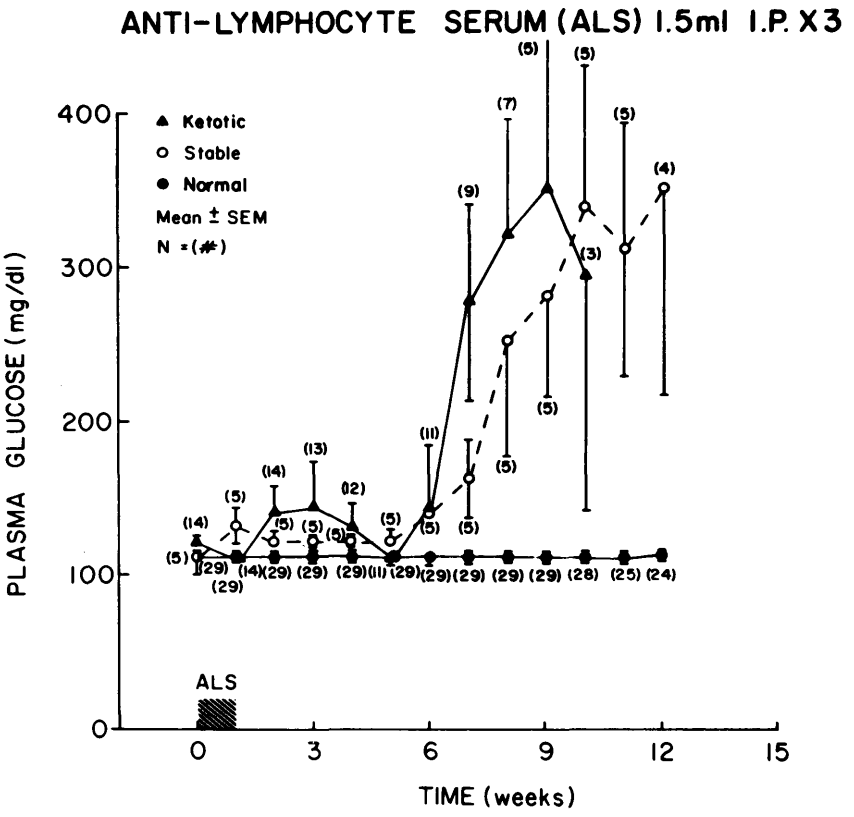

FIGURE 2. Plasma glucose (PG) concentrations (mean ± SEM) in nondiabetic rats treated with three injections of ALS (group E), plotted retrospectively according to outcome. PG concentrations at day 0 were not significantly different in those animals destined to become diabetic. See text for explanation of the large SEMs.

tion of glucocorticoid plus three injections of ALS (group A) was the most effective of the treatment regimens in curing and preventing diabetes. This is not surprising since glucocorticoids and ALS are both very potent lymphocytotoxic agents. The reduction of administered ALS to a single 1.5-ml injection significantly impaired the cure rate among group B diabetic animals, in spite of the addition of the fungal metabolite cyclosporin-A to the treatment regimen. This result, in retrospect, is also not surprising when one considers the fact that CSA is not itself cytotoxic and is generally believed to act by inhibiting T-cell lymphocyte proliferation in response to antigenic stimulation.[16] Hence, once insulitis was present and pronounced enough to have reduced the beta-cell population to the level where insulinopenia and hyperglycemia are evident, the action of CSA to prevent further antigen-induced T-lymphocyte proliferation may be futile. On the contrary, the combination of steroids, ALS, and CSA (group B) significantly reduced the frequency of diabetes among nondiabetic but susceptible littermates. That treatment regimen B was more effective in preventing than curing diabetes is also best explained by the action of CSA in preventing T-lymphocyte proliferation. It is also widely held that CSA action primarily targets helper (inducer) T-cells while sparing suppressor T-cells, with the end result of an enhanced or amplified suppressor cell activity.[17] Hence, it is logical to predict that CSA treatment of prediabetic rats may successfully prevent the onset of diabetes, if treatment is given immediately before or at the time of antigenic stimulation of T-lymphocytes, at which time the sparing of or amplification of suppressor cell activity may abort or minimize the destruction of pancreatic beta-cells. This assumption is supported by the occurrence of transient diabetes among six (12%) of the nondiabetics in group B. These animals generally became diabetic within the first week after entry into the study but characteristically returned to normoglycemic status within 10 days, presumably after sufficient numbers of suppressor T-cells were generated to prevent further pancreatic beta-cell loss.

The above hypothesis of CSA action is being tested in studies currently in progress in our laboratories wherein susceptible animals of different ages are receiving CSA alone for variable periods of time.

Only one additional animal (group E) evidenced transient diabetes. In our previously reported study, wherein ALS was administered to diabetic rats and nondiabetic littermates for a period of 30 days, we did not observe this phenomenon of transient diabetes.[6] In that earlier study, no effort was made to separate diabetics into stable and ketotic groups.

Although the precise mechanism of immune destruction of pancreatic beta-cells remains uncertain, the data presented in this manuscript strengthen our understanding of the requirements of treatment protocols for curing and preventing diabetes in BB/W rats and will be useful for the planning of future studies that will hopefully unravel the basic pathogenesis of this enlightening animal model of autoimmune endocrinopathy.

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