Prevention of Diabetes Mellitus in the BB/W Rat With Cyclosporin-A

ARTHUR A. LIKE, MD, VINCENT DIRODI, BS, SANDRA THOMAS, BS, DENNIS L. GUBERSKI, MS, and ALDO A. ROSSINI, MD

From the Departments of Pathology and Medicine, University of Massachusetts Medical School, Worcester, Massachusetts

Autoimmune diabetes mellitus occurs spontaneously in 40–60% of a colony of BioBreeding/Worcester rats. Pretreatment of susceptible animals for 10-day intervals prior to 70 days of age with Cyclosporin-A (CSA) significantly reduced the frequency and delayed the onset of diabetes. The relatively narrow time frame of successful treatment suggests that effector cells responsible for beta cell destruction in this model of Type I diabetes may be activated during this period of time prior to the onset of overt hyperglycemia. CSA administration did not protect against the occurrence of lymphocytic thyroiditis or autoantibodies directed against smooth muscle or thyroid colloid, suggesting that these BB immunologic phenomena may be controlled by a distinct series of immunologic events. (Am J Pathol 1984, 117:92–97)

AN ACUTE DIABETIC SYNDROME resembling human juvenile onset diabetes occurs spontaneously in approximately 40–60% of a partially inbred colony of BioBreeding/Worcester (BB/W) rats. Salient features of the syndrome include genetic predisposition[1]; abrupt onset of insulin dependent, ketosis-prone diabetes between 60–120 days of age; lymphocytic insulitis with virtually complete destruction of the pancreatic beta cells[2,3]; the occurrence of the syndrome in animals raised in a gnotobiotic environment.[4]

It has been proposed that the BB/W diabetic syndrome is the result of a cell-mediated autoimmune destruction of pancreatic beta cells. Support for an immune pathogenesis includes the predominantly lymphocytic nature of the islet infiltrate; normalization of plasma glucose levels in acutely diabetic rats and the prevention of diabetes in susceptible littermates following injections of rabbit antiserum to rat lymphocytes[5,6]; prevention and cure of diabetes with other immune suppressive agents (glucocorticoids and Cyclosporin-A [CSA])[7,8]; prevention of diabetes by neonatal thymectomy,[9,10] neonatal marrow transfusions[11,12] and transfusions of whole blood[13] or T-lymphocytes.[14]

The reports that susceptibility to diabetes may be linked to the major histocompatibility complex of the BB rat,[15,11] that diabetic-prone BB rats are profoundly lymphopenic, predominantly of thymus-derived lymphocytes,[16–19] and that diabetes can be adoptively transferred to young diabetes-prone BB/W rats,[20] Wistar-Furth animals,[21] and a control (nondiabetic) line of BB/W rats[22] provide additional and persuasive support for an immunologic pathogenesis of the syndrome. Finally, the observations that BB/W rats evidence spontaneous lymphocytic thyroiditis[23] and autoantibodies to smooth muscle, thyroid colloid, and gastric parietal cells[24,25] are not only consistent with an autoimmune pathogenesis but suggest that more than one cell type or antigenic determinant are under immunologic attack. Thus, it has been suggested that the predisposing defect in BB/W rats is more likely to be an abnormal immune response rather than antigenically altered target cell(s).[22,25]

We have previously reported that certain clinically useful immune suppressive agents modify the course of the diabetic syndrome in BB/W rats. Antiserum to rat lymphocytes (ALS) given alone for 1 month normalized plasma glucose levels in 36% of acutely diabetic animals and prevented the occurrence of diabetes in susceptible nondiabetic animals.[5] The combination

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Address reprint requests to Arthur A. Like, MD, Department of Pathology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01605.

of a potent long-acting glucocorticoid and three injections of ALS was somewhat less effective than prolonged ALS administration, but also prevented as well as cured the diabetic syndrome. In contrast, the combination of glucocorticoids, a single injection of ALS, and 30 doses of the fungal metabolite CSA, while effective in reducing the frequency of diabetes when given to nondiabetic susceptible animals, was ineffective after the onset of diabetes.[7] We explained the latter results as follows. Since CSA is not lymphocytotoxic, but is thought to act by inhibiting T-lymphocyte activation in response to antigenic stimulation,[26-28] it would not be effective in already diabetic animals in which lymphocytic insulitis and beta-cell destruction are well established. We also speculated that CSA administration prior to the onset of diabetes and at the time of antigenic stimulation of T-lymphocytes might have spared and/or amplified suppressor T-cell numbers which subsequently prevented or reduced the intensity of effector cell destruction of pancreatic beta cells.[7] The former hypothesis is consistent with the report that prolonged administration of CSA prevents diabetes in susceptible animals.[8]

Earlier reports of immunologic interventions in the BB rat were focused on the effects of a particular pharmacologic agent or immunologic maneuver on the incidence or persistence of diabetes. To date, we are not aware of any reports of the effects of immune suppression therapy on the frequency of lymphocytic thyroiditis or autoantibody synthesis in the BB rat.

In this communication, we present the results of experiments designed to determine whether properly timed short duration administration of CSA alone will prevent or delay the occurrence of diabetes among susceptible BB/W rats. We also report the effects of CSA treatment on the incidence of lymphocytic thyroiditis and the frequency of autoantibodies to smooth muscle and thyroid colloid in the sera of treated animals.

Materials and Methods

Experimental Animals

BB/W rats were obtained from the University of Massachusetts Medical School breeding facility. These animals have an expected frequency of diabetes of at least 40–60% between 60 and 120 days of age. Thirty, 40-, 50-, 60-, and 70-day-old animals were used. In all experiments, litters were randomly divided into CSA and olive oil, or CSA and Intralipid groups.

Treatment Protocols

In the first experiment, groups of 30-, 40-, 50-, 60-, and 70-day-old animals were given 10 consecutive daily doses of CSA, 1 mg/100 g body weight, dissolved in 0.2 ml olive oil by stomach tube. Randomly chosen littermates received an equivalent quantity of olive oil alone for the same duration of time.

In the second series of experiments, 30-, 40-, 50-, 60-, and 70-day-old animals received 10 consecutive daily intraperitoneal injections of CSA dissolved in Intralipid, 2 mg/100 g body weight, or an equivalent volume of Intralipid alone.

Reagents

CSA was a gift of Sandoz LTD, Basel, Switzerland. Olive oil was purchased in a local food store. Intralipid was purchased from Cutter Laboratories, Inc. Testape was a gift of the Eli Lilly Company.

Animal Care and Disposition

All experimental animals were tested for glycosuria twice weekly from 55 to 120 days of age. Rats were defined as diabetic if their urine glucose indicated 2–4+ with Testape and plasma glucose concentrations exceeded 200 mg/dl.

Diabetic animals were sacrificed within 1 week after detection. Nondiabetic rats were sacrificed at 120 days of age. Animals that died prior to 120 days of age without the diagnosis of diabetes were excluded.

Postmortem Procedures

At sacrifice, pancreas and thyroid tissue samples were fixed in Bouin's solution, and paraffin-embedded sections were stained with hematoxylin and eosin. Pancreatic and thyroid sections were evaluated (AAL) for the presence of insulitis and thyroiditis without knowledge of the animal's physiologic or treatment status. Serum samples obtained at autopsy were stored at −20 C and subsequently examined for the presence of autoantibodies to thyroid colloid and smooth muscle, by methods previously described.[25] The indirect immunofluorescence evaluations were performed by one or two observers on coded serum samples obtained from animals treated either with Intralipid alone or Intralipid with dissolved CSA.

Statistical analyses of 2 × 2 tables used the chi-square or Fisher Exact statistic. Analyses of the age of onset of diabetes used the nonparametric Mann–Whitney U test. All analyses were calculated by computer with the Statistical Package for the Social Sciences.

Results

Table 1 illustrates the results of the first study, wherein 30-, 40-, 50-, 60- and 70-day-old BB/W rats received

Table 1 – Effect of Cyclosporin-A on the Frequency of Diabetes and Thyroiditis*

	Frequency of diabetes		Thyroiditis	
Age (days)	CSA	Olive oil	CSA	Olive oil
30	2/10	3/10	2/10	0/10
40	7/14†	6/14†	3/14†	4/14†
50	10/15	10/15	5/15	6/15
60	7/30	22/30	16/30	13/30
70	11/30	13/29†	11/30	10/29†
	60–70-day CSA versus Olive oil			
	$\chi^2 = 15.02$			
	$P < 0.001$			

* All animals received 10 treatments (1 mg/100 g body weight).
† One animal in this group died without diabetes prior to 120 days of age.

10 daily oral doses of CSA (1 mg/100 g body weight) or olive oil. The data clearly indicate that CSA administered between 60 and 70-days of age significantly reduced the frequency of diabetes (CSA versus olive oil, $\chi^2 = 15.02$, $P < 0.001$). Treatment during this age range afforded significantly greater protection than that achieved when animals were treated with CSA during the earlier (30–60 days) and the later (70–80 days) age periods. The data also indicate that CSA did not protect against lymphocytic thyroiditis. The larger number of animals in the 60–70- and 70–80-day age groups is due to the fact that groups of rats were studied on two separate occasions so that we could be certain that the selective protection afforded by CSA administration was reproducible.

Table 2 illustrates the results of the second study, wherein 30-, 40-, 50-, 60- and 70-day-old BB/W animals received 10 consecutive intraperitoneal injections of CSA (2 mg/100 g BW) dissolved in Intralipid or an equivalent volume of Intralipid alone. The data clearly indicate that the larger quantity of CSA injected intraperitoneally afforded significant protection against diabetes when administered to 30-, 40- and 50-day-old rats as well as 60-day-old rats. Animals treated between 60 and 70 days of age evidenced the highest level of protection, whereas those treated between 70 and 80 days of age were not significantly protected against diabetes. The data contained in Table 2 indicate that this dose and method of administering CSA, while more protective than orally administered CSA against diabetes, again did not protect against lymphocytic thyroiditis.

Tables 3 and 4 illustrate the effects of CSA on the age of onset of diabetes in the several experimental groups. The data clearly suggest that both the oral and intraperitoneal routes of administration delayed the onset of diabetes. Although statistical significance was achieved in only two individual treatment groups, the data clearly suggest that CSA administration prior to 70 days of age afforded greater protection. Furthermore, when the data of the CSA and control animals were pooled prior to statistical analysis, CSA significantly delayed the age of onset of diabetes.

The pancreatic islets of the diabetic animals revealed lymphocytic insulitis with loss of beta cells and persistence of small aggregates of nonbeta cells (alpha, delta, and pancreatic polypeptide cells). The islet morphology of diabetic rats was identical in CSA and control animals. Nondiabetic animals of all experimental groups sometimes evidenced moderate degrees of lymphocytic insulitis. The frequency of insulitis in nondiabetic rats was not affected by the dose, route, or timing of CSA administration (data not illustrated).

CSA dissolved in Intralipid and administered intraperitoneally did not significantly alter the frequency of smooth muscle (ASM) or thyroid colloid (ATC) autoantibodies in any of the CSA-treated animals, when compared with animals receiving Intralipid alone. This

Table 2 – Effect of Cyclosporin/Intralipid Injected Intraperitoneally on Frequency of Diabetes, Thyroiditis, and Autoantibodies*

	Frequency of diabetes		Thyroiditis		Autoantibodies	
Age (days)	CSA	Intralipid	CSA	Intralipid	CSA	Intralipid
30	8/29†	17/32	17/29†	17/32	23/29	23/32
	$P < 0.05$					
40	4/30	13/27†	24/30	15/27†	29/30	26/27†
	$P < 0.01$					
50	4/28‡	17/31‡	18/28‡	18/31	27/28‡	24/31‡
	$P < 0.01$					
60	2/26‡	17/28†	22/26‡	15/28†	25/26‡	23/28†
	$P < 0.001$					
70	9/29	16/31	19/29	20/31	24/29	27/31
	NS					

* All animals received 10 injections (2 mg/100 g body weight).
† Two animals in this group died without diabetes prior to 120 days of age.
‡ One animal in this group died without diabetes prior to 120 days of age.

Table 3—Effect of Cyclosporin on Age of Onset of Diabetes (Cyclosporin versus Olive Oil)

Age of administration (days)	Age of diabetes onset (days) (mean ± SEM)	
	CSA	Olive oil
30-40	94.5 ± 8.5 (n = 2)	69.3 ± 5.9 (n = 3)
40-50	93.3 ± 5.2 (n = 7)	84.3 ± 7.8 (n = 6)
50-60	96.6 ± 4.8 (n = 10)	89.9 ± 5.1 (n = 10)
60-70	103.4 ± 3.7 (n = 7)	86.9 ± 3.1 (n = 22)
	$P < .01$	
70-80	89.0 ± 4.1 (n = 11)	87.5 ± 7.0 (n = 13)
All animals	94.9 ± 2.2 (n = 37)	86.3 ± 2.5 (n = 54)
	$P = .015$	

was apparent if one compared the incidence of ATC or ASM alone or in combination, among diabetic or nondiabetic animals separately or in combination (data not illustrated). Table 2 illustrates only the data of autoantibody frequency wherein an animal was considered to be autoantibody-positive if the serum evidenced either or both ATC and ASM. Furthermore, diabetic and nondiabetic animals were grouped together according to their treatment regimen. These data clearly illustrate that CSA did not decrease the frequency of autoantibody synthesis.

A total of 12 animals died without diabetes prior to 120 days of age and were excluded from the study (see Tables 1 and 2). An additional 5 animals were discarded after they were given the wrong treatment (n=4) or were noted to be pregnant (n=1).

Discussion

The fungal metabolite CSA has received considerable attention in recent years as an extremely promising clinical and experimental immune suppressive agent.[29,30] Clinically, CSA inhibits allograft rejection[31] and graft-versus-host reactions.[32] Experimentally, CSA inhibits *in vitro* as well as *in vivo* immune responses.[33] Of particular interest to the present study are the reports that continuous, long-term administration of CSA prevents diabetes in the BB rat,[8] that CSA inhibits experimental autoimmune uveitis,[34] and the recent suggestion that CSA-induced immunologic unresponsiveness might result from activation and/or sparing of suppressor cells rather than their specific induction.[27]

Table 4—Effect of Cyclosporin on Age of Onset of Diabetes (Cyclosporin versus Intralipid)

Age of injections (days)	Age of diabetes onset (days) (mean ± SEM)	
	CSA	Intralipid
30-40	100.8 ± 6.4 (n = 8)	83.1 ± 2.3 (n = 17)
	$P < .01$	
40-50	100.3 ± 9.5 (n = 4)	93.5 ± 3.9 (n = 13)
50-60	86.7 ± 9.7 (n = 4)	80.3 ± 4.4 (n = 17)
60-70	110.0 ± 8.0 (n = 2)	87.9 ± 3.9 (n = 17)
	$P = .063$	
70-80	100.8 ± 2.1 (n = 9)	97.4 ± 3.6 (n = 16)
All animals	99.8 ± 2.8 (n = 27)	88.2 ± 1.7 (n = 80)
	$P < .001$	

We have previously reported that CSA in combination with long-acting glucocorticoids and a single injection of ALS significantly reduced the frequency and severity of diabetes in BB/W rats treated prior to the onset of hyperglycemia.[7] The relative ineffectiveness of this combination of agents in reversing already established diabetes prompted the present study. The results presented above demonstrate that CSA *alone* will significantly reduce the frequency of diabetes in susceptible animals if it is administered orally in 10 consecutive doses between 60 and 70 days of age. The results presented above also demonstrate that larger doses of CSA when dissolved in Intralipid and administered intraperitoneally will protect against diabetes when given in 10-day intervals to animals between 30 and 70 days of age. Previous reports[1-3] that the incidence of diabetes is most frequent between 60 and 120 days of age and infrequently detected prior to 60 days suggest that effector cell stimulation occurs during this time period. The observation that 10 *oral* doses of CSA did not reduce the frequency of diabetes when given to 30-, 40-, and 50-day-old rats might be explained by the relative lack of lymphocyte activation during those time periods, the dose of CSA given, and/or the relatively short half-life and rapidly reversible effects of CSA.[30] The fact that *intraperitoneally* administered CSA afforded protection over a wider age range than did oral CSA might be explained by the larger quantity of CSA injected and/or the likelihood that a greater fraction of the intraperitoneally administered CSA was absorbed. In fact, orally administered CSA may not be completely absorbed because of CSA- and/or olive oil-induced diarrhea or other gastrointestinal disturbances. It is also possible that Intralipid-dissolved CSA was retained intraperitoneally as a depot source of the agent, with prolonged absorption and a longer duration of action than that given orally. These theoretic explanations might be tested by measuring CSA blood levels after oral and intraperitoneal administration. In the absence of CSA serum level data, it is not possible to exclude the possibility that the protection afforded by CSA administration to younger animals, well before the peak onset of diabetes, is the result of a long lived interference with T-cell antigenic sensitization or the result of CSA's hypothetical ability to enhance suppressor T-cell function.[27] This explanation is supported by the observation that CSA administration delayed the age of onset of diabetes. The absence of complete protection of

susceptible animals treated prior to 70 days of age might be explained by the fact that the median age of detection of diabetes in the Worcester colony is 84 days.[1] Hence, it might be argued that the animals destined to become diabetic at a later age (about 85–120 days) were less likely to be experiencing lymphocyte activation during the 30–70-day period and not likely to be protected by CSA injected at those times. The latter suggestion is, of course, speculative, because the optimum therapeutic tissue levels for CSA are not known. Nor is it known whether effector cell antigenic stimulation occurs over a wide or narrow time frame in the BB/W rat. The presence of insulitis among CSA-treated nondiabetic animals suggests that this agent is not completely effective in preventing the immune attack on the pancreatic islets. Alternatively, because nondiabetic animals were sacrificed at 120 days of age, long after cessation of CSA administration, insulitis might also be explained by the fact that CSA action is reversible.[30] This latter explanation is consistent with the observation of Stiller et al, that diabetes occurred with some frequency in BB rats after cessation of long-term (90 days) CSA administration.[35]

The absence of a reduction in the frequency of thyroiditis and autoantibodies among the treated animals is of particular interest. These are the first available data that reveal a possible dissociation of diabetes, thyroiditis, and autoantibody synthesis in BB/W rats. These results suggest that the regulatory T cells that modulate the induction of diabetes, lymphocytic thyroiditis, and B-lymphocyte synthesis of ATC and ASM may be distinct populations of lymphocytes. In this regard, it is worth reemphasizing the following: 1) Thyroiditis appears and becomes increasingly severe well after the peak onset of insulitis and diabetes (M. C. Appel, unpublished data). 2) Serum autoantibodies to smooth muscle and thyroid colloid appear prior to the peak onset of diabetes.[25] 3) Autoantibodies and thyroiditis are frequently observed in nondiabetic BB/W rats.[25] It remains to be determined whether a different dosage schedule or a later period of CSA treatment will prevent thyroiditis and autoantibody synthesis.

Although the results detailed above strengthen the hypothesis of the autoimmune pathogenesis of the BB/W diabetic syndrome, they do not resolve all of the syndrome's enigmas. For example, because there is a consistent association between the presence of T-lymphopenia and the susceptibility to diabetes, and because it is well documented that the helper/inducer subset of T cells is dramatically reduced among diabetic and diabetes-prone animals,[16–19] it is not clear why a CSA-mediated further inhibition of T-cell stimulation should protect against diabetes. On the other hand, the results reported in this communication are not in conflict with the twin hypotheses that susceptibility to BB/W diabetes is the result of deficient suppressor T-cell activity and that the effectiveness of CSA as an immune suppressive agent is the result of its ability to enhance suppressor T-cell function. The results reported above are also consistent with the inhibition by CSA of the effector arm of the BB (anti-beta cell) immune response, possibly by interfering with the release of lymphokines from activated T cells.[36]

Finally, the results reported above suggest that the mechanism responsible for beta-cell destruction in the BB/W rat is being activated during a relatively narrow time frame. These results should therefore help in the design of future studies of the pathogenesis of immune destruction of pancreatic beta cells in this model of spontaneous Type I diabetes.

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