Virus-induced Murine Diabetes
Enhancement by Immunosuppression

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SUMMARY

Adult, male ICR Swiss mice are susceptible to the diabetogenic effects of the D-variant of encephalomyocarditis virus (EMC-D) in contrast to adult C3H/HeJ male mice, which are relatively resistant. To date, experimental evidence suggests that the immune system plays a role in the pathogenesis of this infection. We have investigated the potential involvement of the immune system in the pathogenesis of EMC-D-induced diabetes using cyclosporin-A (CyA), a potent immunosuppressive drug. The data show that treatment with CyA results in increased severity and incidence of diabetes in susceptible ICR Swiss mice and induction of diabetes in resistant C3H/HeJ mice. It is concluded that immune mediation probably is not involved in the early pathogenesis of EMC-D-induced diabetes in mice. DIABETES 1985; 34:1217–21.

Juvenile-onset (type I) diabetes mellitus has been described as a complex, heterogenous group of diseases characterized by the occurrence of glucose intolerance, early age of onset, lymphocytic infiltration of the pancreatic islets of Langerhans suggesting involvement of the immune response, and eventual absolute deficiency of endogenous pancreatic insulin.[1,2] The etiology of the destruction of beta cells in this disease has not been clearly established. Clinical, epidemiologic, and experimental evidence support the hypothesis that cumulative insults, including virus infections and beta cell-specific autoimmunity, may all be involved in the development of diabetes in genetically predisposed individuals.[3] Recently, Stiller and colleagues demonstrated that treatment of patients with recently diagnosed juvenile-onset diabetes with cyclosporin-A (CyA), a potent, nonmyelotoxic immunosuppressing agent, results in a 50% frequency of recovery to a non-insulin-requiring state.[4] These data suggest that an immunologic process may be a major component in the pathogenesis of this disease in humans.

The immunologic role in the pathogenesis of virus-induced diabetes is presently a matter of controversy. The D-variant of encephalomyocarditis virus (EMC-D) selectively infects and destroys pancreatic beta cells in susceptible mouse strains, producing a disease syndrome similar to human insulin-dependent diabetes.[5,6] During the course of infection, the presence of inflammatory cellular infiltration within the pancreatic islets suggests the involvement of autoimmunity in beta cell destruction.[3,7] Results from other studies support this hypothesis.[8–10]

Several studies using immunosuppression to evaluate the potential contribution of the immune system in the pathogenesis of EMC-induced diabetes have yielded conflicting results.[8,10,11] In these investigations, use of the EMC-M strain, a mixture of the diabetogenic EMC-D and the nondiabetogenic EMC-B variants, has probably contributed to the variable results. Furthermore, high mortality in test animals, absence of uninfected, immunosuppressed controls, and an inadequate evaluation of immunosuppression have made the interpretation of such experiments difficult. We have initiated experiments to define more conclusively the role of the immune system in the development of diabetes in the murine host in response to infection by EMC-D. In the present study, the effect of CyA-mediated immunosuppression on the production of diabetes by EMC-D in susceptible ICR Swiss and resistant C3H/HeJ mice was determined. The data show that immunosuppression exacerbated the pathogenesis of the virus in ICR Swiss mice and allowed the development of diabetes in the C3H/HeJ strain.

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MATERIALS AND METHODS

Virus. The D-variant of encephalomyocarditis virus (EMC-D) was obtained from J. Yoon, NIH, Bethesda, Maryland. The virus was propagated and titrated by methods previously

TABLE 1
The effect of cyclosporin-A (CyA) on virus-induced diabetes in ICR Swiss mice

Group	Treatment at day*		GTT (mg/dl)†	% Diabetic‡
	0	1		
1	CyA	HBSS	191 ± 14	0
2	CyA	EMC-D	450 ± 206	80§
3	HBSS	EMC-D	286 ± 200	20
4	HBSS	HBSS	182 ± 18	0

*Test groups of male ICR Swiss mice (10/group) received daily i.p. injections of CyA (10 mg/kg body wt), HBSS, or EMC-D (760 PFU/mouse) at day 1. Control groups of 10 mice each received HBSS at day 0, and EMC-D or HBSS at day 1.
†At day 7 postinfection, mice were given an i.p. injection of glucose (2 mg/g body wt). The animals were bled 1 h later and sera were assayed for glucose content.
‡Animals with GTT levels at least 3 SD above control means were considered to be diabetic.
§Mice exhibited overt symptoms of severe encephalomyocarditis.

described.[12] The virus was passed five times through L929 (L) cells, twice through BHK 21 cells, and once again through L-cells. The resulting stock virus is diabetogenic, produces large, diffuse plaques in L-cells, and does not induce the production of interferon in vitro. Stock virus was diluted in Hanks' balanced salt solution (HBSS) containing 2% calf serum. Mice were given a single intraperitoneal (i.p.) injection (0.2 ml) of the diluted virus at a dose of 760 plaque-forming units (PFU).

Animals. Male ICR Swiss and C3H/HeJ mice were purchased from Harlan Laboratories, Indianapolis, Indiana, and Jackson Laboratories, Bar Harbor, Maine, respectively. The animals were housed in groups of 10, and at the time of infection were 9 wk of age.

Immunosuppression. Cyclosporin-A (CyA) was kindly provided by Dr. David Winter, Sandoz Corp., East Hanover, New Jersey. It was dissolved in 95% ethanol (0.5 ml) and Tween-80 (0.2 ml) and diluted with HBSS to a final concentration of 2 mg/ml. Mice received daily i.p. injections of 10 mg CyA/kg body wt. This concentration of CyA was used because it suppresses the immune response without toxic side effects.[13]

Virus replication in mouse tissues. Pancreata from C3H/HeJ and ICR Swiss mice were collected 5 days postinfection. In a separate experiment, pancreatic and brain tissues were collected from ICR Swiss mice 7 days postinfection. The tissues were homogenized and sonicated for 30 s (Heat Systems Sonicator Model 220F) to free intracellular virus. Cellular debris was removed by centrifugation (2000 × *g* for 15 min) and the clarified fluids were filter-sterilized (Millipore, 0.45 μm) and stored at −70°C until assayed for PFU content. The relative amount of virus replication in each of the tissues is expressed in terms of replication index,[14] which is defined as the number of PFU per gram of tissue divided by the challenge dose of virus. The index was calculated for the purpose of normalizing the results and providing more relevant values for comparison. Virus replication is indicated by indices >1.0.

Neutralizing antibody titers. Heat-inactivated pooled sera from each treatment group were evaluated for neutralizing antibody titers. A 50% plaque reduction (PR_{50}) method was done using EMC-D as the challenge virus. Titers are expressed as the reciprocal of the serum dilution required to reduce the number of PFU by 50%.

Glucose tolerance test (GTT). At 5 and 7 days postinfection, a nonfasting GTT was done. Each mouse received an i.p. injection of glucose at a concentration of 2 mg/g body wt. After 1 h, the animals were bled and the serum glucose levels determined using a YSI model 32A glucose analyzer (Yellow Springs Instruments, Yellow Springs, Ohio). It has been shown that nonfasting glucose levels demonstrate a greater sensitivity to reduction of insulin secretion than do fasting glucose levels.[15] Mice with GTT levels at least 3 SD above control means were considered to be diabetic.

RESULTS

Effect of CyA on the induction of diabetes. ICR Swiss mice were divided into four groups of 10 mice each. At day 0, two groups of animals received CyA while the other two groups received HBSS. CyA-treated mice were given daily injections of the drug throughout the 8-day test period. At day 1, one of the CyA-treated groups and one group that received HBSS were challenged with EMC-D. Glucose tolerance tests were done at day 7 postinfection. The data (Table 1) show that EMC-D produced diabetes in 20% of the untreated, infected control animals (group 3). In contrast, 80% of the CyA-treated mice developed diabetes in response to virus infection (group 2). Immunosuppressed animals in group 2 exhibited symptoms of severe encephalomyocarditis, including gen-

TABLE 2
The effect of CyA treatment on virus-induced diabetes in ICR Swiss and C3H/HeJ mice

Treatment at day*		ICR Swiss		C3H/HeJ	
		GTT (mg/dl)†	% Diabetes‡	GTT (mg/dl)†	% Diabetes‡
0	3				
CyA	HBSS	215 ± 15	0	221 ± 17	0
CyA	EMC-D	521 ± 197	90§	286 ± 172	50§
HBSS	EMC-D	327 ± 133	50	171 ± 37	0‖
HBSS	HBSS	182 ± 19	0	191 ± 21	0

*Test groups of male ICR Swiss or C3H/HeJ mice (10/group) received daily i.p. injections of CyA (10 mg/kg body wt) and either HBSS or EMC-D (760 PFU/mouse) at day 3. Control groups of 10 mice each received HBSS at day 0 followed by EMC-D or HBSS at day 3.
†At day 5 postinfection, mice were given an i.p. injection of glucose (2 mg/g body wt). The animals were bled 1 h later and sera were assayed for glucose content.
‡Animals with GTT levels at least 3 SD above control means were considered to be diabetic.
§All mice exhibited symptoms of encephalomyocarditis.
‖Fifty percent of the mice had symptoms of encephalomyocarditis.

TABLE 3
Effect of CyA on antibody titers and virus replication

Group	Treatment	Neutralizing antibody titers*		Virus content in the brain and pancreas (replication index)†	
		Titer	% of control	Brain	Pancreas
ICR Swiss	EMC-D controls				
	day 5 postinfection	1667	—	ND§	7.7
	day 7 postinfection	1750	—	7.1	0.0
	EMC-D + CyA				
	day 5 postinfection	660	40	ND	52.3
	day 7 postinfection	550	30	29.4	4.7
C3H/HeJ‡	EMC-D controls	1837	—	ND	1.6
	EMC-D + CyA	1350	70	ND	3.2

*Titers are expressed in PR_{50} units.
†Replication index is virus PFU/g tissue divided by the challenge dose of virus.
‡Five days postinfection. The 5-day test period was used because most of the animals were moribund.
§ND, not done.

eralized weakness, loss of appetite, ruffling of fur, uncontrolled disoriented movements, and hindleg paralysis. As in previous studies,[16] virus-infected controls did not demonstrate overt signs of disease. The data also show that CyA treatment had no effect on the GTT (group 1).

In another study, the effect of CyA on the induction of diabetes in normally resistant C3H/HeJ and susceptible ICR Swiss mouse strains was determined. For each strain, animals were divided into four groups of 10 mice each. Two groups were treated with CyA for three consecutive days while two groups received HBSS. On the third day, one CyA-treated group and one HBSS-treated group received EMC-D. The other two groups received HBSS. Daily treatment with CyA was continued throughout the remaining test period. Glucose tolerance tests were determined 5 days postinfection.

The data in Table 2 show that, as in the first experiment (Table 1), CyA treatment increased the severity and incidence of diabetes in ICR Swiss mice. It is also evident that EMC-D produced diabetes in the resistant C3H/HeJ strain only when the animals were treated with CyA. In both strains, mice receiving CyA and EMC-D exhibited severe encephalomyocarditis. The C3H/HeJ-infected control mice also had symptoms of encephalomyocarditis, but the incidence and severity were not as pronounced as that observed in the CyA-treated group.

Immunosuppression by CyA. The immunosuppressive effects of CyA were evaluated by measuring virus-specific neutralizing antibody titers in sera and the extent of virus replication in pancreatic tissues. The data in Table 3 show that CyA decreased circulating neutralizing antibody titers and enhanced virus replication in pancreatic tissues of both strains of mice. These effects were more pronounced in ICR Swiss mice. Analysis of brain tissue from ICR Swiss mice 7 days postinfection revealed that treatment with CyA resulted in a fourfold increase in virus content when compared with infected controls (Table 3).

DISCUSSION

Although the exact mechanism of CyA-induced immunosuppression is not known, in vivo and in vitro experiments demonstrate that CyA may suppress the effector limb of the immune response by impairing the release of interleukin-2 by activated T-helper populations and the release of interleukin-1 by macrophages.[17,18] CyA has a therapeutic advantage clinically in that primary immune responses such as macrophage phagocytosis, chemotaxis, and enzyme secretion are spared.[19]

The data in the present study show that immunosuppression by CyA in EMC-D-infected animals increases the incidence and severity of diabetes in susceptible ICR Swiss mice and induces diabetes in the resistant C3H/HeJ strain (Tables 1 and 2). It should be noted that, since the ICR Swiss strain is outbred, they respond with greater variation to the diabetogenic effects of EMC-D, thus 20% diabetes was observed in experiment 1 while 50% was observed in experiment 2. The lower titers of virus-specific neutralizing antibody and higher titers of virus in the pancreata of CyA-treated, virus-infected mice, compared with virus-infected controls, confirmed that the animals were immunosuppressed.

Results from a previous study by Vialettes and colleagues indicated that CyA does not significantly influence the incidence or evolution of diabetes in susceptible DBA/2 male mice although mortality was increased.[11] However, they used EMC-M virus, a mixture of EMC-D and nondiabetogenic EMC-B, and discontinuous CyA treatment to evaluate the induction of diabetes.

Yoon et al. have shown that the ratio of EMC-B to EMC-D

TABLE 4
Long-term analysis of glucose tolerance tests in EMC-D-infected ICR Swiss mice

Time postinfection*	GTT (mg/dl)	Number of diabetics/group†
1 wk	433 ± 172	10/12
2 wk	310 ± 154	8/10
3 wk	253 ± 164	1/9
2 mo	296 ± 178	1/8

*Twelve ICR Swiss male mice received an i.p. injection of EMC-D (760 PFU). At designated times postinfection, glucose tolerance tests were done as described in Table 1.
†During the course of study, 4 mice died as a result of complications from the periorbital bleeding process.

in the inoculum affects the induction of diabetes in SJL/J mice.[6] The same observation was made in our laboratory using male BALB/cByJ mice (unpublished observation). Thus, EMC-B appears to interfere with the ability of EMC-D to produce diabetes. In the present study, use of a relatively pure EMC-D virus preparation in CyA-treated mice resulted in enhanced incidence and severity of diabetes in both mouse strains tested. Preliminary studies using EMC-B in CyA-treated mice indicate that this virus does not produce diabetes even in immunosuppressed mice (data not shown). This observation suggests that the pathogenesis of EMC-B is self-limiting in the presence of the CyA-spared, nonspecific primary immune response and clinical manifestations are not evident.

Discontinuous CyA treatment may have been insufficient to adequately immunosuppress the mice. This notion is supported by studies showing that the termination of CyA treatment results in an overshoot in T-cell priming, as evidenced by higher numbers of T-helper cells,[20] and suggest that continuous CyA treatment is required for adequate immunosuppression. In our laboratory, analysis of concanavalin A-induced blastogenesis in spleen cells prepared from virus-infected controls and from CyA-treated infected mice supports these findings. In the absence of CyA in vitro, blastogenic responses in the CyA-treated group were increased compared with virus-infected controls. Based on these observations, discontinuous CyA treatment may actually enhance immune responses.

The dosage of CyA used to immunosuppress may also be of critical importance. In the rat model, researchers report that doses in excess of 10 mg/kg body wt result in severe renal disease.[13] In the present study, we evaluated the toxic effects of the drug alone. At CyA doses of 10 mg/kg body wt, which appear to provide adequate immunosuppression, we observed no overt signs of toxicity in CyA-treated controls and 10% mortality in infected, CyA-treated mice.

Although the drug itself was not toxic to the mice, experiments were terminated within the first week postinfection because the immunosuppressed mice were exhibiting symptoms of severe encephalomyocarditis. The C3H/HeJ mice appeared to be particularly susceptible to virus-induced encephalomyocarditis, as 50% of the infected control group and 100% of the CyA-treated, infected animals exhibited this symptomatology. These observations are consistent with those reported by Vialettes and suggest that immunosuppression enhanced virus replication in these animals.

Other studies in our laboratory show that insulitis occurs after day 4 and before day 7 post EMC-D infection in male ICR Swiss mice (unpublished observation). However, overt diabetes was observed at day 4 postinfection. The occurrence of glucose intolerance very early in the course of infection before the onset of insulitis does not support the hypothesis that immune mechanisms are involved in the pathogenesis of EMC-D-induced diabetes.

Long-term analysis of blood glucose levels in EMC-D-infected ICR Swiss mice also does not implicate the immune system in the pathogenesis of virus-induced diabetes. During the first 2 wk postinfection, 80% of these animals were diabetic (Table 4). However, by 3 wk and 2 mo postinfection, only 1 of 9 and 1 of 8 animals, respectively, remained diabetic. These results indicate that the remaining beta cells were able to compensate for the damage sustained during virus infection (Table 4).

In a recent report, it was suggested that viruses could, in rare instances, destroy pancreatic beta cells, but because of the long period of latency of juvenile-onset diabetes in humans, other mechanisms are more likely involved.[21] Clinical studies tend to substantiate this speculation. In an investigation of 250 children who died of fatal virus infections, only 28 demonstrated pathologic changes in the islets,[22] indicating that even in severe viral infections, only a small percentage of viruses have a tropism for pancreatic tissue.

The results presented here do not support the hypothesis that immune mechanisms are involved in the early pathogenesis of EMC-D-induced diabetes in the murine model. Virus destruction of pancreatic beta cells appears to be the major cause of diabetes observed in both mouse strains tested.

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