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Suppression of Development of Diabetes in NOD Mice by Lactate Dehydrogenase Virus Infection

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It has been reported that lactate dehydrogenase virus (LDV) selectively infects a subpopulation of macrophages, thereby affecting the immune system. We studied the effects of LDV infection on the development of diabetes in non-obese diabetic (NOD) mice.

Five-week-old female NOD mice were infected with LDV (10^8 ID_{50}/mouse) and observed until 23 weeks of age. None of the 21-LDV-infected mice developed diabetes, whereas 10/14 (71.4%) uninfected mice did. Although the subpopulations of T cells and the percentage of Mac1-positive cells in the NOD murine spleen and the number of harvested peritoneal macrophages were unaffected by LDV infection, the proportions of Ia-positive peritoneal macrophages were significantly decreased in LDV-infected compared with uninfected mice ($1.1 \pm 0.2\%$, $6.5 \pm 2.9\%$; $P < 0.01$). In LDV-infected NOD mice, insulitis of the same grade as that seen in uninfected NOD mice was observed. In another experiment, 3, 5, 10 or 16-week-old female NOD mice were infected with LDV. None of the mice infected with LDV at 3, 5 or 10 weeks of age developed diabetes and only one of six infected at 16 weeks of age did.

These findings indicate that LDV infection suppresses the development of diabetes in female NOD mice by reducing the capacity of Ia-positive macrophages, and suggest that the development of human type 1 diabetes may be suppressed by certain viral infections.

Introduction

Viruses have been implicated as possible causes of pancreatic β cell destruction in type 1 diabetes mellitus [1]. A Coxsackie B4 virus was isolated from the pancreas of a

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10-year-old child who died during an episode of diabetic ketoacidosis [2]. The isolated virus induced diabetes in SJL mice by infecting and destroying islets. It has been reported that some viruses, such as rubella [3], encephalomyocarditis virus [4], Coxsackie B group viruses [5] and reovirus [6], can induce diabetes in mice or hamsters. However, it has recently been reported that some viruses reduce the incidence of diabetes in NOD mice [7–9] and BB rats [10], which are excellent models of type 1 diabetes mellitus. These findings indicate that viral infection has the potential not only to induce diabetes but also to prevent it.

Lactic dehydrogenase virus (LDV) causes a non-lethal infection in mice and the infection persists for the life of the animals. In LDV-infected mice, serum lactate dehydrogenase (LDH) levels increase and immunological disturbances can be detected [11]. Moreover, it has been reported that LDV selectively infects a subpopulation of macrophages [12] and prevents the development of experimental allergic encephalitis, an autoimmune disease affecting the central nervous system [13]. Therefore, we studied the effects of LDV infection on the development of diabetes in NOD mice.

Materials and methods

Viruses

Lactate dehydrogenase virus (LDV) in serum, was obtained from Dr K. E. K. Rowson. Stock virus preparations of LDV were made by collecting serum from mice that had been infected 24 h previously. The sera were collected by heart puncture and stored at −70°C. The pooled serum contained a median infectious dose (ID_{50}) of approximately 10^{11} per ml. Virus titers were estimated from measuring plasma LDH activity 5 days after the mice had been inoculated with 10-fold dilutions of the viruses. LDH levels were measured utilizing a spectrophotometric analyser (Hitachi 736-60, Japan).

Mice

Five-week old female NOD mice were obtained from Clea Japan Co. (Tokyo, Japan) and were kept under specific pathogen-free conditions. Thirty-five female NOD mice were used in the first experiment. Twenty-one mice were inoculated intraperitoneally (ip) with LDV (10^8 ID_{50}/0.1 ml/mouse) at 5 weeks of age and metabolic changes were studied until 23 weeks of age. Five mice were inoculated with an equivalent dose of heat-inactivated virus and 14 uninfected mice served as controls. In the second experiment, 3, 5, 10 and 16-week-old female NOD mice were infected with LDV as described in the first experiment and the incidence of diabetes after infection was determined. The animals were tested weekly for urinary glucose by Tes-tape (Eli Lilly Co., Indianapolis, USA). Blood samples were collected from the retro-orbital vein plexus of all non-fasting mice that showed positive glycosuria, and plasma glucose levels were measured by a stix-reflectometer. A glucose level higher than 13.9 mmol/l was classified as overtly diabetic. At 20–23 weeks of age, the mice used in the first study were killed and examined as described below.

Demonstration of persistent LDV infection

Plasma lactate dehydrogenase (LDH) titers, which reflect persistent LDV infection, were measured utilizing a spectrophotometric analyser (Hitachi 736-60, Tokyo, Japan).

Detection of LDV infection using anti-LDV serum

Cryostat sections of pancreas and peritoneal macrophages fixed with acetone were stained with anti-LDV serum followed by incubation with FITC-labelled goat anti-mouse IgG. Anti-LDV hyperimmune serum was prepared in mice by ip injection of 10^9 to 10^{10} ID_{50} of LDV at 2-weekly intervals for 6 weeks. One week after the last injection the sera were collected. Virus and virus-immune complexes in the sera were for the most part removed by ultracentrifugation at $10{,}500 \times \boldsymbol{g}$ for 1 h and the sera were inactivated at 56°C for 30 min, pooled and stored at −20°C.

Spleen cell analysis

The spleen cells were mechanically minced in RPMI 1640 medium containing 2% heat-inactivated fetal calf serum. The samples were overlayed on 3 ml of sodium metrizoate Ficoll (JIMRO Co., Takasaki, Japan) and centrifuged at $1{,}200 \times \boldsymbol{g}$ for 20 min. The mononuclear cell layer was collected and washed three times with RPMI 1640 medium. The samples were then incubated with monoclonal antibodies (Thy1.2, Lyt2, L3T4; Becton Dickinson Co., Tokyo, Japan and anti-asialo GM1 antibody; Wako Pharmacy, Tokyo, Japan) and analysed by flow cytometry.

Preparation of peritoneal macrophages and Ia antigen staining

Non-stimulated peritoneal exudate cells from control or LDV-infected NOD mice were collected by peritoneal lavage and incubated in minimal essential medium (MEM) with 10% FCS and 1% bicarbonate in ring cultures at 37°C with 5% CO_2. After 3 h, cultures were washed vigorously with warmed PBS to remove non-adherent cells. For detection of Ia antigen on the cell surface, the cells were fixed with 1% paraformaldehyde for 10 min at room temperature, washed in PBS and stained with monoclonal anti-Ia antibody (OX-6) [14] (kindly donated by Dr A. F. Williams) and fluorescein isothiocyanate (FITC)-labelled anti-mouse IgG. OX-6 is a monoclonal anti-rat antibody which cross-reacts with murine Ia antigen specific for Ia 17, 18 of I-A coded molecules. Anti-Ia antibody (H-10, 81, 10) [15], kindly donated by Dr M. Pierres, was used as negative control. The total number of peritoneal macrophages was counted by a hemocytometer and the percentage of Ia-positive peritoneal macrophages was determined.

Histological examination

Pancreata were fixed in Bouin's solution, embedded in paraffin and then stained with hematoxylin and eosin and examined by light microscopy. Insulitis was graded as follows: 0, no evidence of insulitis; 1, mononuclear cells occupy 1–25% of single islets

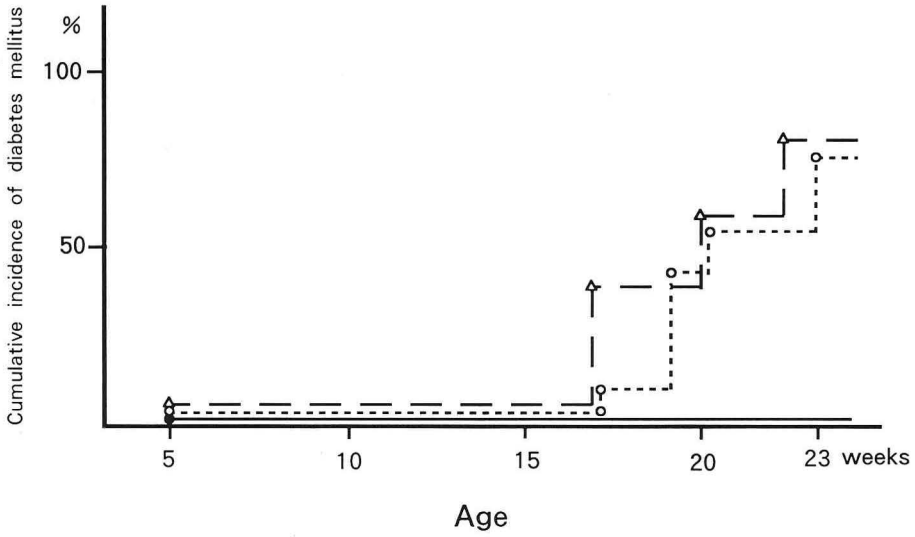

Figure 1. Cumulative incidence of diabetes mellitus in lactate dehydrogenase virus-infected female NOD mice. LDV ($10^8 ID_{50}$/mouse) was administered at 5 weeks of age. ●, LDV-infected female NOD mice ($n=21$); ○, uninfected female NOD mice ($n=14$); △, female NOD mice infected with heat inactivated LDV ($n=5$).

(peri-insulitis); 2, mononuclear cells occupy 26–50% (moderate insulitis); 3, mononuclear cells occupy 51–100% (extensive insulitis). The degree of insulitis was determined by a pathologist who was unaware of the protocol for this experiment.

Insulin concentrations in pancreatic tissues

Pancreata were harvested and lympholized and their dry weight was measured. Tissues were homogenized and the insulin was extracted from the pancreata in acid ethanol. Insulin was measured by a competitive inhibition radioimmunoassay with human insulin as standard and ^{125}I-A-14 porcine insulin as tracer.

Statistical analysis

Statistical significance of the differences in the incidence of diabetes between the two groups was tested by Fisher's exact test and that between the means of sample groups was tested by unpaired Student's *t*-test.

Results

Effects of persistent LDV infection on the incidence of diabetes in NOD mice

In the first experiment, no LDV-infected mice developed diabetes, while 10 of the 14 (71.4%) uninfected mice did ($P<0.01$) (Figure 1). NOD mice inoculated with inactivated LDV developed diabetes with the same incidence as uninfected NOD

Table 1. *Grade of insulitis in female NOD mice after LDV infection*

Age at examination		Number of islets	Grade of insulitis 0	1	2	3
10 weeks	Uninfected	38	21	12	4	1
	LDV-infected	46	21	15	7	3
22 weeks	Uninfected	16	4	9	1	2
	LDV-infected	58	24	18	12	4

Table 2. *Incidence of diabetes in female NOD mice infected with LDV at various ages*

Ages of infection	Incidence of diabetes (diabetic mice/total mice)	Period of observation
Uninfected	10/14 (71.4%)	23 weeks
Infected at 3 weeks	0/5 (0%)*	22 weeks
5 weeks	0/5 (0%)*	25 weeks
10 weeks	0/6 (0%)†	26 weeks
16 weeks	1/6 (16.7%)	26 weeks

Three, 5-, 10- and 16-week-old female NOD mice were inoculated with 10^8 ID_{50} of LDV. The mice were observed for the development of diabetes until 22 and 26 weeks of age.
*$P<0.05$, †$P<0.01$ *vs* uninfected mice.

mice. Blood glucose levels in LDV-infected and uninfected NOD mice at 21 and 23 weeks of age were 6.7 ± 1.1 mmol/l and 17.8 ± 9.4 mmol/l, respectively ($P<0.05$). Body weights in LDV-infected mice were significantly heavier in LDV than in uninfected mice (22.5 ± 1.3 g *vs* 18.4 ± 1.7 g; $P<0.01$). On the other hand, mononuclear cell infiltrations of pancreatic islets in LDV-infected mice were of the same degree as in uninfected NOD mice of the same age (Table 1). Serum LDH levels were significantly elevated in all LDV-infected NOD mice compared with those in control NOD mice (526 ± 125 U *vs* 33.3 ± 26.8 U, $P<0.01$). LDV was detectable in peritoneal macrophages but not in pancreatic tissue.

In the second experiment, none of the NOD mice infected at 3, 5 or 10 weeks of age developed diabetes. Only one of six mice infected at 16 weeks of age developed diabetes (Table 2).

Reduction of Ia-positive peritoneal macrophages in LDV-infected NOD mice

Seven days after LDV infection, the number of harvested peritoneal macrophages and the percentage of Ia-positive macrophages were determined. As shown in Table 3, there were no differences between LDV-infected and uninfected mice with regard to the number of peritoneal macrophages. However, the proportions of Ia-

Table 3. *Reduction of the percentage of Ia-positive macrophages by LDV infection in female NOD mice*

	Number of peritoneal macrophages	Percentage of Ia-positive macrophages (%)
LDV-infected NOD mice	$3.7 \pm 1.1 \times 10^6$	1.1 ± 0.2*
Uninfected NOD mice	$3.2 \pm 0.9 \times 10^6$	6.5 ± 2.9

Female NOD mice were infected with 10^8 ID_{50} of LDV. Seven days after infection, non-stimulated peritoneal macrophages were harvested and the number of peritoneal macrophages and percentages of Ia-positive macrophages were determined.
*$P < 0.01$ *vs* uninfected NOD mice.

Table 4. *The effects of LDV infection on subpopulations of spleen cells in female NOD mice*

	Thy1.2	L3T4	Lyt2	Mac1
LDV-infected* NOD mice	65.1 ± 6.3	32.1 ± 4.2	14.7 ± 1.5	9.9 ± 1.4
Uninfected NOD mice	57.5 ± 6.9	31.1 ± 5.6	16.6 ± 1.6	10.8 ± 6.3

*Five-week-old female NOD mice were infected with 10^8 ID_{50} of LDV. At 16 weeks of age, mice were killed and subpopulations of spleen cells were determined. There were no differences between the data from LDV infected and uninfected mice.

positive peritoneal macrophages were significantly decreased in LDV-infected compared with uninfected NOD mice ($1.1 \pm 0.2\%$, $6.5 \pm 2.9\%$, $P < 0.01$).

Effects of LDV infection on the spleen cell populations of female NOD mice

Table 4 shows the proportion of T-cell subsets and percentage of Mac1-positive cells in the spleen of 16-week-old mice that had been inoculated with LDV at 5 weeks of age. There were no significant differences in the percentage of Thy1.2, L3T4, Lyt2 and Mac1-positive cells between LDV-infected and uninfected mice. The diabetic control NOD mice showed low levels of pancreatic insulin (0.015 ± 0.012 U/pancreas dry weight) compared with LDV-infected NOD mice (0.034 ± 0.012 U/pancreas dry weight, $P < 0.01$) at 16 weeks of age.

Discussion

We have demonstrated that persistent lactate dehydrogenase virus infection suppresses the development of diabetes in female NOD mice regardless of age at the time of infection. The NOD mouse is an excellent model of human type 1 diabetes

[16]. The development of diabetes in this model is characterized by mononuclear cell infiltration in the pancreatic islets (insulitis) and by autoimmune islet cell destruction [17, 18]. The central role of T cells in the pathogenesis of the disease is suggested by the effects of thymectomy [19] and treatment with anti-T-cell monoclonal antibodies [20]. Furthermore, the disease can be transferred to irradiated non-diabetic NOD mice with T cells from overtly diabetic donor mice [21]. Recently, islet specific autoreactive T-cell clones were also isolated from mononuclear cells in the setting of insulitis [22]. These cells are thought to destroy islet cells. On the other hand, it has been shown that regulatory T cells, which suppress the autoimmune reaction in NOD mice, play a part in the onset of diabetes in NOD mice [23]. Moreover, it has been suggested that macrophages [24], natural killer cells [25] or cytokines [26] are also involved in the destruction of islet cells in NOD mice. It has recently been reported that LDV infects a subpopulation of macrophages and that the percentage of Ia-positive macrophages decreases in LDV-infected mice [12]. It has also been reported that LDV infection suppresses the antigen presenting ability of macrophages [27]. We therefore speculate that LDV suppressed the production of autoreactive effector cells by suppressing the antigen-presenting function of macrophages. This speculation cannot, however, completely account for our findings in that insulitis in NOD mice is not suppressed by LDV infection and the onset of diabetes is suppressed in NOD mice infected with LDV at 10 weeks of age, when antigen presentation of autoantigen has presumably finished. Further investigations are necessary to elucidate the mechanisms involved in the suppression of diabetes in NOD mice by LDV infection.

Recently, another virus, lymphocytic choriomeningitis virus (LCMV), has been shown to prevent autoimmune diabetes in BB rats [10] and NOD mice [7]. It has been reported that LCMV infects T lymphocytes, primarily helper T cells [28]. In LCMV-infected BB rats, depletion of T lymphocytes was detected and no insulitis was observed. Also, spleen cells transferred from NOD mice infected with LCMV were not able to induce either insulitis or diabetes in recipient NOD mice. On the other hand, in LDV-infected NOD mice, T-cell subsets from the spleen were unchanged and insulitis was not suppressed. This suggests that the mechanisms of LDV-mediated suppression of diabetes in NOD mice may be different from those of LCMV.

Persistent viral infection may be important for the suppression of diabetes in NOD mice. Recently, persistent infection with murine hepatitis virus (MHV) was observed in a NOD mouse colony. The incidence of diabetes decreased after the appearance of antibodies to MHV and increased again after treatment by caesarian delivery [8]. These findings indicate that persistent MHV infection suppressed the development of diabetes in female NOD mice. The common characteristic of these three viruses is persistent infection. Persistent viral infection may non-specifically affect the incidence of diabetes in NOD mice.

In this report, we have demonstrated that LDV infection suppressed the onset of diabetes in mice predisposed to develop type 1 diabetes. Although it was formerly widely believed that viral infection only induced the development of diabetes, our data and the reports on LCMV and MHV suggest that viral infection may be capable not only of inducing diabetes, but also of suppressing the onset of human type 1 diabetes. These findings may shed additional light on the significance of viral infection in the onset of human type 1 diabetes.

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