

# Virus-Induced Diabetes Mellitus: Mengovirus Infects Pancreatic Beta Cells in Strains of Mice Resistant to the Diabetogenic Effect of Encephalomyocarditis Virus

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In mice, Mengovirus produces a fatal encephalitis. Plaque purification of the virus resulted in the isolation of a clone (Mengo-2T), which in addition to encephalitis caused diabetes. Microscopic examination of pancreases from infected mice revealed necrosis in the islets of Langerhans and infiltration of inflammatory cells. By immunofluorescence viral antigens were found in the islets, and radioimmunoassays demonstrated a substantial decrease in pancreatic immunoreactive insulin. Studies on susceptibility among inbred strains of mice showed that whereas the D variant of encephalomyocarditis virus caused diabetes only in SJL/J mice, Mengo-2T caused diabetes in strains of mice resistant to encephalomyocarditis-induced diabetes (i.e., CBA/J, C3H/HeJ, CE/J, AKR/J, C57BL/6J). The ability of Mengo-2T to induce diabetes in encephalomyocarditis-resistant mice was found to be due to the greater capacity of Mengo-2T as compared to the D variant of encephalomyocarditis virus to replicate in and destroy the islets of these animals. Although Mengo-2T and the D variant of encephalomyocarditis virus are antigenically indistinguishable by hyperimmune sera, our studies show that these viruses have different host ranges and tissue tropisms.

Encephalomyocarditis (EMC) virus, a member of the cardiiovirus genus of the picornaviridae, produces encephalitis and myocarditis in mice (2). The D variant of this virus, designated EMC-D, is generally nonlethal but has a tropism for the pancreatic islets of Langerhans and causes diabetes mellitus (10). In contrast, Mengovirus, another member of the cardiiovirus genus, produces a rapidly fatal encephalitis in mice (2, 8). Recently, we isolated and studied a clone of this virus, designated Mengo-2T, which also was highly lethal for mice (4). Despite the marked differences in lethality between EMC-D and Mengo-2T, these two viruses could not be distinguished antigenically by hyperimmune sera (2, 4, 7). Moreover, these two viruses showed an estimated 20% difference in nucleotide sequences by cDNA-RNA hybridization, and receptor binding experiments suggest that they may use different receptors on the surface of cells (4).

The present study, which was initiated to see whether Mengo-2T infects pancreatic beta cells, shows that not only can this virus cause diabetes, but it can do so in strains of mice resistant to the induction of diabetes by EMC-D.

## MATERIALS AND METHODS

**Animals.** SJL/J, C57BL/6J, CBA/J, CE/J, C3H/HeJ, and AKR/J mice were purchased from Jackson Laboratory (Bar Harbor, Maine), and all animals were maintained on Purina-NIH mouse ration containing 5.0% fat and 2.3% protein. Only male mice were used, and at the time of infection, the animals were 5 to 6 weeks old. Unless otherwise indicated, mice were inoculated intraperitoneally (i.p.) with virus.

**Viruses.** All of the experiments reported here used EMC-D virus (10) and Mengo-2T virus (4). Mengovirus, passaged in

neonatal mouse brain, was plaque purified three times by methods described elsewhere (10) and passaged in secondary mouse embryo cells. Virus titer was determined by plaque assay on secondary mouse embryo cells.

**Blood glucose assays.** Each animal was bled on day 3 or 4 or on both days for glucose tolerance tests (GTTs). GTTs were performed by i.p. injection of 2 mg of glucose per g of body weight, and glucose levels were determined 60 min later (6). Mice with plasma glucose concentrations 3 standard deviations above the mean of the uninfected animals were considered diabetic.

**Extraction and measurement of insulin.** Insulin was extracted from the pancreas by methods described elsewhere (1). The concentration of immunoreactive insulin (IRI) in the pancreas and plasma was measured by using the Phadebas insulin radioimmunoassay kit (Pharmacia Fine Chemicals, Piscataway, N.J.). Data were expressed in total nanograms of IRI per milliliter of plasma and total micrograms of IRI per gram of pancreas (11).

**Histopathology and immunofluorescence.** Mice were killed 4 to 7 days after infection; the pancreas and brain were removed and fixed in 10% buffered Formalin. The material was embedded in paraffin, cut in 6- $\mu$ m sections, and then stained with hematoxylin and eosin. For the detection of viral antigens, indirect immunofluorescent staining was carried out on cryostat sections of pancreas (10).

**Isolation of islets and determination of virus titer.** SJL/J or C3H/HeJ mice were infected with Mengo-2T or EMC-D. At different times after infection, pancreases were removed, and ca. 100 islets from each pancreas were isolated with the aid of a stereomicroscope (11; J. W. Yoon, C. J. Bachurski, S. Y. Shin, and J. A. Archer, in S. L. Pohl and J. Larner, ed., *Methods in Diabetes*, in press). The islets were weighed, suspended in 10% Eagle minimal essential medium (wt/vol), homogenized, appropriately diluted, and assayed for infectious virus. Protein concentration was determined

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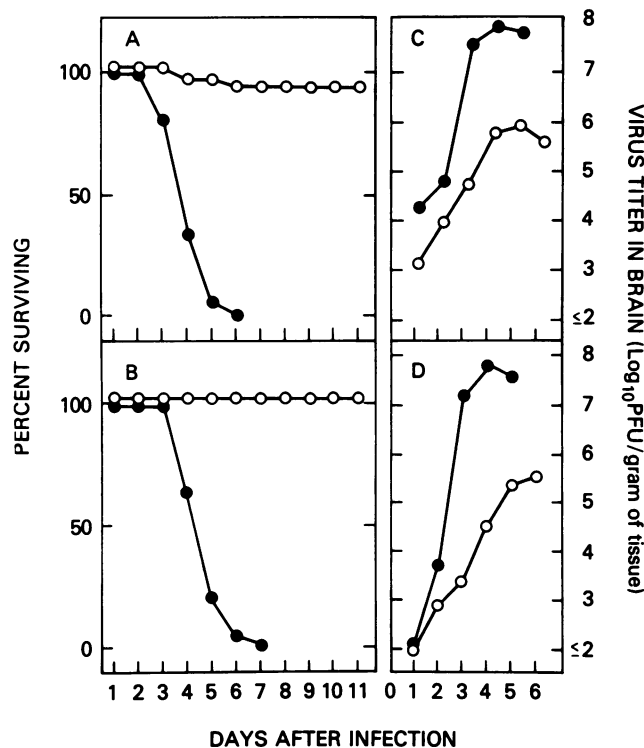


FIG. 1. Mortality and virus replication in the brains of mice infected with Mengo-2T or EMC-D. SJL/J mice were infected intracerebrally (A and C) with 50 PFU of Mengo-2T (●) or EMC-D (○) per mouse or i.p. (B and D) with 500 PFU per mouse. Thirty mice were used in each group. Each point in C and D represents the mean virus titer in brain homogenates from groups of three mice.

(3) by using a 10% (wt/vol) homogenate of pancreatic islets in phosphate-buffered saline (pH 7.2).

## RESULTS

**Differences in the neurotropism of Mengovirus and EMC virus.** SJL/J mice were inoculated intracerebrally with 50 PFU of Mengo-2T or EMC-D, and lethality and viral titers were determined. All of the mice inoculated with Mengovirus developed neurological symptoms (e.g., limb paralysis, hunched posture, and ataxia) within 3 days and died several

days later (Fig. 1A). In contrast, only a few (20%) of EMC-infected mice showed transient limb paralysis, and only 2 of 30 mice died.

Evidence suggesting that this difference in mortality was due to differences in viral replication came from measuring viral titers in the brains of mice at various times after infection. Mengo-2T replicated rapidly, reaching a maximum titer on day 4 of close to  $10^8$  PFU/g of brain (Fig. 1C). In contrast, the titer of EMC-D was 100-fold lower. Similar results were obtained when mice were inoculated i.p. with 500 PFU of Mengo-2T or EMC-D (Fig. 1B and D). Thus, regardless of the route of inoculation, Mengo-2T caused a lethal encephalitis, whereas EMC-D did not.

To determine the site of virus involvement, sections of brains from infected SJL/J mice were examined for histopathological changes. EMC-D produced only minimal pathological changes in the hippocampus (Fig. 2A) and little or no evidence of pathology in the cerebellum (Fig. 2C) or neocortex (not shown). In contrast, Mengo-2T caused severe nerve cell damage and infiltration of inflammatory cells. In the hippocampus, the pyramidal cell layer, in particular, was extensively damaged (Fig. 2B). In the cerebellum, the Purkinje cells showed severe necrotic changes, and focal necrosis was observed in the molecular layer (Fig. 2D). Focal necrosis of nerve cells also was found in the neocortex and the anterior horn of the spinal cord (not shown). Neither Mengo-2T nor EMC-D virus produced observable changes in the leptomeninges, ependyma, choroid plexus, or vascular endothelium.

**Mengovirus-induced diabetes.** We previously showed that EMC-D could infect and destroy pancreatic beta cells, resulting in insulin deficiency and diabetes mellitus (10). To see whether Mengo-2T also was beta cell-tropic, tests for IRI and nonfasting blood glucose were performed after i.p. inoculation of SJL/J mice with 500 PFU of Mengo-2T. The concentration of IRI in the plasma increased on days 1 and 2 (probably reflecting release of insulin from damaged beta cells) and then declined (Fig. 3A). Nonfasting blood glucose levels were inversely related to insulin levels and showed an increase on days 4 and 5. These observations suggested that, if more sensitive methods were used (e.g., GTTs), glucose abnormalities might be more readily detected. Four days after viral inoculation, 60-min GTTs were performed. Approximately 80% of the infected mice showed distinctly abnormal GTTs (Fig. 3B).

**Host differences in the induction of diabetes.** Earlier studies showed that only certain inbred strains of mice developed diabetes when infected with EMC-D (5, 9-12). To see

TABLE 1. Relationship between GTT and IRI<sup>a</sup>

Strain	Blood glucose (GTT) (mg/dl) ± SD			IRI (μg/g) ± SD		
	Uninfected	Infected		Uninfected	Infected	
		Mengo-2T	EMC-D		Mengo-2T	EMC-D
SJL/J	162 ± 25	390 ± 83	421 ± 56	102 ± 7	31 ± 10	19 ± 7
C57BL/6J	172 ± 14	327 ± 135	157 ± 16	109 ± 9	59 ± 18	112 ± 13
CBA/J	166 ± 51	370 ± 49	139 ± 15	110 ± 11	51 ± 7	109 ± 9
CE/J	144 ± 35	334 ± 98	160 ± 23	112 ± 10	58 ± 10	116 ± 10
C3H/HeJ	153 ± 38	368 ± 121	149 ± 16	117 ± 8	54 ± 16	107 ± 9
AKR/J	149 ± 12	576 ± 66	151 ± 15	104 ± 7	45 ± 7	101 ± 7

<sup>a</sup> Mice were infected i.p. with Mengo-2T ( $10^3$  PFU) or EMC-D ( $5 \times 10^5$  PFU). At 4 days after infection, GTTs were performed, pancreases were removed, and the concentration of IRI was measured.

<sup>b</sup> Each group consisted of four or five uninfected mice and 5 to 11 infected mice.

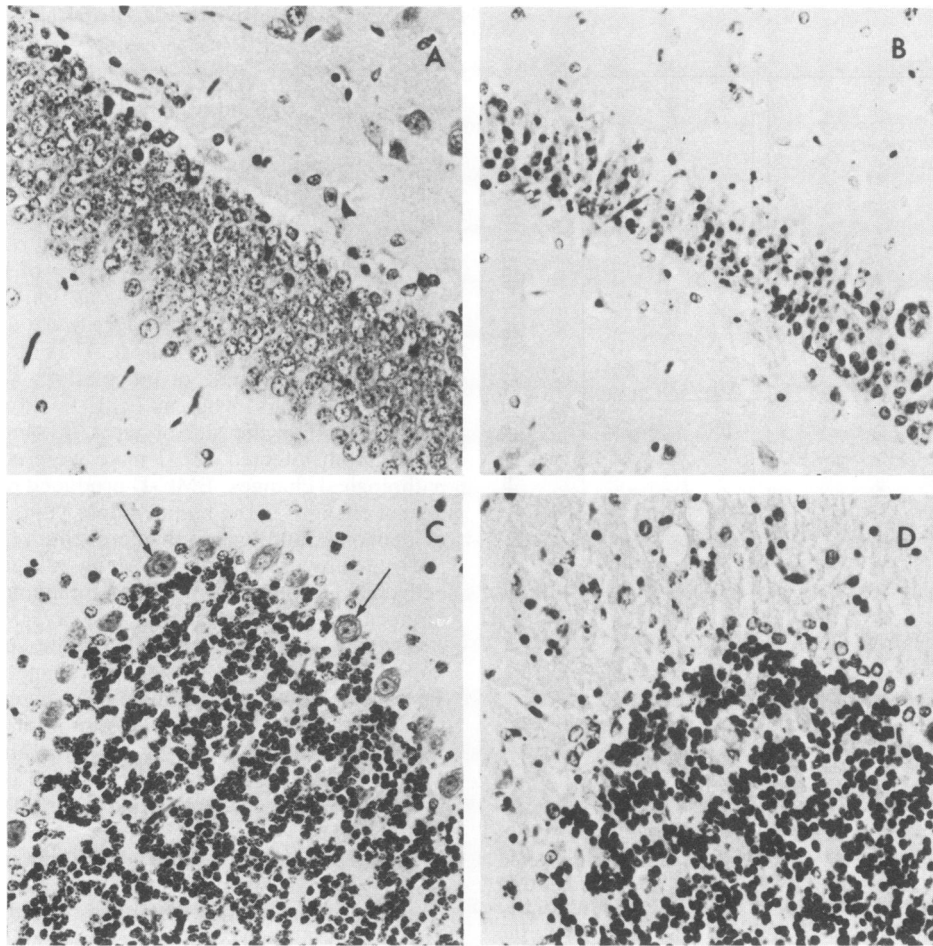


FIG. 2. Histopathological changes in brain after viral infection. Brain sections were obtained from SJL/J mice 4 days after i.p. infection with 500 PFU of Mengo-2T or EMC-D. (A) Pyramidal cells (diagonal layer) in the hippocampus from an EMC-D-infected mouse showing only minimal pathological changes ( $\times 330$ ). (B) The same area from a Mengo-2T-infected mouse demonstrating pyknotic nuclei, extensive necrotic changes, and loss of cells ( $\times 330$ ). (C) Section of cerebellum from an EMC-D-infected mouse with essentially normal Purkinje cells (arrows) ( $\times 430$ ). (D) The same area from a Mengo-2T-infected mouse showing extensive loss of Purkinje cells and focal necrosis in the molecular cell layer (upper third of photomicrograph) ( $\times 430$ ). A through D were stained with hematoxylin and eosin.

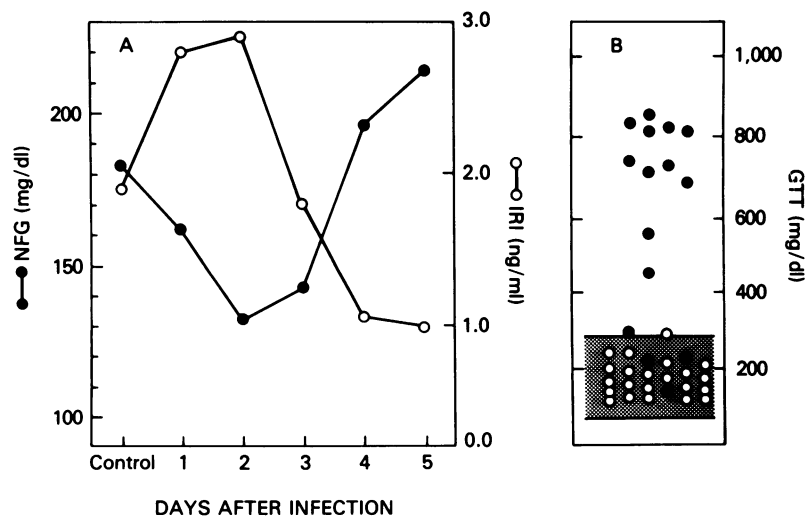


FIG. 3. Effect of Mengo-2T infection on plasma IRI, nonfasting glucose (NFG) and GTTs. SJL/J mice were infected i.p. with 500 PFU of Mengo-2T. (A) Each point represents the mean nonfasting glucose (—●—) or IRI (—○—) level on more than 20 mice. (B) GTTs were performed 4 days after infection. Each point represents an individual animal. Shaded area is the mean GTT of 22 uninfected mice  $\pm$  3 standard deviations. Symbols: ●, infected with Mengo-2T; ○, uninfected.

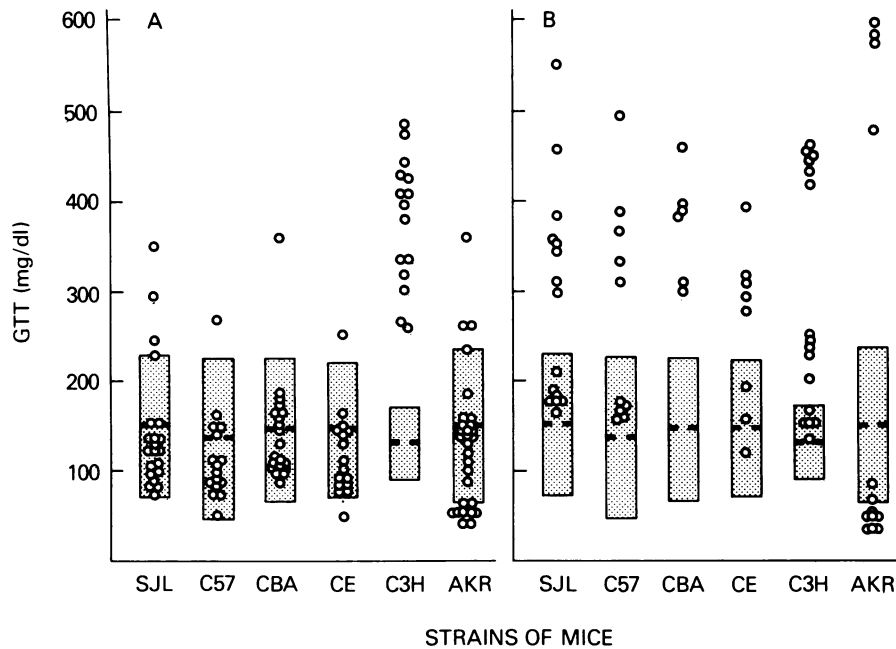


FIG. 4. Mengovirus-induced glucose abnormalities in different strains of mice. Male mice were infected i.p. with  $10^3$  PFU of Mengo-2T. GTTs were performed 3 and 4 days later. Shaded areas represent the mean glucose level (horizontal bars)  $\pm$  3 standard deviations of 20 uninfected mice of each strain. Data from two experiments were pooled. Each point represents an individual animal. (A) Three days after infection. (B) Four days after infection.

whether the induction of diabetes by Mengo-2T was influenced by the host, six inbred strains of mice were infected, and GTTs were performed. Male SJL/J, C57BL/6J, CBA/J, CE/J, C3H/HeJ, and AKR/J mice developed abnormal GTTs as early as 3, and clearly by 4, days after infection (Fig. 4A and B). In contrast, except for SJL/J, the same strains of

mice failed to develop abnormal GTTs when infected with EMC-D (Fig. 5A and B).

To see whether there was any relationship between virus-induced glucose abnormalities and IRI levels in the pancreas, different strains of mice were infected with Mengo-2T or EMC-D, and 4 days later GTTs were done and the concen-

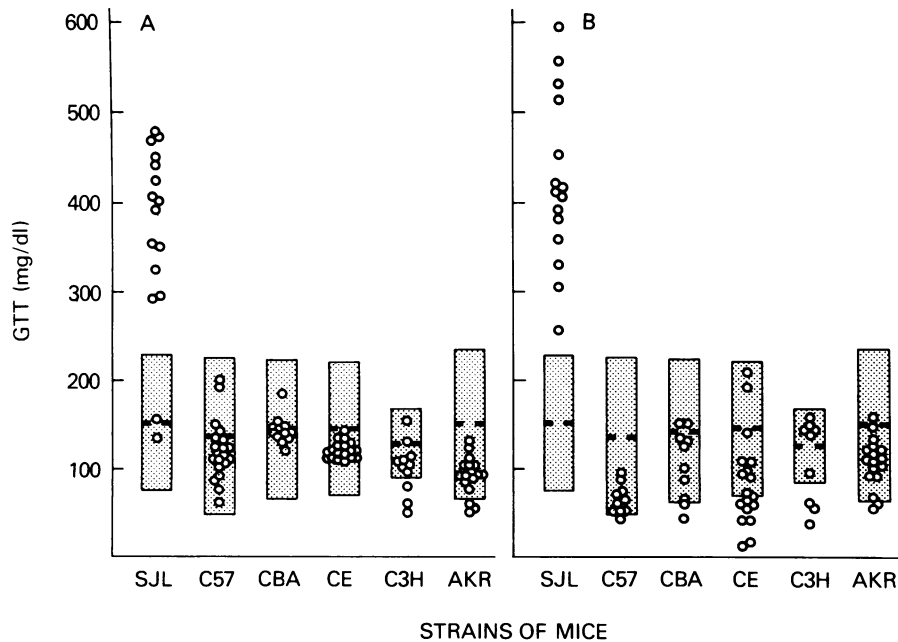


FIG. 5. EMC virus-induced glucose abnormalities in different strains of mice. Male mice were infected i.p. with  $5 \times 10^5$  PFU of EMC-D. GTTs were performed 3 and 4 days later. Shaded areas represent the mean glucose level (horizontal bars)  $\pm$  3 standard deviations of 20 uninfected mice of each strain. Each point represents an individual animal. (A) Three days after infection. (B) Four days after infection.

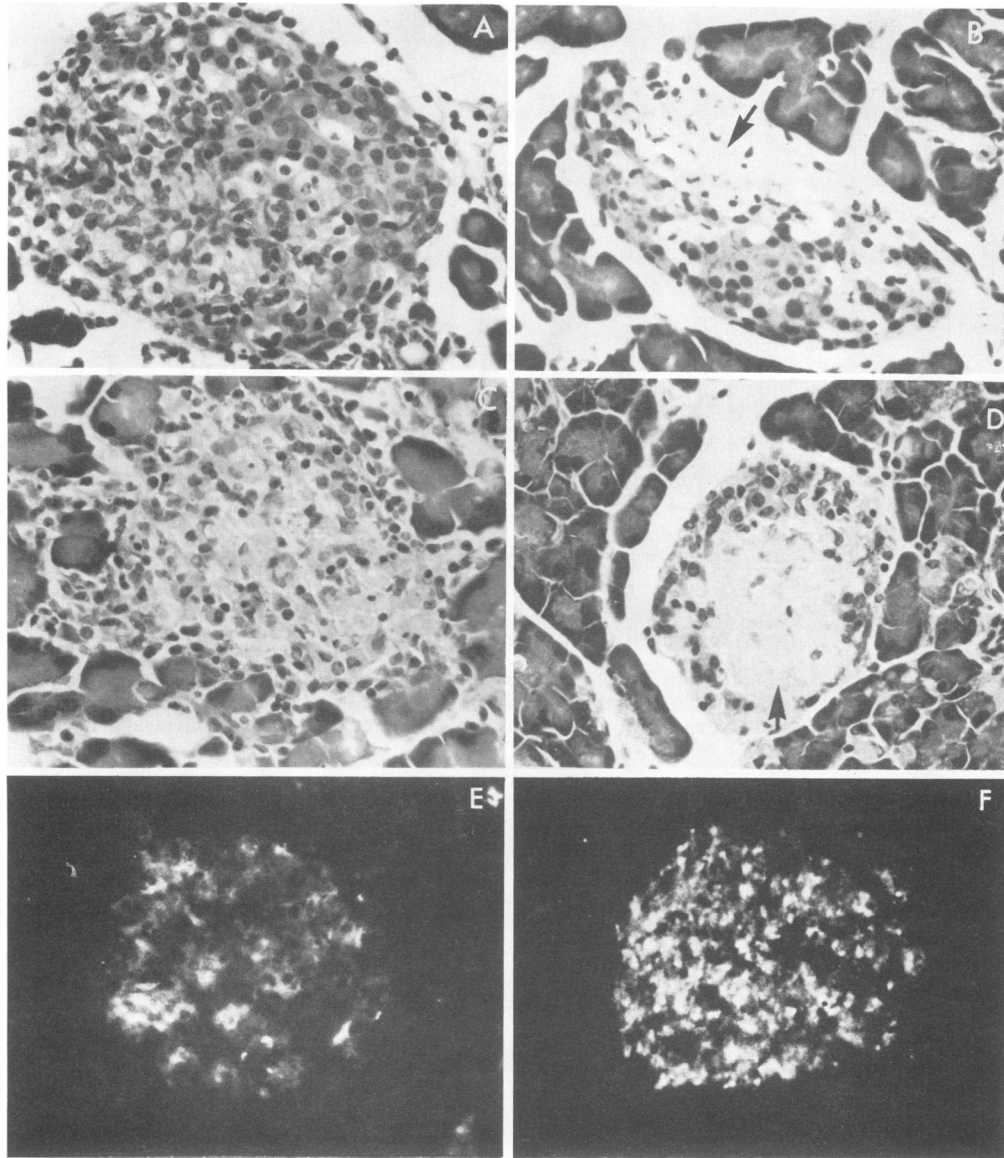


FIG. 6. Representative sections of pancreas from different strains of mice infected 5 to 7 days earlier with Mengo-2T ( $10^3$  PFU). (A) Infected mouse (CE/J) showing extensive infiltration of almost the entire islet with inflammatory cells ( $\times 550$ ). (B) Infected mouse (C3H/HeJ) showing focal necrosis in approximately one-third of the islet (arrow) ( $\times 550$ ). (C) Infected mouse (C57BL/6J) showing inflammatory infiltrate in the islet and extensive necrosis ( $\times 550$ ). (D) Infected mouse (SJL/J) showing complete loss of normal islet architecture and severe coagulation necrosis (arrow) ( $\times 550$ ). (E) Immunofluorescence microscopy of a pancreatic section showing an islet with scattered cells containing viral antigens at 2 days after infection. (F) Immunofluorescence microscopy of a pancreatic islet with viral antigens in most of the cells. A through D were stained with hematoxylin and eosin.

tration of IRI in the pancreas was determined (Table 1). The GTT values of uninfected mice ranged from  $144 \pm 35$  to  $172 \pm 14$  mg/dl. GTTs were clearly abnormal in all strains infected with Mengo-2T, whereas only SJL/J mice showed abnormal glucose levels after infection with EMC-D. The mean IRI in the pancreas of uninfected mice ranged from  $102 \pm 7$  to  $117 \pm 8$   $\mu\text{g/g}$  of pancreas. Strains of mice infected with Mengo-2T had between  $31 \pm 10$  and  $59 \pm 18$   $\mu\text{g}$  of IRI per g of pancreas. After inoculation of EMC-D, the IRI content of SJL/J pancreas decreased from  $102 \pm 7$  to  $19 \pm 7$   $\mu\text{g/g}$  but remained within the normal range in all the other strains. Thus, the glucose abnormalities induced by these viruses showed an inverse correlation with the pancreatic insulin content.

**Destruction of pancreatic islet cells and demonstration of viral antigens.** To see whether the abnormalities in glucose and insulin were related to the degree of Mengovirus-induced histopathology, sections of pancreas were prepared 5 to 7 days after infection. Approximately 100 islets from each of six inbred strains were examined microscopically. Slightly over 40% of the islets from strains of mice infected with Mengo-2T showed moderate to severe damage (2+ to 3+) (Table 2). Nearly 50% of the islets displayed mild alterations (1+), and the remaining 2 to 10% showed no evidence of pathology.

The nature and severity of the pathological changes in the six inbred strains infected with Mengo-2T were very similar (Table 2 and Fig. 6A–D). In the case of inbred mice infected

TABLE 2. Histopathological changes in the islets of Langerhans<sup>a</sup>

Mouse strain	% of islets showing a damage level of: <sup>b</sup>			
	0	1+	2+	3+
SJL/J	7	54	27	12
C57BL/6J	10	49	33	8
CBA/J	2	57	37	4
CE/J	4	51	33	12
C3H/HeJ	8	47	32	13
AKR/J	5	53	31	11

<sup>a</sup> Mice were infected i.p. with Mengo-2T ( $10^3$  PFU per mouse), and 5 to 7 days later sections of pancreas were examined microscopically.

<sup>b</sup> The percentage of islets showing damage is expressed on a 0 to 3+ scale: 0, no damage; 1+, scattered, mild inflammatory infiltrates; 2+, moderate to severe inflammation; 3+, severe islet inflammation and necrosis. Seven mice from each strain were sacrificed, and ca. 100 islets (82 to 126) were examined.

with EMC-D, little or no pathology was observed except in the diabetes-susceptible SJL/J mice (data not shown) (10).

Further evidence that Mengo-2T actually infected beta cells was obtained by immunofluorescence (Fig. 6E and F). Two to 3 days after infection, viral antigens were seen in the cytoplasm of beta cells. The severity of the infection varied considerably, with some islets showing only a few cells (Fig. 6E) and others showing almost all the cells containing viral antigens (Fig. 6F). The surrounding acinar cells were relatively free of viral antigens.

**Virus replication in islets of Langerhans.** To see whether there was any difference in the capacities of Mengo-2T and EMC-D to replicate in islet cells of SJL/J mice (susceptible to both EMC- and Mengo-induced diabetes) as compared to C3H/HeJ mice (resistant to EMC- but not to Mengo-induced diabetes), islets were obtained at different times after infection and assayed for infectious virus. Figure 7A shows that EMC-D and Mengo-2T replicated equally well in the islets of SJL/J mice. In contrast, Mengo-2T grew to a 100-fold higher virus titer than did EMC-D in the islets of C3H/HeJ mice (Fig. 7B). A second experiment (Fig. 7C and D) yielded similar results, except that the Mengo-2T titer was only 10- to 50-fold higher than the EMC-D titer in C3H/HeJ mice. Virus titers calculated per microgram of islet protein (Fig. 7E and F) gave comparable results.

DISCUSSION

We have shown for the first time that Mengovirus produces abnormal GTTs in mice. Plaque-purified Mengo-2T virus infects and destroys pancreatic beta cells as demonstrated by immunofluorescence and histopathology. The destruction of beta cells results in a decrease in the insulin content of the pancreas. This in turn leads to hypoinsulinemia and the subsequent development of hyperglycemia. Although destruction of beta cells appears to be the primary mechanism for the induction of diabetes, the fact that Mengo-2T also causes lethal encephalitis makes it difficult to exclude the possibility that neurologically regulated hormones may contribute to some extent to the abnormalities in glucose homeostasis.

Earlier studies with a neurovirulent Mengovirus pool failed to demonstrate beta cell damage or hyperglycemia in mice (7). One explanation is that a variant with diabetogenic potential was isolated during plaque purification of Mengovirus. In the case of EMC virus, we now know that our virus pool originally contained a mixture of variants and that one

of these, the nondiabetogenic B variant, could actually inhibit the development of diabetes by the diabetogenic D variant (10). By analogy, a similar situation might very well explain our previous failure to detect beta cell damage with Mengovirus. Thus, it is possible that many viruses common in both human and animal populations may be complex mixtures of variants, including some with diabetogenic potential.

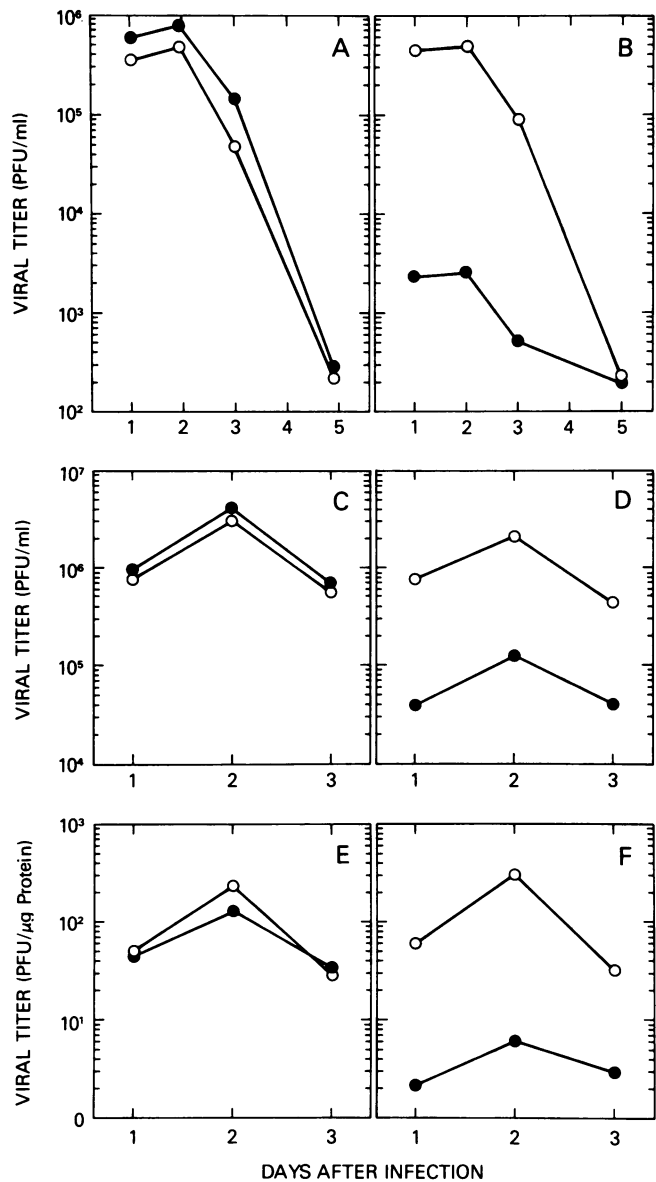


FIG. 7. Comparison of viral replication in the islets of Langerhans of mice infected with Mengo-2T and EMC-D. SJL/J mice and C3H/HeJ mice were inoculated i.p. with  $5 \times 10^3$  PFU of virus. At the times indicated, pancreases were removed from two mice and the islets (ca. 100 per pancreas) were isolated, weighed, suspended, homogenized, and assayed for infectious virus on secondary mouse embryo cells. The data represent the mean of duplicate individual determinations. Symbols: ○, Mengo-2T; ●, EMC-D. Experiment I: (A) islets from SJL/J mice; (B) islets from C3H/HeJ mice. Experiment II: (C) islets from SJL/J mice; (D) islets from C3H/HeJ mice; (E) data from C expressed per microgram of protein; (F) data from D expressed per microgram of protein.

Since there were similarities between the diabetes-like syndromes produced by EMC-D and Mengo-2T, we were concerned that Mengo-2T might have been contaminated inadvertently with EMC-D. Two lines of evidence argue against this. First, Mengo-2T was plaque purified three times. The resulting subclones induced both diabetes mellitus and lethal encephalitis with the same time course and severity as the parental clone. In contrast, EMC-D did not cause lethal encephalitis. Second, parallel experiments showed that EMC-D produced diabetes in SJL/J mice, but not in C57BL/6J, CBA/J, CE/J, C3H/HeJ, or AKR/J mice. In contrast, Mengo-2T produced diabetes in all six inbred strains of mice tested. Thus, the spectrum of host susceptibility to diabetes is strikingly different when Mengo-2T and EMC-D are compared.

The precise mechanism by which Mengo-2T infects pancreatic beta cells in the strains of mice resistant to EMC-D is not known. One possibility is that interferon may account for the difference in host range. Such a hypothesis would require that EMC-D be more sensitive than Mengo-2T to interferon and that SJL/J beta cells be less responsive to the antiviral actions of interferon than beta cells from other strains of mice. Both assumptions seem to be necessary to explain the host range differences. A second possibility is that Mengo-2T and EMC-D are distinct viruses that bind to different receptors on the surface of beta cells. In different mouse strains, there may be quantitative differences in the expression of these receptors or in the ability of the receptors to aid in penetration of these two viruses. Support for such a mechanism comes from recent studies (4) which showed that neuronal cells possess different receptors for Mengo-2T and EMC-D. Thus, receptors specific for Mengo-2T may be broadly expressed on the beta cells of mice, whereas those for EMC-D may be restricted to a few strains.

Regardless of the mechanism that accounts for differences in susceptibility, it is clear that Mengo-2T produces diabetes in strains of mice resistant to the diabetogenic effect of EMC-D. The present experiments show that this is due to the fact that more beta cells are destroyed by infection with Mengo-2T than by infection with EMC-D. This in turn appears to be related to the greater capacity of Mengo-2T to replicate in the islets of EMC-D-resistant mice. These and other studies (4, 5, 7, 9-12) support the argument that

cardiovirus variants have different host ranges and tissue tropisms.

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