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## Virus-Induced Diabetes Mellitus. X. Attachment of Encephalomyocarditis Virus and Permissiveness of Cultured Pancreatic $\beta$ Cells to Infection

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Monolayers of pancreatic  $\beta$ -cells from strains of mice susceptible (SJL/J) and resistant (C57BL/6J) to the development of virus-induced diabetes mellitus were inoculated with the M variant of encephalomyocarditis (EMC) virus. Immunofluorescence showed that viral antigens appeared in up to 10 times more  $\beta$  cells from susceptible SJL/J mice than from resistant C57BL/6J mice. Infectious center assays revealed that 10-30 times more SJL/J  $\beta$  cells contained infectious virus than C57BL/6J  $\beta$  cells. Viral attachment experiments showed no difference in the binding of EMC virus when embryonic fibroblasts, pancreatic fibroblasts, and kidney cells from SJL/J and C57BL/6J mice were compared. However, at least twice as much virus attached to the pancreatic  $\beta$  cells from susceptible than from resistant strains of mice. Our data suggest that genetically determined differences in viral receptors on the surface of  $\beta$  cells may be one of the factors controlling susceptibility to EMC-induced diabetes mellitus.

The M variant of encephalomyocarditis (EMC) virus produces a diabetes-like syndrome in mice by infecting and destroying pancreatic  $\beta$  cells (1-5). Only certain strains of mice develop this syndrome and susceptibility to EMC-induced diabetes is inherited as an autosomal recessive trait (6). The genetic factors controlling susceptibility operate at the level of the  $\beta$  cells, and whether a particular strain of mouse develops diabetes appears to be related to differences in the permissiveness of the  $\beta$  cells to infection with EMC virus (7, 8).

Recently pancreatic  $\beta$  cells from strains of mice that develop EMC-induced diabetes (susceptible) and pancreatic  $\beta$  cells from strains of mice that do not develop EMC-induced diabetes (resistant) were grown in culture and infected with EMC virus (8). Examination of these cultures revealed that  $\beta$  cells from susceptible mice produced up to 50 times more virus than did  $\beta$  cells from resistant mice. However, it had not been determined whether the

higher viral yield in cultures from susceptible mice was due to more virus being produced per cell or more cells being infected. The present investigation was initiated to resolve this question and, in addition, to see whether there was any difference in the attachment of EMC virus to  $\beta$  cells from susceptible as compared to resistant strains of mice.

The M variant of EMC virus was grown and assayed on CAF-1 mouse embryo fibroblasts (MEF) (8). Monolayers of MEF, kidney cells, and pancreatic fibroblasts were prepared by routine methods. Monolayer cultures enriched for pancreatic  $\beta$  cells were prepared as described previously (8). Based on staining with fluorescein-labeled anti-insulin antibody, between 50 and 85% of the cells in these cultures were  $\beta$  cells.

<sup>3</sup>H-labeled EMC virus was prepared by infecting MEF monolayers with 10 plaque-forming units (PFU) of virus/cell. After a 1-hr incubation, Eagle's minimal essential medium (MEM) with 1% calf serum and 10  $\mu$ Ci/ml of [<sup>3</sup>H]uridine was added. When

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approximately 80% of the cells showed cytopathology, the medium containing the labeled virus was harvested, and the virus was purified essentially by the methods used by Ziola and Scraba for Mengo virus (9). The virus banded at a bouyant density of  $1.33 \text{ g/cm}^3$  in CsCl and contained  $1.6 \times 10^5$  cpm/ml with an infectious titer of  $8.4 \times 10^7$  PFU/ml.

Infectious center assays were performed by inoculating monolayers of  $\beta$  cells (35-mm petri dishes) with EMC virus at a multiplicity of infection (m.o.i.) of 100. At the end of 60 min, the unattached virus was removed by washing the cells three times. The monolayers then were briefly trypsinized (5 min at  $37^\circ$ ), and the cells were resuspended and incubated at  $37^\circ$  for 40 min in MEM containing antibody to EMC virus (neutralization titer,  $>300$ ) to neutralize any infectious virus in the medium or on the surface of the cells. The cells were then washed three times, resuspended, and counted, and the appropriate numbers were added to confluent MEF monolayers. The monolayers then were overlaid with methylcellulose and the number of cells that produced plaques (infectious centers) was determined 72 hr later.

The number of cells containing viral antigens was determined by immunofluorescence.  $\beta$  cells grown on coverslips were stained with fluorescein isothiocyanate-(FITC) labeled anti-EMC antibody as described previously (8).

Viral attachment assays were performed on monolayers (60-mm petri dishes) of MEF, pancreatic fibroblasts, and kidney cells containing approximately  $1.5 \times 10^6$  cells and on monolayers enriched for pancreatic  $\beta$  cells containing approximately  $8.0 \times 10^5$  cells. Based on preliminary experiments in which different concentrations of the virus and cells were tested optimal attachment resulted when monolayers were inoculated with 0.15 ml of labeled virus ( $1200 \text{ cpm} = 6 \times 10^5 \text{ PFU}$ ). At various times after viral inoculation, 0.85 ml of cold 0.1 M phosphate-buffered saline (PBS), pH 7.5, was added. The medium then was removed and assayed for infectivity (8) and radioactivity (10). The

percentage of virus bound to cells at any given time was determined by calculating the difference between the amount of virus recovered in the medium and the amount of virus inoculated. Petri dishes incubated with MEM but not containing cells served as controls.

The permissiveness of  $\beta$ -cell cultures from strains of mice that develop diabetes (SJL/J, NIH-Swiss) and from strains of mice that do not develop diabetes (C57BL/6J) to EMC infection is illustrated in Table 1. Immunofluorescence showed that up to 10 times more SJL/J cells contained viral antigens than did C57BL/6J cells. At 24 hr after infection (m.o.i., 10), 5% of the C57BL/6J cells were positive for viral antigens, while 46% of the NIH-Swiss cells and 54% of the SJL/J cells contained viral antigens. When a higher m.o.i. (100) was employed, immunofluorescence was seen in 19% of the C57BL/6J cells and in 91% of the SJL/J cells. In contrast, no difference was observed in the number of cells containing viral antigens when MEF cultures were infected with EMC virus; approximately 95% of the cells in C57BL/6J and SJL/J monolayers contained viral antigens at 18 hr after infection. Viral antigens were not detected in uninfected  $\beta$ -cell monolayers.

The difference in susceptibility was even more apparent by infectious center assay. The data in Fig. 1 show that at each of the cell concentrations tested, 10–30 times more SJL/J than C57BL/6J cells contained infectious virus. In the C57BL/6J cultures, fewer than 3 cells per 500 cells were infected, while in the SJL/J cultures, 60 cells per 500 cells were infected. In the experiment illustrated in Fig. 1, an m.o.i. of 100 was used. In other experiments in which a lower m.o.i. (10) and a higher m.o.i. (1000) were used, similar types of results were obtained (data not shown).

To see whether the difference in susceptibility could be related to the rate at which EMC virus attached to SJL/J as compared to C57BL/6J cells,  $^3\text{H}$ -labeled virus was added to monolayers and at different times thereafter the amount of virus that attached was determined. The attachment of  $^3\text{H}$ -labeled EMC virus to

TABLE 1  
NUMBER OF CELLS CONTAINING VIRAL ANTIGENS IN PANCREATIC MONOLAYERS PREPARED FROM STRAINS OF MICE SUSCEPTIBLE AND RESISTANT TO EMC-INDUCED DIABETES<sup>a</sup>

Hours after inoculation	Inoculum (PFU/cell)	Strain	Type of culture	Number of cells observed	Cells showing viral antigens (%)
Uninfected	—	C57BL/6J	$\beta$	179	0
Uninfected	—	SJL/J	$\beta$	245	0
18	20	C57BL/6J	$\beta$	304	6
18	20	SJL/J	$\beta$	360	52
24	10	C57BL/6J	$\beta$	913	5
24	10	NIH-Swiss	$\beta$	531	46
24	10	SJL/J	$\beta$	301	54
24	100	C57BL/6J	$\beta$	314	19
24	100	SJL/J	$\beta$	71	91
18	20	C57BL/6J	MEF <sup>b</sup>	69	95
18	20	SJL/J	MEF	81	97

<sup>a</sup> Monolayers were inoculated with EMC virus and stained with FITC-labeled anti-EMC antibody, and the number of cells containing viral antigens was determined.

<sup>b</sup> Mouse embryo fibroblast.

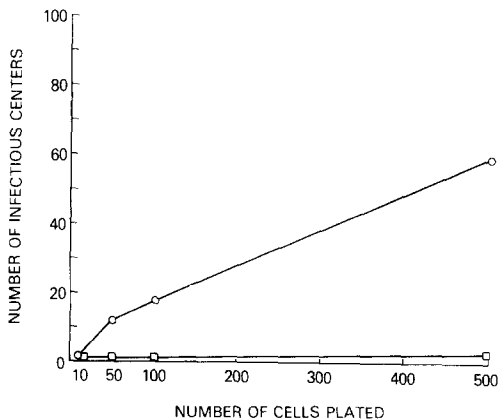


FIG. 1. Number of cells containing infectious virus in pancreatic  $\beta$ -cell monolayers prepared from strains of mice susceptible and resistant to the development of EMC-induced diabetes mellitus. Monolayers were inoculated with EMC virus using a multiplicity of infection of 100. After incubation at 37° for 60 min, the unattached virus was removed and the monolayers were trypsinized. The individual cells were resuspended and incubated (37° for 40 min) in antibody to EMC virus to neutralize extracellular virus. The cells then were washed, counted, and plated on MEF monolayers overlaid with methylcellulose. The number of cells that produced plaques was determined 48 hr later. ○—○, SJL/J; □—□, C57BL/6J.

MEF, pancreatic fibroblasts, kidney cells, and pancreatic  $\beta$  cells is illustrated in Fig. 2. Viral attachment ranged from 9 to 22%, depending on the cell type. No significant difference in binding of <sup>3</sup>H-labeled virus was observed when cells from susceptible and resistant strains of mice were compared. The attachment of virus as measured by radioactivity leveled off at 16 to 32 min after inoculation. In contrast, the attachment of virus as measured by infectivity continued for at least 64 min. Although the total amount of infectious virus which attached varied depending on the cell type, there was no significant difference in attachment when MEF, pancreatic fibroblasts, and kidney cells from susceptible and resistant strains were compared. However, in the case of pancreatic  $\beta$  cells, more infectious virus attached to cells from the susceptible than resistant strain. The data in Fig. 2h represent an average of four separate experiments employing duplicate plates at each point. Binding of infectious virus to C57BL/6J  $\beta$ -cell monolayers reached 20% at 16 min and increased to only 22.5% at 64 min. The attachment of infectious virus to SJL/J

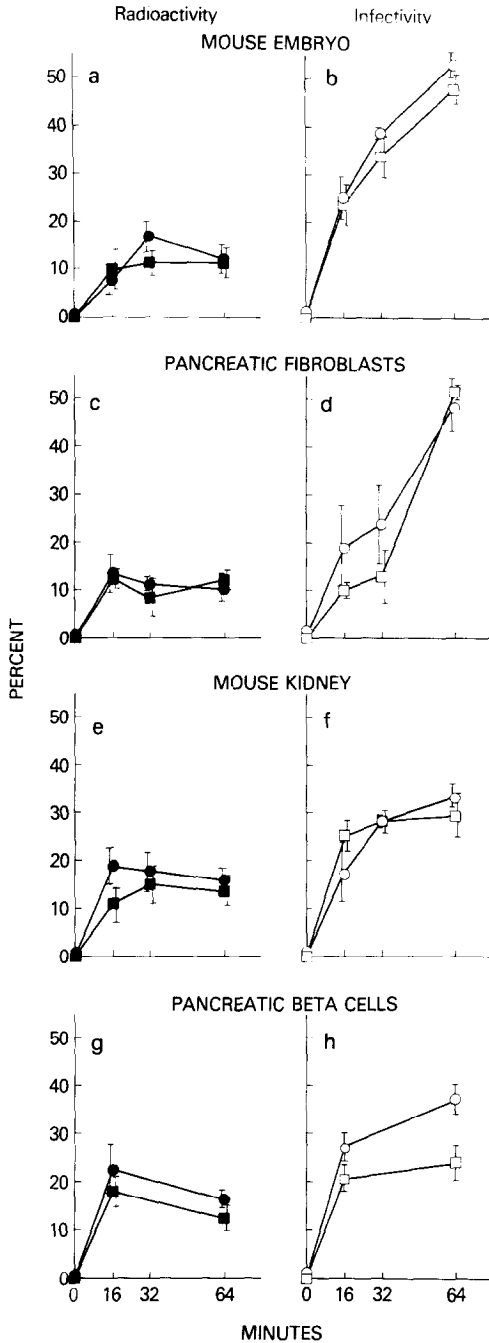


FIG. 2. Attachment of EMC virus to cells from susceptible (SJL/J) and resistant (C57BL/6J) strains of mice. Monolayers were inoculated with EMC virus ( $1200 \text{ cpm} = 6 \times 10^5 \text{ PFU}$ ) and incubated at  $37^\circ$ . At the end of 16, 32, or 64 min, the amount of virus bound was determined and expressed as a percentage of the input virus. Each point represents the arithmetic mean  $\pm$  the standard deviation of pooled data of two to four experiments, in which

monolayers, however, continued for at least 64 min, at which time 38.5% of the input virus had bound. Paired *t* tests of the slopes of the regression lines from the pooled data at 0, 16, and 64 min revealed a significant difference at the 95% confidence level ( $P < 0.05$ ). In another experiment in which a different virus pool was used (data not shown), 2.5 times more virus attached to the SJL/J monolayers than to the C57BL/6J monolayers.

The immunofluorescence and infectious center data on cultured  $\beta$  cells reported here is consistent with earlier *in vivo* findings (8) which showed that SWR/J  $\beta$ -cells were more susceptible to infection than C57BL/6J  $\beta$ -cells. Moreover, the present findings indicate that the difference in susceptibility of  $\beta$  cells observed *in vivo* is retained under *in vitro* cultivation conditions. Blocks at any one of several sites in the viral cycle (e.g., attachment, uncoating, or replication) could account for the observed differences in susceptibility. In the case of the picornaviruses, it is known that only certain cell types have receptors for these viruses (11-13), and differences in the concentration of these receptors could influence the susceptibility of the various organs within the host. Our experiments with the M variant of EMC virus showed that more of this virus attached to  $\beta$  cells from mice that developed diabetes (SJL/J) than to  $\beta$  cells from mice that did not develop diabetes (C57BL/6J). In contrast, no difference in attachment was observed when other cell types (e.g., kidney, embryonic fibroblast, pancreatic fibroblast) from these two strains of mice were tested. These findings are compatible with earlier observations which showed differences in viral replication in  $\beta$ -cell cultures but not kidney or embryo cell cultures, when SWR/J and C57BL/6J mice were compared (8). In this connection, it is known that some viruses can grow in monolayer cultures derived from organs

duplicate or triplicate determinations were made. Panels a, c, e, and g: Attachment of  $^3\text{H}$ -labeled virus to SJL/J cells (●—●) or C57BL/6J cells (■—■). Panels b, d, f, and h: attachment of infectious virus to SJL/J cells (○—○) or C57BL/6J cells (□—□).

which in the host are ordinarily resistant to these viruses. The development of susceptibility in culture presumably is due to induction of receptors (11, 12, 14, 15). It appears, however, that at least in the case of our  $\beta$ -cell system, differences among strains in susceptibility to the M variant of EMC virus persist in culture.

A significant difference in the attachment of EMC virus was observed only when attachment was assayed by infectivity. The failure to demonstrate any difference when radioactivity was measured may in part be explained by the fact that the assay for  $^3\text{H}$ -labeled virus detects both defective particles and virus particles which have eluted from the cell and have lost their infectivity (10, 16-18). Other investigators have reported results similar to our findings: Viral attachment (e.g., poliovirus type 1, rhinovirus-14, and human coronavirus) when measured by radioactivity may give lower values than when measured by infectivity (10, 18; K. Holmes, personal communication).

The actual attachment of the M variant of EMC virus to pancreatic  $\beta$  cells in fact may be greater than the apparent twofold difference observed here. Recent immunofluorescence studies on sections of pancreas from several strains of mice inoculated with EMC virus but resistant to the development of diabetes (i.e., C57BL/6J, CBA/J, AKR, and A/J) revealed a considerable difference in the number of  $\beta$  cells that became infected (19). Although neither C57BL/6J nor CBA/J mice developed diabetes when inoculated with EMC virus, approximately 12% of the  $\beta$  cells from C57BL/6J mice contained viral antigens at 72 hr after inoculation, as compared to 3% of the  $\beta$  cells from CBA/J mice (19). The fact that so few CBA/J  $\beta$  cells became infected suggested that it might be possible to detect even greater differences in viral attachment if the  $\beta$  cells from this strain were compared with  $\beta$  cells from the susceptible SJL/J strain. Recent experiments using these two strains showed that three to four times more virus attached to SJL/J cells than to CBA/J  $\beta$  cells (unpublished data).

Another factor that might contribute to

differences in viral attachment is the source of the virus. Recently, it was found that EMC virus which had been passaged in tissue culture several times was less diabetogenic than EMC virus which had been passaged in mice (19). Whether this was due to a reduced tropism for pancreatic  $\beta$  cells is not known, but the possibility that virus passaged in mice might show better attachment than the tissue culture-passaged ( $^3\text{H}$ -labeled) virus used in the present experiments merits investigation. Moreover, it is known that different strains of virus show very different rates of attachment. For example, Mak *et al.* (20), found that 95% of a small plaque variant of Mengo virus bound to L cells, while only 20% of a large plaque variant of this virus bound to L cells. Of the various members of the EMC virus group studied thus far (i.e., Mengo, Columbia SK, Columbia MM, and Kissling), only the M variant produced diabetes when inoculated into mice (21).

Still another factor that could influence viral attachment is the actual percentage of  $\beta$  cells in the enriched cultures. Our cultures usually contained about 65%  $\beta$  cells, but it is still not technically possible to obtain large quantities of  $\beta$  cells which are absolutely free of other pancreatic cells (e.g., fibroblasts, ductal cells, or acinar cells). Moreover, certain cell types (e.g., Fig. 2, pancreatic fibroblasts) may bind more virus than  $\beta$  cells. Thus, the greater binding of EMC virus to non- $\beta$  cells that contaminate the enriched cultures may have obscured an even larger difference in the specific binding of EMC virus to  $\beta$  cells than the apparent twofold difference observed in our studies.

In conclusion, differences favoring the attachment of EMC virus to  $\beta$  cells from strains of mice that develop diabetes now have been observed in seven out of seven experiments performed. Whether restriction at levels other than attachment (e.g., transcription and/or translation) also contributes to the degree of permissiveness of  $\beta$  cells from different strains of mice to EMC replication is not known. However, based on present information, it appears that genetically determined differences in

viral receptors may at the least be one of the factors controlling susceptibility to EMC-induced diabetes mellitus.

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