Differing Attachment of Diabetogenic and Nondiabetogenic Variants of Encephalomyocarditis Virus to β-Cells

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The D variant of encephalomyocarditis (EMC-D) virus does not induce the production of interferon (IFN) and produces an insulin-dependent diabetes mellitus (IDDM)–like syndrome in certain mouse strains. In contrast, the B variant (EMC-B) virus, which is serologically identical to EMC-D virus, is a good inducer of IFN and is nondiabetogenic. It has been postulated that IFN may play a major role in determining the ability of these two viruses to infect pancreatic β-cells. However, recent studies have shown that ICR Swiss and BALB/cByJ male mice are not protected by IFN against EMC-D virus–induced IDDM. Furthermore, treatment of these two strains of mice with anti-IFN γ-globulin before infection with EMC-B virus does not result in diabetes. These observations suggest that mechanisms other than the IFN system are involved in determining the ability of the viruses to infect and destroy β-cells. Studies were initiated to identify other mechanisms of action. In this communication, we show that up to six times more EMC-D than EMC-B virus attaches to primary β-cells extracted from male ICR Swiss mice. This difference in ability to attach to β-cells may account for the difference in the diabetic potential of this mouse strain. *Diabetes* 38:1103–108, 1989

Certain strains of mice, and with few exceptions only males, develop a diabetes mellitus–like syndrome after infection with the diabetogenic variant of encephalomyocarditis (EMC-D) virus (1–3). Mice infected with the B variant of EMC (EMC-B) virus, which is serologically indistinguishable from EMC-D virus (1), do not develop diabetes or exhibit overt evidence of disease. Because it has been shown that EMC-B virus is a good inducer of interferon (IFN), whereas EMC-D virus induces little or no IFN (1), it has been suggested that IFN produced during the early stages of EMC-B virus infection plays a role in protecting the β-cells from viral destruction. Several studies support the hypothesis that IFN may play a role in the pathogenesis of these two virus variants. For example, Gadzik et al. (4) reported that a single injection of the IFN-inducer polyinosinic-polycytidylic acid (poly I:C) protects SWR/J mice against the diabetogenic effects of EMC-M virus, an EMC virus from which both the D and B variants were isolated (1). Yoon et al. (5) reported that pretreatment with multiple doses of either L929 (L)-cell–produced IFN or poly I:C reduced the incidence of diabetes in SJL/J mice infected with EMC-D virus. More direct evidence that IFN is involved in the nondiabetogenicity of EMC-B virus was provided in the same study by data showing that 40% of mice given antibody to IFN-α and -β 1 h before infection with EMC-B virus developed mild diabetes. However, the mildness of the diabetic state, coupled with the fact that only 13% of the islet cells had viral antigens present, suggested that IFN in and of itself cannot account for the different biologic effects of the two virus variants. Indeed, we have evidence and have reported that exogenously administered IFN-α and -β or poly I:C protects SWR/J but not ICR Swiss mice against the diabetogenic effects of EMC-D virus (6). This observation indicates that the protection against virus-induced diabetes by IFN is strain dependent and suggests that other mechanisms are involved that determine the diabetogenic properties of these two viruses. In this study, data are presented that show that EMC-D virus adsorbs to β-cells to a greater extent than does EMC-B virus and might explain the difference in their diabetogenic potential.

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RESEARCH DESIGN AND METHODS

Viruses. The EMC-D and EMC-B virus variants were propagated and titrated by methods previously described (7). In our laboratory, the B variant is nondiabetogenic, produces small, discrete plaques on L-cells, and induces up to 2000

PR_{50} (50% plaque-reduction) units of IFN/ml in L-cell cultures, whereas the D variant is highly diabetogenic and produces large, diffuse plaques and no detectable levels of IFN (8). Each virus was diluted in Hanks' balanced salt solution (HBSS) to give the indicated multiplicity of infection (MOI).

For preparation of radiolabeled EMC-B virus stock, 75-cm^2 tissue-culture flasks of confluent baby hamster kidney (BHK21) cells were infected with EMC-B virus (1.0 ml) at an MOI of 10. After a 1-h adsorption, the cells were washed with HBSS and fed with methionine-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% fetal bovine serum (FBS) for 2 h. The cells were then washed twice with 10 ml of HBSS and refed with 8 ml of DMEM containing 0.1 mCi/ml [^{35}S]methionine (ICN Radiochemicals, Irvine, CA). After 16–24 h of incubation at 37°C, cells and supernatant fluids were harvested and centrifuged at 1400 × *g* for 10 min. Supernatants were collected and added to 36-ml ultracentrifuge tubes containing 1.0 ml of a 30% sucrose solution and centrifuged at 100,000 × *g* at 4°C for 90 min. The supernatant fluids were decanted from each tube and the pellets washed with 20 ml HBSS. The pellets were resuspended in 0.1 ml of 50 mM Tris-HCl (pH 7.6). The suspensions were further diluted in HBSS and stored at −70°C in 0.1-ml aliquots. It was determined that the virus plaque-forming unit (PFU) to counts per minute (cpm) ratio of these preparations was 15:1.

Cell cultures. Baby mouse pancreas (BMP) cells, an SV40-transformed β-cell line from newborn CD-1 mice, were kindly provided by J.V. Hallum (Oregon Health Sciences University, Portland, OR). Mouse L-cells and BHK21 cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were grown and maintained in DMEM supplemented with 10% FBS and 50 μg/ml gentamicin sulfate.

Primary β-cells were isolated and purified from male ICR Swiss mice (9–10 wk old) according to procedures described previously (9,10). Briefly, pancreases were aseptically removed from 10 animals at a time, washed three times in phosphate-buffered saline (PBS), and minced. The tissue fragments were washed for 5 min in PBS with continuous stirring. The supernatant was discarded, and 10 ml of prewarmed (37°C) trypsin-collagenase solution was added. After 12 min of incubation at 37°C with gentle stirring, the tissue fragments were allowed to settle, and the supernatant was collected. Trypsin-collagenase solution (10 ml) was added to the remaining tissues. The trypsinization procedure was repeated until all pancreatic tissue was digested. Each supernatant was added to 20 ml cold medium 199 and centrifuged at 260 × *g* for 10 min. After decantation of the supernatant, the cell pellet was washed with PBS, and the cells were resuspended in 5 ml ice-cold 25% Ficoll and added to a 13-ml conical tube. The cell suspension was overlaid with 5 ml of cold 20% Ficoll and centrifuged for 10 min at 1000 × *g*. The portion of the gradient formed from the interface to the top of the pellet was removed and washed twice with cold medium to remove residual Ficoll. The cells were resuspended in 2 ml medium 199 supplemented with 300 mg/dl glucose, added to a 35-mm tissue-culture dish, and incubated overnight (37°C) to remove fibroblasts, which adhere to the dish. Nonadherent islets were collected and added to a 10-ml Erlenmeyer flask and disrupted by stirring in prewarmed (37°C) trypsin-collagenase solution for 12 min. The cells were washed, resuspended in medium 199, and added to a 12-well tissue-culture plate. Most (85–90%) of the cells were identified as β-cells by fluorescein isothiocyanate–labeled anti-insulin antibody. The yield of β-cells was consistent with that reported previously (11,12).

Virus-attachment assay. All virus-attachment studies were done in 1.5-ml polystyrene microtubes (Sarstedt, Princeton, NJ). The virus (100–200 μl) was added to 100 μl of cells at the indicated MOI. The same amount of virus was added to vials containing 100 μl DMEM and served as background virus controls. The vials were incubated at 4°C for 1 h with rotation at 80 rpm. Duplicate experimental and background vials were centrifuged at 260 × *g* (4°C) for 5 min and the supernatants aspirated. Cells were washed three times with 1.5 ml of HBSS and centrifuged at 260 × *g* (4°C) for 5 min. Cell pellets were resuspended in 1.5 ml HBSS, frozen, thawed, sonicated for 30 s, and vortexed. Cell debris was removed by centrifugation and the number of PFUs determined. Attachment data are expressed as PFU per cell, which was calculated by dividing the number of PFUs per vial by the original number of cells per vial, and does not necessarily reflect the actual number of PFUs attached to individual cells.

Competition assay. Two hundred microliters of EMC-B virus, EMC-D virus at the desired concentration, or HBSS were added to triplicate 1.5-ml vials containing either 100 μl of BMP cells or 100 μl DMEM supplemented with 2% FCS. Vials were incubated at 4°C for 30 min and rotated at 80 rpm. After centrifugation (260 × *g* for 5 min), the supernatants were aspirated; the cells were resuspended in radiolabeled EMC-B virus at the desired concentration, incubated at 4°C for 30 min, and centrifuged; and the supernatants were aspirated again. After washing twice with 1.5 ml HBSS, the cells were resuspended in 50 μl HBSS, pipetted onto Whatman 3-mm filters, and air dried. The filters were rinsed three times with 10% TCA and three times with 95% ethanol and air dried for 30 min, and the cpm were determined.

Enzyme-linked immunosorbent assay (ELISA). Dilutions of EMC-B and EMC-D viruses were prepared in carbonate-bicarbonate coating buffer (pH 9.6). Two hundred microliters of each dilution were added to triplicate experimental and antigen-control wells in a 96-well ELISA plate (Corning, Corning, NY). Coating buffer was added to antibody-control wells and blanks. After incubation for 24 h at 4°C, the wells were washed with 0.05% PBS-Tween (pH 7.4) and filled with a 1% bovine serum albumin solution in PBS-Tween. After 30 min at room temperature (RT), the wells were washed four times with PBS-Tween, and 200 μl of mouse anti-EMC virus serum diluted in PBS-Tween was added to experimental and antibody-control wells. Antigen-control wells and blanks received PBS-Tween. After 2 h at RT, the wells were washed four times with PBS-Tween, and 200 μl of goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma) diluted 1:500 in PBS-Tween was added to each well. Blanks received PBS-Tween. The plate was incubated for 2 h at RT, and after washing four times with PBS-Tween, each well received 200 μl of *p*-nitrophenyl phosphate (Sigma) diluted to a concentration of 1 mg/ml in 10% diethanolamine buffer (pH 9.8). The enzyme was allowed to react with the substrate for 30 min at RT, after which 50 μl of 3 M NaOH was added to each

well to stop the reaction. After 5 min, absorbance at 405 nm was recorded for each well on a Dynatech Minireader II.

Statistical analysis. Data are expressed as the sample mean ± SD. Student's *t* test was used to evaluate the statistical significance of the data. $P < .05$ was considered significant.

RESULTS

Attachment and penetration of EMC-D virus to BMP cells. The attachment of EMC-D virus to BMP cells at 4°C and RT was compared with the procedure described in RESEARCH DESIGN AND METHODS. At an MOI of 100, ~10 times more virus attached to cells at 4°C compared with RT after a 1-h incubation ($P < .02$; Fig. 1). No loss in titer was observed during the 2-h test for virus incubated at either temperature (data not shown). Based on these data, virus adsorption for subsequent experiments was done at 4°C. The specificity of the attachment was determined by measuring virus penetration (loss of PFUs attached to cell surface) over 2 h. Virus, at an MOI of 100, was adsorbed for 1 h, after which the cells were washed and resuspended in DMEM. Zero-hour samples were collected at this time and the remaining samples transferred to 37°C to allow the virus to penetrate. Experimental and background control samples were taken at the times indicated and frozen. At the time of assay, the samples were thawed and the cells disrupted by sonication, and infectious virus content was determined. Approximately 0.3 PFUs/cell were attached at 0 h (Fig. 2). It is not unreasonable to assume that most of the virus that attaches to specific receptor sites will penetrate the host cell. Based on this assumption, 56, 83, and 93% penetration was observed at 15, 30, and 60 min, respectively. Virus replication was first detected at 2 h postinfection, with titers doubling between 8 and 10 h postinfection, the time for completion of one replication cycle for this virus.

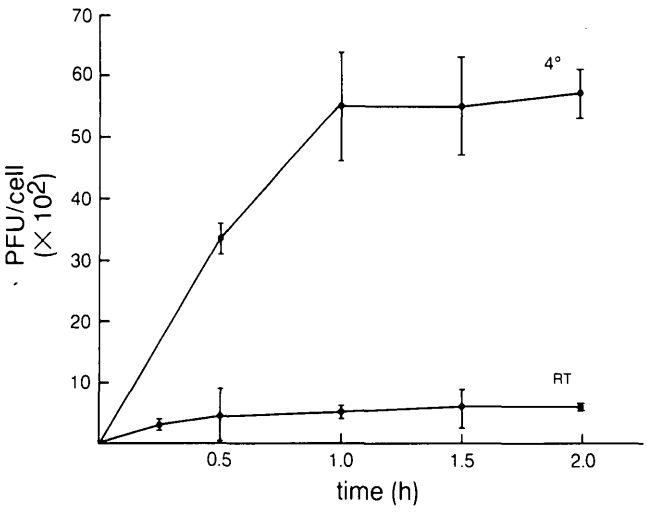

FIG. 1. Attachment of encephalomyocarditis (EMC)-D virus to baby mouse pancreas cells at 4°C and room temperature (RT). Virus was added to cells in suspension at multiplicity of infection of 100 and incubated at 4°C or RT. Samples were taken at times indicated and assayed for number of plaque-forming units (PFUs) adsorbed as described in RESEARCH DESIGN AND METHODS. Each data point is mean PFU/cell ± SD for duplicate samples.

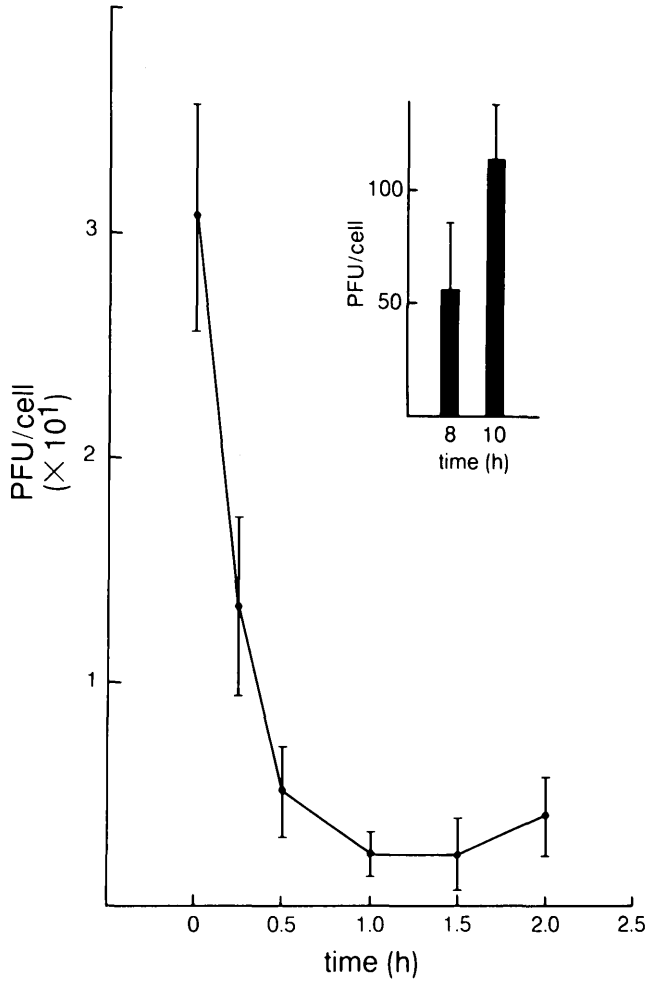

FIG. 2. Penetration of encephalomyocarditis (EMC)-D virus into baby mouse pancreas cells. Cells in suspension were infected with EMC-D virus at a multiplicity of infection of 100. After incubation at 4°C for 1 h, samples were washed 3 times and incubated at 37°C. Samples were taken at times indicated and assayed for virus plaque-forming unit (PFU) content as described in RESEARCH DESIGN AND METHODS. Each data point is mean PFU/cell ± SD for duplicate samples. *Inset*: 8- and 10-h samples showing virus replication.

Attachment of B and D variants to BMP cells. To determine if the attachment of the two virus variants to BMP cells differed, each virus was adsorbed to the cells for 1 h at MOI of 75, 300, or 1200. At an MOI of 1200, more than twice as much EMC-D as EMC-B virus attached to the cells ($P < .05$; Fig. 3). Note that neither curve reached saturation over the range tested, although EMC-D virus was in a more linear phase of attachment than EMC-B virus.

Competition for cell receptor sites. With the techniques described in RESEARCH DESIGN AND METHODS, studies were done to determine if EMC-D and EMC-B viruses compete for common receptor sites on BMP cells. Pretreatment of BMP cells with unlabeled EMC-B at an MOI of 100 reduced the attachment of labeled EMC-B by 50% ($P < .05$; Table 1). Pretreatment with EMC-D virus at the same MOI reduced the attachment of labeled EMC-B virus to about the same extent. These results suggest that attachment of the viruses to these cells is specific and that they compete for common receptor sites.

Virus attachment to primary β-cells. Primary β-cells were

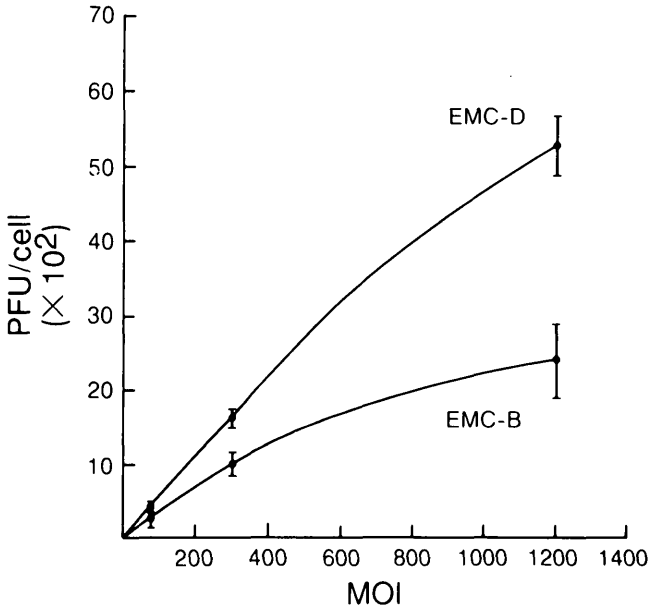

FIG. 3. Attachment of encephalomyocarditis (EMC)-D and EMC-B virus variants to baby mouse pancreas cells. Cells in suspension were infected with each virus variant at multiplicity of infection (MOI) indicated. After 1-h incubation at 4°C, cells were washed and number of virus attached determined as described in RESEARCH DESIGN AND METHODS. Each data point is mean plaque-forming unit (PFU)/cell ± SD for duplicate samples.

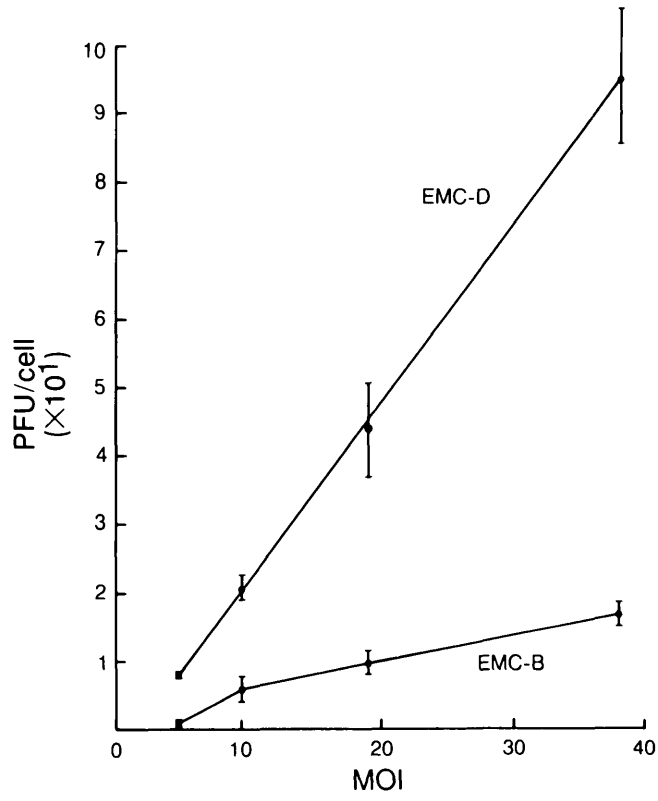

FIG. 4. Attachment of encephalomyocarditis (EMC)-D and EMC-B viruses to primary β-cells. Each virus variant was adsorbed for 1 h at 4°C to primary β-cells in suspension. Number of plaque-forming units (PFUs) adsorbed to cells was determined. Each data point is mean PFU/cell ± SD for duplicate cultures. MOI, multiplicity of infection.

prepared as described in RESEARCH DESIGN AND METHODS. Each of the virus variants was adsorbed for 1 h to the cell preparations at different MOI as indicated. At an MOI of 40, ~5.5 times more EMC-D than EMC-B virus attached to the cells ($P < .01$; Fig. 4). Furthermore, the D variant was in a linear phase of attachment over the range of MOI tested, whereas that of EMC-B virus was nearly saturated at a low MOI. These results are similar to those obtained with BMP cells (Fig. 3). The study was repeated twice at MOI 20 and 50. At each MOI, ~5 times as much EMC-D virus was adsorbed as EMC-B virus ($P < .05$ in each instance; Table 2).

Antigenic content of virus stocks. The antigenic content of each of the virus stocks was determined with the ELISA technique. Over the linear range of the graph, ~2 times as much antigen per PFU was present in the EMC-B virus preparation (Fig. 5).

TABLE 1
Competition between encephalomyocarditis (EMC)-B and EMC-D viruses for receptor sites on baby mouse pancreas (BMP) cells

Experiment	Pretreatment	[^{35}S]methionine-labeled EMC-B (cpm)	cpm (% of control)
1	EMC-B	2400 ± 60*	51
	HBSS	4700 ± 1400	100
2	EMC-B	2300 ± 300*	46
	EMC-D	2700 ± 700*	54
	HBSS	5000 ± 1350	100

cpm, counts per minute; HBSS, Hank's balanced salt solution. BMP cells in suspension were pretreated for 30 min at 4°C with either EMC-B virus, EMC-D virus (multiplicity of infection [MOI] 100), or HBSS as indicated. Cells were then washed and infected with [^{35}S]methionine-labeled EMC-B (MOI 3) for 30 min at 4°C. Cells were washed 3 times, and cpm were determined as described in RESEARCH DESIGN AND METHODS.
*$P < .05$ vs. HBSS control.

DISCUSSION

Previous studies show that certain strains of mice develop a diabetes mellitus–like syndrome after infection with the D variant of EMC virus (1–3). The same strains of mice infected with the B variant of EMC virus, which is serologically indistinguishable from EMC-D virus (1), do not develop diabetes or exhibit overt evidence of disease. Because the EMC-B virus is a good inducer of IFN, it has been postulated that the IFN produced as EMC-B virus replicates protects the β-cells from virus infection. Although there is some evidence that this may be true in at least one mouse strain (5), recent data obtained with ICR Swiss mice are not consistent with

TABLE 2
Attachment of B and D variants of encephalomyocarditis (EMC) virus to primary β-cells

	Experiment 1		Experiment 2	
Virus	MOI	Attachment (PFU/cell)	MOI	Attachment (PFU/cell)
EMC-B	20	0.4 ± 0.02	50	2.2 ± 0.4
EMC-D	20	1.8 ± 0.71*	50	10.0 ± 2.5*

MOI, multiplicity of infection; PFU, plaque-forming unit. β-Cells were isolated from pancreases of 20 male ICR Swiss mice as described in RESEARCH DESIGN AND METHODS. Each virus was added to suspensions of primary β-cells (2.5×10^5 cells) at MOI indicated. Virus was adsorbed to cells in the cold (4°C) for 1 h. After washing, the cells were frozen, thawed, and sonicated to release adsorbed virus. A plaque assay was done to determine the infectious virus content of each sample.
*$P < .05$ vs. EMC-B attachment.

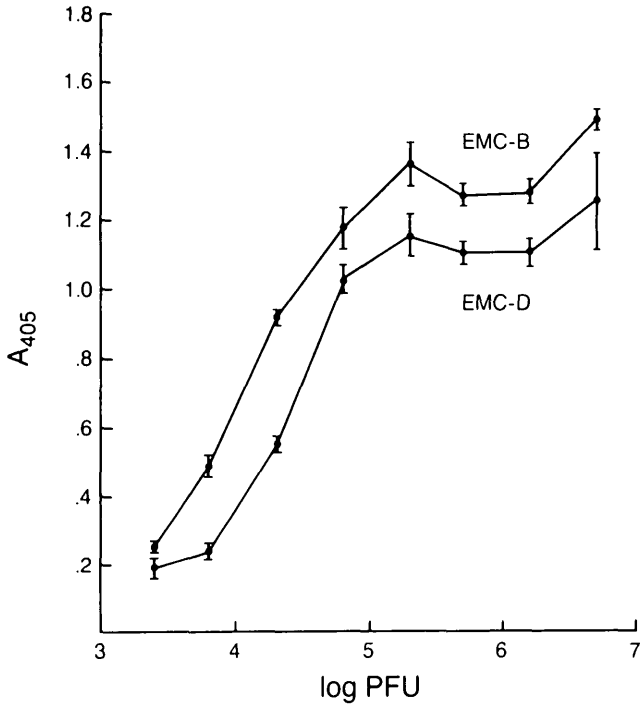

FIG. 5. Antigenic content of encephalomyocarditis (EMC)-B and EMC-D virus stocks. Antigenic content of different dilutions of each virus, ranging from 10^3 to 10^7 plaque-forming units (PFUs)/well, was determined by enzyme-linked immunosorbent assay as described in RESEARCH DESIGN AND METHODS. Each data point is mean absorbance at 405 nm (A_{405}) ± SD for triplicate samples.

this hypothesis (6). A possible explanation for the differences in diabetogenicity of certain strains of mice is that the viruses may have different affinities for β-cells. Our study was done to explore this possibility.

BMP cells provided a good model system for comparing the attachment of the two virus variants. Both viruses replicate in this cell line, with yields of EMC-D virus ~1 log higher than those of the B variant (data not shown). It was determined that ~10 times more EMC-D virus attached to BMP cells at 4°C compared with attachment at RT. This finding agrees with that of McClintock et al. (13), who reported that more EMC-D virus attached to Friend leukemia cells and mouse L-cells at 0°C compared with RT. A penetration assay was done to measure the specificity of attachment; ~90% of the specifically bound virus was internalized by 1 h post-infection (Fig. 2). This assay was repeated several times, and the results were essentially the same. These data are in agreement with those reported by Madshus et al. (14). The burst size of ~400 PFUs/cell (Fig. 2) is within the range reported for other picornaviruses (15).

The initial experiments suggested that BMP cells could be used to determine if specific attachment of the two virus variants differed. Approximately twice as much EMC-D as EMC-B virus attached to the cells at an MOI of 1200 (Fig. 3). However, the data show that the EMC-B virus stock had twice as much antigen per PFU as the EMC-D virus preparation (Fig. 5), which suggests that the EMC-B virus stock could contain up to twice as many noninfectious virus particles and could account for the differences in the attachment of the two viruses (Fig. 3). However, note that the B variant appeared to be nearer to saturation than EMC-D virus over the range in MOI tested. Furthermore, if noninfectious virus was entirely responsible for the difference in attachment, the attachment curves would have paralleled each other. Higher MOI might have shown a greater difference between the attachment of the two viruses to this cell line. Due to the virus stock PFU titers, it was not possible to increase the MOI further. Concentration of the virus would also have concentrated the antigenic content. Because saturation was not achieved, it was not possible to determine affinity constants from Scatchard plots. It is also evident that the viruses competed for a common receptor site (Table 1). The competition for the receptor site was not as great as that reported for EMC viruses in other cell types (13,16).

The data obtained with BMP cells suggest that the affinity of the viruses for β-cells differs. However, because this is a continuous transformed cell line, the possibility that virus receptors were altered by the transformation process could not be ruled out. Therefore, the attachment experiments were repeated with primary β-cells from male ICR Swiss mice. The differences in attachment of the two viruses to these cells were more pronounced than those seen in BMP cells and, even allowing for the twofold difference in antigenic content of the virus preparations, were statistically significant (Fig. 4; Table 2). Although it is possible that the two viruses recognize different receptor sites on primary β-cells, the data showing that the viruses share a common receptor site on BMP cells suggest that it is more likely that EMC-D virus has a higher affinity than EMC-B virus for a shared site. Recent studies in our laboratory show that there is no difference in the adsorption of each virus variant to either mouse L-cells or BHK21 cells (unpublished observations). The same studies show that although the viruses replicate to the same titer in BHK21 cells, the replication of EMC-B virus is greatly reduced in L-cells. Because L-cells are excellent producers of IFN and EMC-B virus is a good inducer of this substance (1), it is probable that the IFN induced as EMC-B virus replicates aborts the infection. This has been shown to occur during the replication of EMC-MM virus (17). The possibility that locally produced INF within the pancreas might abort the replication of EMC-B virus and contribute to its inability to produce diabetes cannot be ruled out. Due to the great amount of enzymatic activity, we have not been able to successfully assay for the presence of IFN in pancreatic extracts.

The data in this study suggest that different degrees of affinity for β-cell receptors may contribute to the different pathogenicities of the B and D variants of EMC virus. The resistance of certain mouse strains to the diabetogenic effects of EMC-D virus could be due to genetically determined modifications in virus receptors on the surface of β-cells.

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