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Primary Virus-Cell Interactions in the Immunofluorescence Assay of Venezuelan Equine Encephalomyelitis Virus

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The conditions under which Venezuelan equine encephalomyelitis (VEE) virus attached to host cells markedly influenced the assay of virus by the fluorescent cell-counting technique. When virus inoculum was centrifuged onto McCoy cell monolayers, approximately 97% of virus was attached to cells within 10 min, in contrast to 34% after stationary incubation at 35 C for 2 hr. Maximal binding of virus occurred only in the presence of 0.1 to 0.15 M NaCl. This salt requirement, added to evidence of *p*H dependence and temperature independence of VEE virus attachment to cells, indicated that the initial union involved electrostatic forces. Virus penetration, measured by the insensitivity of virus-cell complexes to viral antiserum, was complete in 30 min at 35 C. The process was temperature-dependent and unaffected by the ionic content of medium. For assay of VEE virus by the fluorescent cell-counting technique, infected cells may be enumerated as early as 12 hr after infection of cell monolayers. The relationship between virus concentration and cell-infecting units was linear; the distribution of fluorescent cells was random. The virus assay was equivalent in sensitivity but more precise and rapid than that of intracerebral inoculation of mice.

Recently, rapid and quantitative assays were developed for a number of viruses (10, 11, 13) and a rickettsia (12); these assays are based on immunofluorescent staining of infected cell monolayers and enumeration of cells containing fluorescent viral or rickettsial antigens. The feasibility of extending this technique to the assay of Venezuelan equine encephalomyelitis (VEE) virus was investigated, because it was previously demonstrated that cells infected with the virus are amenable to immunofluorescent staining (26). In developing the assay, certain specific requirements were encountered for the attachment of VEE virus to cells; these requirements, in some aspects, may be unique to this arbovirus. The availability of a quantitative assay of the virus and of a technique that promotes efficient, rapid, and almost synchronous virus attachment to cells facilitated kinetic studies on the early reactions between this animal virus and host cells.

This report describes an investigation of initial virus-cell interactions in the course of developing a rapid assay of VEE virus by the fluorescent cell-counting technique. The relevance of the findings to other virus-cell host systems is discussed.

MATERIALS AND METHODS

Virus. The Trinidad strain of VEE virus was used throughout this study; its history has been recorded elsewhere (15). A working suspension of virus was prepared by inoculating monolayers of McCoy cells grown in milk dilution bottles with a 10^{-1} dilution of 20% chick embryo suspension of virus. After adsorption of inoculum at 35 C for 2 hr, cell monolayers were washed, overlayed with 5 ml of maintenance medium, and incubated at 35 C. Widespread destruction of cell monolayers occurred within 40 hr. The cell cultures were then frozen and thawed, and the culture fluid was distributed in 1-ml portions into glass vials and stored at −60 C. The suspension contained $10^{9.1}$ mouse intracerebral LD_{50} units of virus per ml. Prior adaptation of VEE virus to the cell line was not a requisite, because mouse brain or embryonated egg suspensions of virus readily infected cell monolayers.

Cell line and cultivation. The established cell line, McCoy, derived from human synovial tissue (8) was used in the assay of virus. Preliminary tests indicated that more than 99% of the cells were susceptible to the virus. Nutrient medium for the cell line consisted of mixture 199 containing 0.5% lactalbumin hydrolysate, 10% heat-inactivated calf serum, and 50 μg of streptomycin and 75 μg of kanamycin per ml. Cells were cultivated on circular cover slips (15-mm diameter) inverted in flat-bottomed glass vials (19 by 65 mm). A 1-ml amount of cell suspension, containing 10^5 to

3×10^5 cells, was introduced onto cover slips which were then incubated at 35 C for 24 hr, or until a complete cell monolayer was formed. Cell monolayers were washed with an appropriate diluent before the addition of virus inoculum.

Virus assay. Determinations were usually carried out in triplicate. Virus dilutions were prepared in phosphate-buffered saline (PBS), *p*H 7.1, free of calcium or magnesium ions. This consisted of 8.5 g of NaCl, 1.07 g of Na_2HPO_4 (anhydrous), and 0.39 g of $NaH_2PO_4 \cdot 2H_2O$ per liter of distilled water. Inoculum in 0.2-ml volume was introduced directly onto cover-slip cell monolayers after their transfer from glass vials to rotor chamber inserts (11). The latter were employed because they withstand the high centrifugal force required to sediment VEE virus. Rotor chamber inserts placed in a swinging-bucket SW 25.1 rotor were centrifuged in a model L Preparative Ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.) at 19,642 to 29,432 $\times$ *g* for 15 min at 30 C. The residual inoculum was removed after centrifugation, the cover-slip cell monolayers were placed into glass vials, and 1 ml of maintenance medium was then added to each vial. After incubation at 35 C for 1 hr, the maintenance medium was replaced with 1 ml of a 1:40 dilution of virus antiserum in mixture 199 (serum neutralization index, 4.8 $\log_{10}$ for undiluted serum). The rationale for this procedure is presented in Results. After further incubation at 35 C for 20 to 24 hr, cover-slip cell monolayers were rinsed twice with cold PBS, fixed with cold (−60 C) acetone, and either prepared immediately for immunofluorescent staining and cell-counting or stored at −60 C. Fluorescence of viral antigens in fixed cell cultures was not diminished when they were stored under these conditions for 8 weeks.

The method of intracerebral inoculation of mice for assay of virus has been described previously (15).

VEE antiserum conjugate and immunofluorescent staining. Aerogenic vaccination of rhesus monkeys was employed to make VEE antiserum. One month after vaccination, the animals were bled. The antiserum was conjugated with fluorescein isothiocyanate by the method of Riggs et al. (35). To remove unbound dye, the conjugated antiserum was passed through a column of Sephadex G-25.

The direct fluorescent-antibody method was used to demonstrate immunofluorescence of viral antigens in infected cells. Fixed cell cultures were washed three times with PBS and stained with conjugated antiserum for 30 min at room temperature. Cover-slip cell monolayers were then rinsed in two changes of PBS (to remove excess conjugate) and mounted in a semipermanent medium (36).

Fluorescence microscopy and cell-counting. Cover-slip cell cultures were examined with an American Optical microscope equipped with a Fluorolume illuminator (model 645), Corning no. 5840 and Schott BG-12 exciter filters, and an E. K. No. 2A barrier filter. With this optical system at a magnification of 430 $\times$, the number of microscopic fields contained in the area of a 15-mm cover slip was 1,064. For each cover-slip cell monolayer, 50 microscopic fields were examined for fluorescent cells. To calculate the number of cell-infecting units (CIU) of virus per milliliter, the average number of fluorescent cells per field was multiplied by the number of fields per cover slip, the reciprocal of the dilution of virus inoculum, and a volume factor (for conversion to milliliters).

Determination of virus attachment. Attachment was measured by following the disappearance of virus from inoculum after its addition to cell monolayers. Cover-slip monolayers were inoculated with virus suspension (multiplicity of infection, 0.1) in 0.2-ml volume. After designated intervals of incubation or centrifugation, residual inoculum was removed and the cell cultures were immediately washed twice with PBS. Residual inoculum was then introduced onto fresh cell monolayers to measure unattached virus. For this, the residual inoculum was adsorbed onto cell cultures by centrifugation at 19,642 to 29,432 $\times$ *g* for 30 min. Cover-slip cell monolayers exposed to initial or residual inocula were then treated in the manner described earlier for virus assay. The amount of virus that was attached to cells at a given time was expressed as a percentage of the virus input. The latter was the sum of the amounts of attached and free virus.

Determination of virus penetration. Virus penetration into cells was measured by the insensitivity of attached virus to antiserum. Inoculum was attached to cell monolayers as described above. Cell cultures were washed twice with PBS, overlaid at designated intervals with 1 ml of a 1:40 dilution of virus antiserum, and then incubated at 35 C for 20 hr. The quantity of virus that penetrated cells at a given time was expressed as a percentage of the input virus.

Calculation of attachment and penetration constants. The attachment and penetration rate constants (*k*) were calculated from the relationship, $\ln (V_0/V_t)nt$, where V_0 = the input virus concentration, V_t = unattached or unpenetrated virus at time *t*, and *n* = the number of cells per cm^3 determined by resuspension of cover-slip cell cultures and enumeration.

RESULTS

In this study, the terms employed to denote the early stages of virus-cell interactions are defined as follows. "Adsorption" is a general term referring to the various interactions and processes involved in the initial binding of virus to cells, leading to the loss of identity of the former. Its implications of physicochemical nonspecificity, however, limit its descriptive usefulness (30, 39). "Attachment" is the initial, specific union between virus and cells, which may or may not be reversible. "Penetration" refers to the progressive insensitivity of virus-cell complexes to viral antiserum.

Virus attachment. Previous findings indicated that centrifugal force was highly efficient in promoting the attachment of virus to cells (10, 11, 13). An experiment was performed to determine the rate of VEE virus attachment onto cell monolayers during stationary incubation (35 C) and

during centrifugation (19,642 to 29,432 × *g*, 30 C). The concentration of cells per cover-slip culture was 5.3 × 10^5 per milliliter. Inocula employed for stationary and centrifugation experiments were 10,853 and 5,171 CIU, respectively. Additional experimental details are described under Materials and Methods.

The rate of virus attachment with each treatment is shown in Fig. 1. Within 10 min, 97% of the virus inoculum was attached when aided by centrifugal force. After stationary incubation at 35 C for 2 hr, only 34% of the virus inoculum was attached. The attachment rate constant (*k*) was 3.3 × 10^{-7} cm^3 min^{-1} with centrifugation and 7.3 × 10^{-9} cm^3 min^{-1} with stationary incubation. The latter is consistent with findings reported for Newcastle disease and polioviruses under similar circumstances (3, 21). Because centrifugation was highly efficient and rapid for virus attachment to cells, it was adopted as the standard procedure.

In preliminary experiments, erratic virus assay values resulted when either McCoy cell maintenance medium or PBS containing $CaCl_2$ and $MgCl_2$ was used for dilution of virus inoculum.

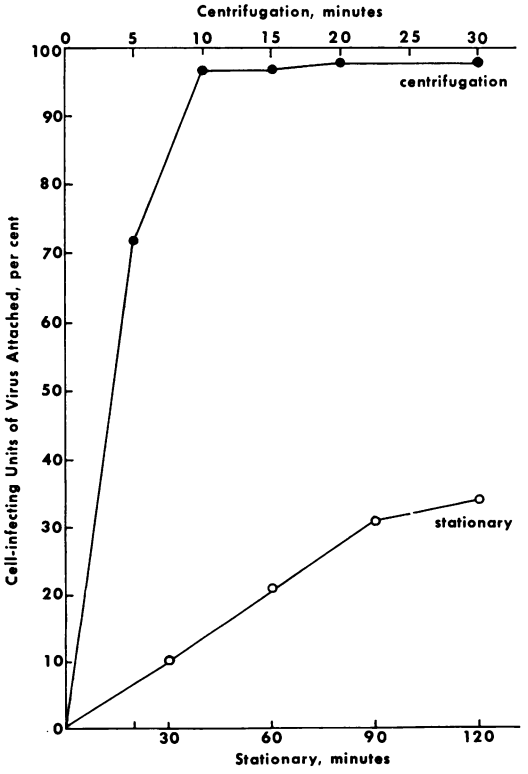

FIG. 1. *Attachment of VEE virus onto cover-slip cultures of McCoy cells by centrifugation (19,642 to 29,432 × g, 30 C) and stationary incubation (35 C).*

TABLE 1. *Effect of virus diluent on the attachment and assay of VEE virus*

Diluent	Virus attached	Virus titer[a]
	%	*CIU/ml*
PBS[b]	99.0	1.7 × 10^9
PBS + 0.0009 M $CaCl_2$ and 0.0005 M $MgCl_2$	20.9	3.5 × 10^8
PBS + 0.0009 M $CaCl_2$	23.3	3.9 × 10^8
PBS + 0.0005 M $MgCl_2$	5.9	1.0 × 10^8
Mixture 199 + 5% calf serum	31.7	5.3 × 10^8
Mixture 199 + 10% calf serum	19.1	3.2 × 10^8
Mixture 199 + 20% calf serum	8.9	1.5 × 10^8
Heart Infusion Broth	25.1	4.2 × 10^8

[a] Virus input, 6,800 cell-infecting units (CIU) with 0.2-ml inoculum.

[b] NaCl (0.15 M) buffered by 0.01 M phosphate buffer.

In addition, the values were often as much as 1.0 log unit less than that obtained by the method of intracerebral inoculation of mice. To test the hypothesis that these events may be related to the attachment of virus to cells, different diluents were employed to prepare virus inoculum. The molarity of $CaCl_2$ and $MgCl_2$ in diluents was similar to that usually found in cell cultivation reagents. Immediately after centrifugation of virus inoculum onto cells, monolayers were rinsed twice with PBS and treated in the prescribed manner. Virus attachment and, consequently, virus assay values were significantly affected by the composition of the diluent (Table 1). The largest quantity of virus that attached to cell monolayers occurred with PBS containing a monovalent cation; smaller amounts of virus attached in the presence of divalent cations. Magnesium ions were more marked in impeding virus attachment than were calcium ions. In the presence of calf serum, virus attachment decreased as the concentration of serum increased. In view of these findings, PBS free of calcium and magnesium ions was employed routinely as the virus diluent.

The possibility that the low assay values obtained with diluent containing divalent cations might be the result of virus elution from cells after initial attachment was investigated. Virus inoculum was prepared in PBS containing calcium and magnesium ions and in PBS free of these cations. Immediately after centrifugation of inoculum, cell monolayers were rinsed twice, and a 0.2-ml volume of the corresponding diluent was added to cell monolayers. At 30-min intervals, the diluent was removed and assayed for virus in the usual manner. Although virus appeared to elute more rapidly in PBS containing $CaCl_2$ and

$MgCl_2$ than in PBS free of the divalent salts, the amount that eluted in the presence of either diluent was less than 0.1% within 2 hr (Table 2). Comparable results were noted with mixture 199 containing 5% calf serum. Virus elution from cells was not a significant factor contributing to low virus assay values. An additional factor considered was that of virus aggregation. Virus suspended in PBS containing divalent salts was treated for 5-, 10-, and 15-sec intervals with an ultrasonic probe, model BP-1 (Blackstone Ultrasonics, Inc., Sheffield, Pa.). Assay values were comparable between sonic-treated virus suspensions and untreated controls. There was no indication from the results that virus aggregation occurred in the presence of divalent cations.

To ascertain whether the electrolyte requirement for maximal attachment of VEE virus to cells is dependent on the presence of a specific cation, other monovalent cations were substituted for sodium in 0.01 M phosphate buffer. Maximal attachment of virus was attained only with NaCl (Table 3). Less effective in promoting virus attachment were the monovalent cations potassium and ammonium. The findings that a small quantity of virus is attached to cells in the presence of sucrose solution is similar to previous observations with herpes and polioviruses (7, 18, 21).

TABLE 2. *Elution of VEE virus at 35 C after attachment to McCoy cells*

Attachment time	PBS[a] + Ca^{++} and Mg^{++}		PBS free of Ca^{++} and Mg^{++}	
	CIU[b]	Virus eluted	CIU	Virus eluted
min		%		%
0	42	0.01	0	0
30	106	0.04	0	0
60	127	0.05	42	0.01
90	170	0.07	84	0.03
120	234	0.09	216	0.09

[a] PBS consisted of 0.15 M NaCl buffered by 0.01 M phosphate solution plus 0.0009 M $CaCl_2$ and 0.0005 M $MgCl_2$.

[b] Cell-infecting units of virus eluted. Virus input, 2.4×10^5 CIU with 0.2-ml inoculum.

TABLE 3. *VEE virus attachment to McCoy cells in the presence of monovalent cations*

Diluent	Virus attached	Virus titer[a]
	%	*CIU/ml*
PB[b] + 0.1 M NaCl	99.00	1.8×10^9
PB + 0.1 M KCl	5.20	9.5×10^7
PB + 0.1 M K_2SO_4	0.55	1.0×10^7
PB + 0.1 M NH_4Cl	0.83	1.5×10^7
PB + 0.25 M sucrose	4.11	7.4×10^7

[a] Virus input, 7,565 CIU with 0.2-ml inoculum.

[b] Phosphate buffer (0.01 M).

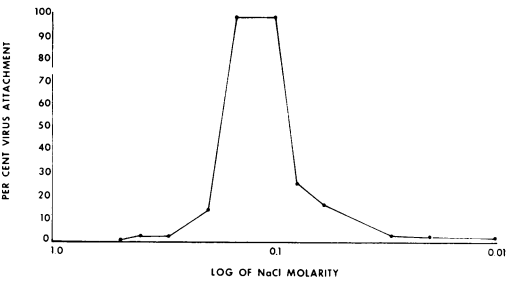

FIG. 2. *Attachment of VEE virus to McCoy cells in the presence of different NaCl concentrations.*

The quantity of virus attached to cells as a function of NaCl concentration was investigated. Virus dilutions were prepared in 0.01 M phosphate buffer containing concentrations of NaCl ranging from 0.01 to 0.5 M. The range of molarity for testing was limited because cell monolayers exhibited plasmolysis or plasmoptysis beyond the designated range. Virus attachment to cells was carried out and determined in the usual manner. Concentrations of NaCl from 0.1 to 0.15 M were optimal for achieving maximal binding of virus to cells (Fig. 2). Greater or lesser salt concentrations markedly inhibited attachment. These findings are compatible with the proposed role of salt concentration in attaining an electrostatic complementary configuration between virus and cells for maximal attachment (33).

In previous studies, it was demonstrated that the attachment response of bacteriophage to its bacterial host (34, 40), and Newcastle disease virus to host cells (21), was affected by variations in the *p*H of the suspending medium. This suggested that chemical groups participate in the binding of virus to cells. To determine whether *p*H of the suspending medium was important in the virus-host cell system currently under study, Sorensen's phosphate buffer containing 0.15 M NaCl, adjusted to give *p*H values ranging from 4.3 to 8.7, was used as the attachment medium. That chemical groups (carboxyl and amino) are also involved in the attachment of VEE virus to cells is suggested by the *p*H dependence of the reaction (Table 4). Maximal binding of virus occurred near neutrality. At both acidic and basic conditions, corresponding to intervals at which ionization of carboxyl and amino groups is depressed, virus attachment decreased markedly.

The effect of temperature on the attachment of VEE virus to cells during centrifugation was studied by sedimenting inoculum onto cell mono-

layers at temperatures that ranged from 30 to 4 C. With the exception of this variable, the procedure for virus attachment and assay was similar to that described earlier. The findings that initial binding of virus to cells was independent of temperature is further evidence of the electrostatic nature of the reaction (39). Similar results were obtained by others with bacteriophage (9), Newcastle disease virus (21), poliovirus (3), herpesvirus (7), and fowl plague and vaccinia viruses (2).

Virus penetration. It became necessary to determine the rate of virus penetration into cells when it was discovered that enumeration of individual fluorescent cells was precluded by the appearance of microplaques between 12 and 16 hr after virus infection of cell monolayers. To provide a more convenient time for counting individual infected cells, virus antiserum was employed to prevent a second cycle of infection of cell monolayers by extracellular virus. The earliest time at which antiserum may be added without neutralizing attached virus was determined by following the rate of virus penetration into cells. The experiment was carried out at temperatures of 35, 28, and 4 C. The results (Fig. 3) indicate that virus penetration into cells is temperature-dependent. At 35 C, there was a short, initial period of approximately 10 min when virus attached to the cell surface was highly sensitive to antiserum. Thereafter, virus penetration proceeded at a linear rate, with a velocity constant (k) of 6.9×10^{-8} cm^3 min^{-1}, and was complete in approximately 30 min. At 28 C, virus penetration also appeared to be linear but only 50% of attached virus penetrated into cells in 56 min. The quantity of virus that penetrated into cells at 4 C was less than 5% in 60 min. For routine assay of virus, cell cultures were incubated at 35 C for 1 hr after virus attachment. Antiserum was then added to prevent subsequent infection of cell monolayers by newly released virus.

TABLE 4. *Effect of pH on attachment of VEE virus to McCoy cells*

pH of attachment medium[a]	Virus attached	Virus titer[b]
	%	*CIU/ml*
4.3	2.8	0.3×10^8
5.1	5.7	0.6×10^8
6.1	8.5	0.9×10^8
7.2	98.0	11.0×10^8
8.2	40.9	4.5×10^8
8.7	18.0	2.0×10^8

[a] Sorensen's phosphate buffer with 0.15 M NaCl.
[b] Virus input, 2,234 CIU with 0.2-ml inoculum.

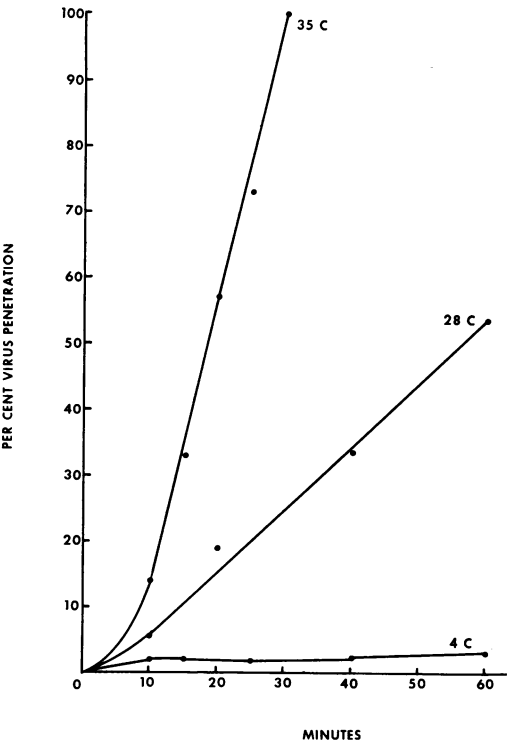

FIG. 3. *Effect of temperature on the penetration of VEE virus into McCoy cells as measured by insensitivity to viral antiserum.*

Earlier, it was demonstrated that a specific monovalent cation was required to attain maximal attachment of VEE virus to cells. The presence of divalent cations appeared to affect the process. To ascertain whether specific cations were needed to affect virus penetration into cells, cell monolayers were incubated at 35 C for 1 hr with medium of different cationic content immediately after virus attachment. The test media for virus penetration were then replaced with 1 ml of an appropriate dilution of virus antiserum, and the cell cultures were incubated for 20 hr. From the comparable assay values obtained with the different media (Table 5), it is evident that the presence of electrolytes was not a prerequisite for virus penetration. Since cell monolayers were routinely overlaid (with mixture 199 containing 5% calf serum) after virus attachment, this practice was continued.

Incubation period. The rapid rate of VEE virus multiplication in cell cultures (27) was indirectly substantiated by observations on the initial fluorescence of infected cells. The earliest visual signs of cellular infection by virus, in the form of faint, cytoplasmic fluorescence, appeared 8 hr after adsorption of inoculum. By 12 hr, the

TABLE 5. *Role of cations on the penetration of VEE virus into McCoy cells*

Medium for virus penetration	Virus titer[a]
	CIU/ml
Deionized water + 0.25 M sucrose	1.0×10^{10}
PB[b] + 0.0009 M $CaCl_2$, 0.0005 M $MgCl_2$	1.0×10^{10}
PB + 0.0009 M $CaCl_2$, 0.0005 M $MgCl_2$, 0.02 M EDTA[c]	1.0×10^{10}
PB + 0.15 M NaCl	1.1×10^{10}
Mixture 199 + 5% calf serum	1.2×10^{10}

[a] An alternate virus preparation was used in this experiment.
[b] Phosphate buffer (0.01 M).
[c] Disodium ethylenedinitrilotetraacetate.

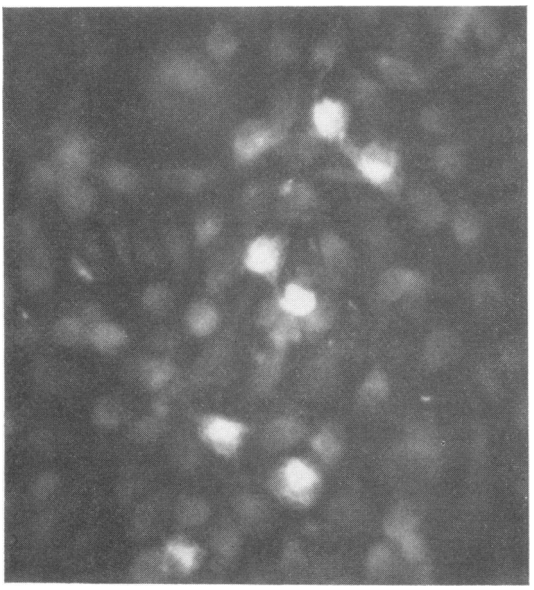

FIG. 4. *Fluorescent VEE viral antigen in McCoy cells 12 hr after infection.* × *126*

amount and intensity of fluorescence had markedly increased, so that infected cells could be easily discerned (Fig. 4). Fluorescent viral antigen was diffuse, granular, and confined to cell cytoplasm. Between 12 and 16 hr, fluorescent microplaques that were indicative of a second cycle of virus infection were noted in cell monolayers. Microplaques usually contained from 5 to 20 fluorescent cells. The number doubled or tripled at 20 hr (Fig. 5). With extended incubation periods, microplaques coalesced to form a fluorescent cell monolayer.

Counts of infected cells were compared between cell cultures incubated with and without antiserum for intervals of 8, 10, 12, 16, 20, and 24 hr. At either condition, infected cell counts were tripled between 8 and 12 hr. Counts of individual infected cells were precluded with the appearance of microplaques in cell cultures incubated for 16 hr without the antiserum overlay. In the presence of antiserum, counts of infected cells were comparable between 12 and 24 hr. From observations on the development of fluorescent viral antigen and infected cell counts, individual infected cells may be enumerated as early as 12 hr after adsorption of virus inoculum. Antiserum must be added to inoculated cell cultures, however, when the incubation time is extended beyond 12 hr. In this study, an incubation period of 20 hr was employed for virus assay.

Quantitative evaluation of assay. Results in Fig. 6 reveal a linear relationship between twofold dilutions of virus over a range of 1.5 log units and the number of CIU of virus. These data suggest that each fluorescent cell resulted from infection by a single infective virus particle or aggregates not divisible by dilution.

In a single experiment, 11 determinations were performed to estimate the precision of the assay of VEE virus. Cover-slip cell monolayers were infected by a standard quantity of virus inoculum and then treated in the prescribed manner. The number of CIU of virus per ml of inoculum ranged from 1.0×10^9 to 1.7×10^9, with a mean of 1.4×10^9 and standard deviation (SD) of $\pm$ 0.19. Expressed as a percentage, the SD was 13% of the mean.

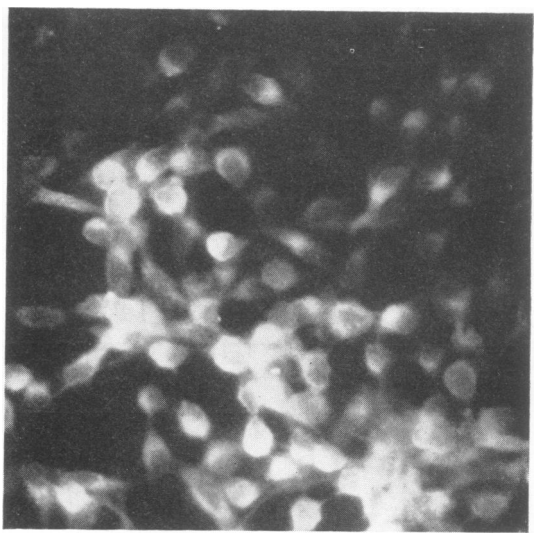

FIG. 5. *McCoy cell monolayer with fluorescent microplaque after injection with VEE virus 20 hr earlier.* × *126.*

The mode of distribution of fluorescent cells on a cover-slip monolayer was determined by examining 200 random microscopic fields. The frequencies of field containing infected cells correspond closely to the theoretical frequency (Fig. 7). The χ^2 test of goodness-of-fit of the observed data to the theoretical Poisson distribution showed no significant deviation (probability = 0.83 with degrees of freedom = 6). Fluorescent cells were randomly distributed in cell monolayers.

The sensitivity of the fluorescent cell-counting assay was compared with that of intracerebral inoculation of mice. Results in Table 6 show that assay values were comparable by either method. The fluorescent cell-counting assay, however, exhibited less variability than the mouse assay. By the former, results were attained within 20 hr; by the latter, an observation time of 10 days was needed before assay values could be estimated.

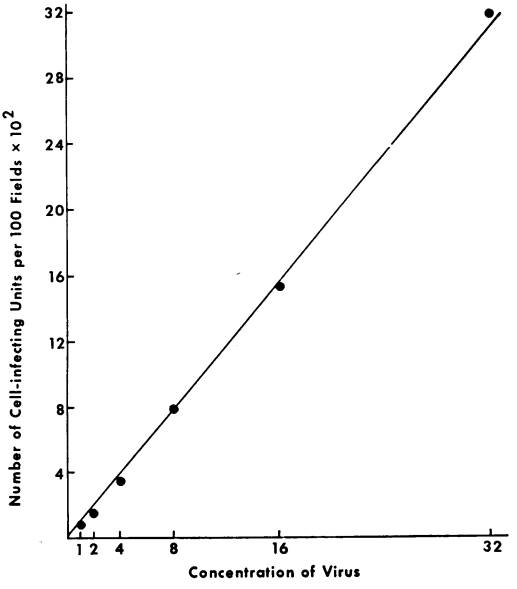

FIG. 6. *Linear function between the number of cell-infecting units and concentration of VEE virus.*

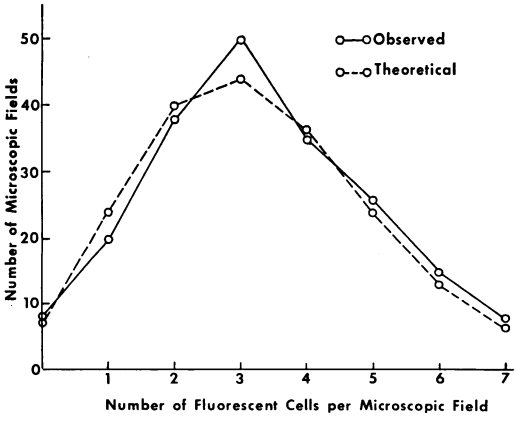

FIG. 7. *Frequency distribution of fluorescent cells on McCoy cell monolayer injected with VEE virus.*

TABLE 6. *Comparison of two procedures for the assay of VEE virus*

Assay procedure	Virus titer	SD	SE
Fluorescent cell-counting	9.1[a]	±0.05	±0.09
Intracerebral inoculation of mice	9.1[b]	±0.18	±0.18

[a] Reciprocal of cell-infecting units of virus ($\log_{10}$) per milliliter, based on six titrations, determined in 20 hr.

[b] Reciprocal of LD_{50} ($\log_{10}$) mouse intracerebral units of virus per milliliter, based on six titrations, 10-day observation period.

DISCUSSION

The mechanism of VEE virus attachment to host cells appeared to be similar in many aspects to that of other animal virus and bacteriophage systems (39). Studies on the primary interactions between virus and cells indicated, however, that maximal initial binding of virus to cells was achieved only under specific circumstances. The conditions under which virus attached to host cells profoundly influenced the assay of VEE virus by the fluorescent cell-counting technique. Centrifugation of virus inoculum onto cell monolayers promoted efficient and rapid attachment of virus. Approximately 97% of virus inoculum was bound to cells within 10 min, in contrast to 34% after stationary incubation at 35 C for 2 hr. These results were comparable to those obtained with another arbovirus (11). Virus attachment mediated by centrifugal force attains added importance in view of the theory of Brownian motion as it relates to the arrival rate of virus particles in suspension at the surface of a cell system. Virus in Brownian motion may take an average of 4 hr to diffuse 0.1 mm, and several hours may elapse before half the virus particles reach the cell surface (41). The effects of thermal inactivation on virus particles during extended intervals of incubation for attachment are minimized by the rapidity of virus binding to cells when augmented by centrifugal force. Furthermore, the short time required to achieve complete and almost synchronous attachment of virus by this procedure helps to delineate that reaction from

virus penetration and, thereby, makes it possible to measure accurately the rate of the latter.

The attachment of VEE virus to cells was highly specific in its salt requirement, in that maximal binding occurred only in the presence of a buffer solution containing defined concentrations of the monovalent salt, NaCl. No substitution for the sodium cation was found in the limited number of monovalent salts that were tested. The presence of divalent cations in the medium appeared to inhibit virus attachment. That divalent cations do not function as bridges between virus and cells is substantiated by these findings. The influence of cations on attachment of viruses to host cells may be interpreted in terms of interactions between particles that carry net negative charges (32). Cations may function by altering the electrostatic configuration of the attachment sites so that complementation occurs between sites on the virus and on the cell surface. The salt requirement, added to evidence of *p*H dependence and temperature independence of the attachment of VEE virus to cells, supports the concept that the initial union involves electrostatic forces. Similar findings were reported with bacteriophages (33, 39) and with other animal viruses (3, 21).

Although divalent cations, usually calcium and magnesium, have been generally found to be necessary for the electrostatic attachment of bacteriophages (43) and animal viruses to host cells (3, 25, 30), there are numerous exceptions. The requirements for maximal binding of different T phages have been found to vary with the valency of cations and the salt concentration (22). Preliminary experiments carried out in this study with another arbovirus, yellow fever (group B member), indicated that its medium requirement for maximal attachment differed from that of VEE virus (group A member). Centrifugation of yellow fever virus inoculum onto McCoy cells revealed that maximal virus attachment occurred in the presence of mixture 199 containing 5% calf serum, and in decreasing amounts in PBS containing $MgCl_2$ and $CaCl_2$ and in PBS containing only NaCl. In contrast, the presence of calf serum in the attachment menstruum, although not affecting the viability of VEE virus, inhibited its attachment to cells. Virus attachment efficiency decreased as the serum concentration increased. In general, these data support the conclusions based on experiments with the bacteriophage system (32) that the requirements for virus attachment are virus-specific and not cell-specific. It is possible, therefore, that the different requirements for maximal attachment of these two arboviruses may be a group characteristic, but additional tests are needed with several representative arboviruses before any generalization is warranted.

The attachment of virus to cells in vivo is not necessarily followed by elution, and, if it does occur, the amount eluted is small (5). Our limited observations with VEE virus tend to uphold this viewpoint. Elution of VEE virus from host cells was minimal (less than 0.1% within 2 hr) and was not affected by the presence of either mono- or divalent cations in the medium. The small quantity of virus that eluted retained its infectivity. Similarly, Newcastle disease, fowl plague, and vaccinia viruses did not elute from host cells under conditions in which they readily elute from red cells (1, 21). No significant elution of rabbitpox virus from HeLa cells could be demonstrated (30). These findings, however, may not have general applicability in view of seemingly contradictory observations. Smith and Sharp (38) reported that part of the attached vaccinia virus subsequently eluted from L cells, and Joklik and Darnell (20) demonstrated that more than half of the poliovirus particles eluted from HeLa cells. The eluted poliovirus was altered so that it could not initiate infectivity. Elution of poliovirus occurred from HeLa cell-plasma membrane preparations incubated at 37 C but not at 0 C (17). It appears that the virus-cell system and the conditions employed to demonstrate virus elution markedly influence the phenomenon.

The finding that VEE virus was initially held at the cell surface for several minutes, where it was susceptible to antiserum, and that virus penetration then proceeded as a first-order reaction, was similar to reported observations with poliovirus (16, 18, 29). Virus penetration, measured by antiserum insensitivity, was complete in approximately 30 min at 35 C. The variable penetration times reported for other animal viruses, ranging from 20 min to several hours (4, 6, 18, 23, 37, 42), may be a reflection of diverse experimental conditions and the difficulty in isolating the penetration stage from the rest of the infective cycle (5). In contrast to the temperature independency of the attachment reaction, the penetration of VEE virus was temperature-dependent. Virus penetration was more rapid at 35 C than at 28 C. Penetration was minimal, less than 5%, at 4 C. These results, together with evidence obtained with other animal viruses (7, 18, 19, 23, 24, 31), suggest that the penetration process may involve cellular enzymes. Although penetration of a variety of bacteriophages has been found to be dependent upon the environmental supply of divalent cations (28, 43), penetration of VEE virus was independent of the ionic content of the medium.

The rapidity of the fluorescent cell-counting

assay of VEE virus described in this report is an outstanding feature. Individual infected cells may be enumerated as early as 12 hr after infection. Because virus multiplication is rapid (27), the early release of virus into the extracellular environment results in a second cycle of infection and the subsequent appearance of microplaques between 12 and 16 hr after initial infection. Their formation may be prevented by overlayering cell cultures with viral antiserum 1 hr after virus penetration. The incubation period may be then extended beyond 12 hr and still permit individual infected cells to be counted. Paralleling the findings of previously described fluorescent cell-counting virus assays (10, 11, 13, 44), the relationship between virus concentration and the number of CIU was linear and the distribution of infected cells was random. The assay was equivalent in sensitivity but exhibited less variability than the highly sensitive assay of VEE virus by the method of intracerebral inoculation of mice (14).

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