Receptor Interaction Between Eastern Equine Encephalitis Virus and Chicken Embryo Fibroblasts

STEPHEN C. MARKER,[1]* DONALD CONNELLY,[1] AND PETER B. JAHRLING

United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701

The attachment of eastern equine encephalitis virus to chicken embryo fibroblasts was studied at 0°C. The binding specifically responsible for initiating infection was studied in the initial experiments by employing plaque-forming ability as the measured response. Results from these initial studies were closely paralleled in studies of binding of radiolabeled virus under the same conditions. Binding that had occurred at the pH optimum, pH 6.5, could be reversed only at higher pH. The observed pH dependence of virus attachment suggested the interaction of at least two ionizable species in the initial binding of virus to cell, and that one to three attachments must occur between virus and cell prior to infection.

Virus-cell receptor interactions have been extensively studied with several bacteria and their associated bacteriophages (1, 12,19) and, more recently, with several animal virus-cell receptor systems (3, 10). Virus receptors may be on a unique organelle, such as the T bacteriophage tail structures (18) or adenovirus fiber (13), which are both readily visible by electron microscopy, or the receptor may be on less conspicuous but equally unique arrangements of protein subunits, such as the influenza hemagglutinin (17) or the group A arbovirus icosahedral surface lattice of protein subunits (21). The chemistry of the receptor sites on these structures is beginning to be elucidated (5).

We present here methods for studying the interactions between the receptor of eastern equine encephalitis (EEE) virus, a group A arbovirus, and chicken embryo fibroblasts to provide the biological correlations prerequisite for chemical characterization of the receptor structures involved. The observed pH dependence of virus attachment provides a readily measured parameter of receptor function, reflecting the chemical structure in or about the active receptor site. The pH dependence of virus attachment is useful for identification and may be utilized for selecting virus variants with structural alterations affecting receptor function. Viruses with such identified alterations in receptor function could be employed in chemical studies of receptor structure.

[1] Present address: Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, MN 55455.

MATERIALS AND METHODS

Viruses. EEE, strain Arth 167 wild virus (provided by Philip Coleman, Medical College of Virginia, Richmond, Va.), was obtained in first duck embryo cell culture passage after two adult mouse brain passages. Venezuelan equine encephalitis virus, strain Trinidad donkey 1-2AC-8, was described previously (6). To prepare stock virus suspensions, these viruses were passed one additional time in duck embryo cell culture.

Cells. Chicken embryo fibroblasts (CEF) were prepared from 9- to 10-day-old embryos. Cells were seeded at 6×10^6 cells per well in 10-cm² wells of plastic six-well plates (Linbro Chemical Co., New Haven, Conn.) and grown in Earle 199 medium with 10% calf serum (Armour and Co., Chicago, Ill.). The monolayers were incubated at 37°C in 5% CO_2 for 3 to 4 days before use.

Adsorption of virus. The six stock buffers for adsorption experiments were 1 M sodium phosphate, pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 at 20°C. Before use, the 1 M stock buffers were diluted 1:20 in 0.15 M NaCl and filtered. The six diluted, buffered solutions were used to dilute the viruses prior to the 1-h adsorption period.

For the adsorption studies, virus stock solutions were diluted with a mixture of Hanks balanced salt solution (HBSS), 2% calf serum, and 0.025 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Ultrol, Calbiochem, Los Angeles, Calif.), pH 7.4, to contain approximately 5×10^4 PFU/ml (titrated under optimal conditions) and then were further diluted 1:100 into the buffered solutions at 0°C. The six-well plates of CEF monolayers were cooled to 0°C in ice; the medium was aspirated; and 0.2 ml of diluted virus was applied. The inoculated monolayers were kept in ice for the 1-h adsorption period; the unadsorbed virus was aspirated; and the infected monolayers were overlaid with 2 ml of

Eagle basal medium with Earle salts, 1% agar (Ionagar, Difco Laboratories, Detroit, Mich.), 2% calf serum, 50 μg of gentamicin per ml (Schering Laboratories, Bloomfield, N.J.), and 0.25 M HEPES (pH 7.4).

To determine the number of unadsorbed PFU remaining in the supernatant, the adsorption was performed as described above; the supernatant was diluted with a solution containing 1.8 ml of HBSS, 2% calf serum, and 0.025 M HEPES, pH 7.4; and the diluted virus was plated on 10 fresh CEF monolayers.

To ascertain the adsorption buffers effect on the ability of the CEF monolayers to show virus plaques, the monolayers were preincubated for 1 h at 0°C in the buffers without virus. The buffers were removed and replaced with 0.2 ml of virus inoculum containing 100 PFU in a solution of HBSS (0.2 ml), 2% calf serum, and 0.025 M HEPES at pH 7.4. The test inoculum was adsorbed for 1 h and removed; then the monolayer was overlaid with agar. This preincubation did not affect the number of plaques that developed

The effect of incubation in the adsorption buffers alone on the plaque-forming efficiency of the virus was investigated by incubating 10^4 PFU/ml in the adsorption buffers at 0°C for 1 h; the virus was then diluted 1:100 in a solution of HBSS, 2% calf serum, and 0.025 M HEPES (pH 7.4) and plaqued on fresh CEF monolayers. The plaquing efficiency of the viruses was not altered by the preincubation.

Preparation of radiolabeled virus. CEF monolayers were prepared in 800-cm² 0.5-gallon (ca. 1.89-liter) roller bottles (Bellco Glass, Inc., Vineland, N.J.), seeded at 4.5×10^8 cells per bottle in 150 ml of medium 199-10% calf serum, and rolled at 0.4 rpm for 2 days prior to use. The media were then replaced with HBSS-0.025 M HEPES, pH 7.4. After 24 h, the HBSS was discarded, and the roller bottles were infected with 10^9 PFU in 10 ml of HBSS for 1 h at 37°C. The inoculum was removed and replaced with 35 ml of amino acid-free medium 199 containing a 10-μCi/ml [³H]amino acid mixture (New England Nuclear Corp., Boston, Mass.), adjusted to pH 7.4 with 7% NaHCO₃, and rolled for 24 h at 0.4 rpm at 37°C.

The infectious supernatant was clarified at 10,000 rpm for 10 min (T-30 Spinco rotor) and then pelleted at 25,000 rpm for 2 h (T-30 rotor). The pelleted virus was suspended in a small volume of 0.15 M NaCl-0.05 M Tris (pH 7.4) and sedimented in a 10 to 30% sucrose (wt/wt) gradient, containing 0.15 M NaCl and 0.05 M Tris (pH 7.4), in a SW25.1 Spinco rotor, at 25,000 rpm for 2.5 h. The opalescent virus band was harvested by puncturing the side of the tube and was stored at −70°C.

Adsorption experiments with ³H-labeled virus were performed as described for unlabeled virus; labeled virus was diluted 1:100 in the buffered solutions prior to the 1-h adsorption period. The unadsorbed virus was removed and discarded. The monolayers in each well were dissolved in 0.4 ml of 5% sodium dodecyl sulfate and transferred, with one 0.2-ml water rinse, into a 10-ml scintillation vial with 6 ml of Scintilute containing 20% Scintosol GP

(Isolab Inc., Akron, Ohio). ³H counts were corrected for background, quench, and counting efficiency.

To test the reversibility of the adsorption process (Table 1), the labeled virus was adsorbed in the above manner at pH 6.5 for 1 h at 0°C. The unabsorbed virus was removed, discarded, and replaced with 2 ml of one of the following solutions at 0°C for 5 min: 0.15 M NaCl-0.05 M sodium formate, pH 3 or 4; 0.15 M NaCl-0.05 M sodium phosphate, pH 5.5 or 8.0; or 0.15 M NaCl-0.05 M sodium glycine, pH 10. The solution was then removed, and the monolayer with the remaining adsorbed virus was prepared for scintillation counting as before.

All PFU and distintegrations per minute presented are the arithmetic means of three replicate determinations.

RESULTS

Effect of adsorption buffer pH on the binding of virus to cells. We have attempted to identify the receptor binding specifically responsible for initiating productive infection (as opposed to a hypothetical virus-cell attachment that does not proceed to a infection) by using plaque-forming ability as the measure of binding in the initial experiments.

When virus is adsorbed to chicken cells in buffers of different pH values, the number of plaques formed depends strongly on the buffer pH during the first hour of contact between virus and cells. Varying the NaCl and albumin concentrations in the buffer used during this first hour further alters the number of plaques ultimately formed (Fig. 1). However, the overall dependence of adsorption on pH is still readily apparent.

Once the initial buffer containing the unabsorbed virus was removed from the cells, no further alteration in the ultimate plaque number was caused by washing the cells for a few seconds with HBSS-2% calf serum-0.025 M HEPES, pH 7.4.

Effect of virus and cell type on adsorption. By performing the adsorptions with different

TABLE 1. *pH effect on adsorption reversibility of EEE virus*

pH used to remove adsorbed virus[a]	Initial ³H-labeled virus inoculum remaining adsorbed after 5 min at the new pH (%)
3	24
4	24
5.5	18
6.5	21
8	6
10	5

[a] ³H-labeled virus was adsorbed for 1 h at 0°C at pH 6.5; then the pH 6.5 buffer was removed and replaced with a buffer at the new pH for 5 min at 0°C.
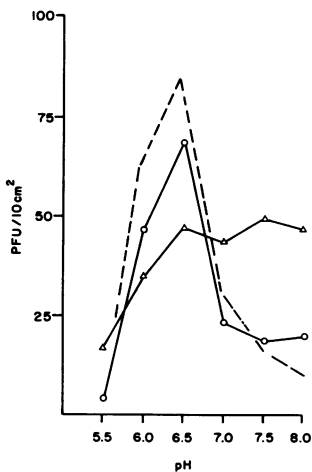
FIG. 1. *Adsorption of EEE to CEF in three different media: 0.15 M NaCl-0.05 M sodium phosphate (Z = 0.24 at pH 8.0; Z = 0.22 at pH 6.0) (- - -); 0.15 M NaCl-0.05 M sodium phosphate-0.1% human serum albumin (○); 0.05 M sodium phosphate-0.1% human serum albumin (Z = 0.096 at pH 8.0; Z = 0.075 at pH 6.0) (△).*

viruses on the same cell, or the same virus on different cells, it may be possible to decide if the effect of buffer pH on ultimate plaque numbers is primarily a result of virus or cell receptor structure. Variation in the amount of bound virus was found to be a function of both virus and cell receptor. The greatest adsorption to CEF of Venezuelan equine encephalitis virus, strain Trinidad, occurred at pH 5.5, the lowest pH tested (Fig. 2A). When EEE, Arth 167, was adsorbed to BHK-21 cells, the adsorption optimum at pH 6.5 was not as peaked as the optimum in chick cells (Fig. 2B).

After establishing that we were measuring mainly a virus-cell receptor interaction that could lead to productive infection, we then measured the binding of radiolabeled virus to CEF. The effect of pH on the binding of radioactive virus is very similar to the binding of virus to cells when measured by its ability to subsequently form a plaque.

The reversibility of binding that had occurred at pH 6.5 was then studied. Virus was bound to CEF for 1 h at pH 6.5 at 0°C. The buffer was removed and replaced with solutions buffered from pH 3 to 10, for an additional 5-min period at 0°C, in an attempt to elute the bound virus. Table 1 shows the percentage of the total inoculum, ^{3}H-labeled virus, remaining adsorbed to the monolayer after the elution attempts. The virus bound at pH 6.5 can be subsequently eluted at higher but not at lower pH.

DISCUSSION

We used the ability of bound virus to form plaques to assure that our early studies would reflect an attachment of virus to cell that could lead to productive infection and not to nonspecific binding. However, attachment is only the first of a series of conditions necessary for a virus to form a plaque.

Pierce et al. (14) have demonstrated the effect of ionic strength on the binding of Sindbis virus to chicken embryo fibroblasts. Increasing ionic strength (Z) beyond 0.17 caused a decrease in virus adsorption. We also saw this effect of ionic strength (Fig. 1). However, the decrease in virus adsorption with increasing ionic strength occurred only at pH values ≥7.0. The effect of ionic strength binding is itself a function of pH. All our remaining studies were performed between Z = 0.24 at pH 8.0 and Z = 0.22 at pH 6.5; thus we looked only at the mechanism of "tightly" bound virus.

The ionic nature of bacteriophage attachment to *Escherichia coli* originally prompted investigations of the effect of pH on virus binding (15, 20). Studies in the T2 phage-*E. coli* B system of the potentially ionizable groups involved in this attachment led to the conclusion that virus amino groups and cellular carboxyl groups were primarily involved. The pH dependence of arbovirus hemagglutination (16) suggested that the attachment of these viruses which lead to productive infection might also be pH dependent.

Several alternative hypotheses could account for a pH dependence in the binding of virus to cell. First, and most obviously, ionizing species of opposite charge may be located on virus and cell, respectively; thus, the pH dependence of
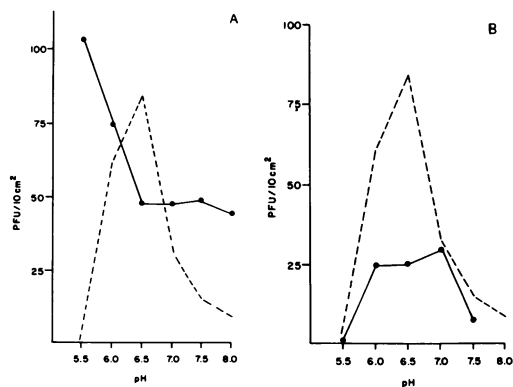

FIG. 2. *(A) Adsorption of Venezuelan equine encephalitis to CEF (●) compared with the adsorption of EEE (- - -). (B) Adsorption of EEE to BHK-21 (●) compared with the adsorption of an equal amount of EEE to CEF (- - -).*

the binding, and the binding itself, may be described by number, pK, and binding affinity of the ionizing species. Second, the ionizing species of opposite charge may be located entirely on the virus, entirely on the cell, or on both, but the actual binding may be accounted for by other interactions. Thus, the ionizing species provides the pH dependence by allowing the conformations necessary for the actual binding by other interactions, i.e., ionic, hydrophobic, or hydrogen bonds.

In either case, the minimum number of types of ionizable species necessary to account for the general form of the attachment curves is readily determined (Fig. 3). A single ionizable species can only describe a curve similar to a simple titration (Fig. 3A). Two types of ionizable species can account for a peaked symmetrical
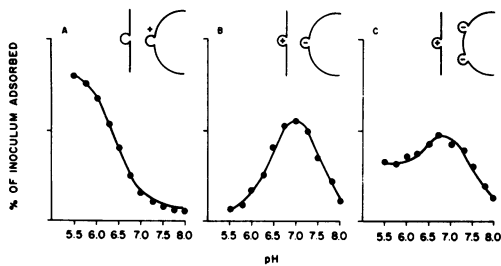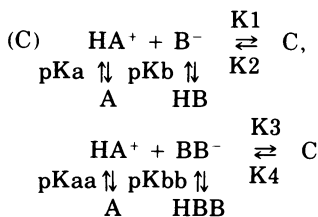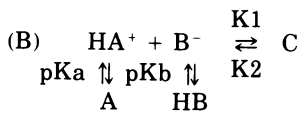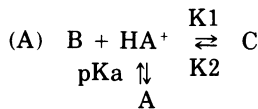

FIG. 3. *Possible adsorption profiles showing effect of the number of ionic species types used for binding reaction. These profiles were constructed by solving differential kinetic equations by computer for the following three model adsorption systems:*

$$(A) \quad B + HA^+ \quad \overset{K1}{\underset{K2}{\rightleftarrows}} \quad C$$
$$pKa \updownarrow$$
$$A$$

$$(B) \quad HA^+ + B^- \quad \overset{K1}{\underset{K2}{\rightleftarrows}} \quad C$$
$$pKa \updownarrow \quad pKb \updownarrow$$
$$A \qquad HB$$

$$(C) \quad HA^+ + B^- \quad \overset{K1}{\underset{K2}{\rightleftarrows}} \quad C,$$
$$pKa \updownarrow \quad pKb \updownarrow$$
$$A \qquad HB$$

$$HA^+ + BB^- \quad \overset{K3}{\underset{K4}{\rightleftarrows}} \quad C$$
$$pKaa \updownarrow \quad pKbb \updownarrow$$
$$A \qquad HBB$$

HA, HB, and HBB are the potentially ionizable groups on the cell or virus. While in the appropriate ionized form, the cell can adsorb to the virus, resulting in a complex (C). The rate constants (K_1, K_2, K_3, K_4), the pK's of the ionizable groups, and starting concentrating of viral receptor (A_0) and cellular receptor (B_0) are variables in the program.

curve (Fig. 3B), since the pK of each group is distributed equal distances on either side of the peak. A small amount of asymmetry may be accounted for in this model by a large inequality in the number of receptors. In this case, if one of the ionizing species is acidic and the other basic, then a simple attraction of opposite charges can account for the pH optimum. Three or more types of ionizable species are necessary to account for the markedly asymmetrical curve of the type shown in Fig. 3C. The number of attachment sites that must participate in the binding of virus to cell also affects the general form of the attachment curves (Fig. 4). In general, the more binding reactions that must occur prior to infection, the greater the effect of pH on the reaction.

The shape of the adsorption curve versus pH seen with EEE virus (Fig. 1) was peaked and asymmetrical, suggesting that at least two ionizing species were involved, perhaps present, in very unequal numbers. The degree of pH dependence suggests that one to three attachments must occur between virus and cell before infection can proceed. The lack of binding reversibility at low pH (Table 1) implies that the binding is not simply an ionic interaction of
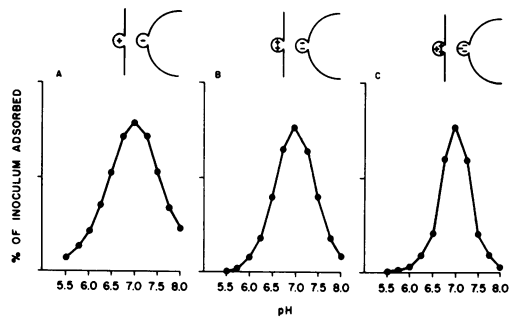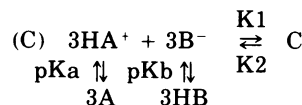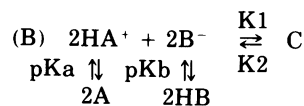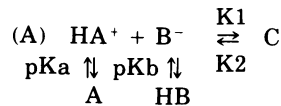

FIG. 4. *Possible adsorption profiles showing effect of the number of attachments that must occur between virus and cell prior to infection. These profiles are constructed by solving differential kinetic equations by computer for the model adsorption systems:*

$$(A) \quad HA^+ + B^- \quad \overset{K1}{\underset{K2}{\rightleftarrows}} \quad C$$
$$pKa \updownarrow \quad pKb \updownarrow$$
$$A \qquad HB$$

$$(B) \quad 2HA^+ + 2B^- \quad \overset{K1}{\underset{K2}{\rightleftarrows}} \quad C$$
$$pKa \updownarrow \quad pKb \updownarrow$$
$$2A \qquad 2HB$$

$$(C) \quad 3HA^+ + 3B^- \quad \overset{K1}{\underset{K2}{\rightleftarrows}} \quad C$$
$$pKa \updownarrow \quad pKb \updownarrow$$
$$3A \qquad 3HB$$

more than two oppositely charged species in an aqueous environment. The reversibility of the binding at higher pH appears to reflect the ionization of a single species, perhaps a group not even involved in the binding optimum. A definite mechanism cannot be described from the available data; however, they provide a basis for formulating binding hypotheses. When further chemical studies provide tentative binding structures, they should account for these data.

These are several possible structural candidates for both the viral and the cellular receptors. The three envelope glycoproteins could provide the viral receptors (2, 4). Even the lipid bilayer (9), under the glycoprotein array, is accessible to small molecules (7) and could conceivably contain receptor sites. Furthermore, liposome models (8, 11) suggest that cellular lipids may provide receptors for virus binding. By establishing a measurable biological correlate of the virus-cell receptor interaction, i.e., the pH dependence of the interaction, we can proceed to correlate structure-function differences between strains of group A arbovirus that in turn may help elucidate the chemical nature of the receptors.

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