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Correlation Between Virus-Cell Receptor Properties of Alphaviruses *in vitro* and Virulence *in vivo*

By

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With 4 Figures

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Summary

Virulent and avirulent clones of Venezuelan, Western, and Eastern equine encephalitis viruses were examined for their *in vitro* attachment characteristics to the surface of cultured cell monolayers. These attachment characteristics were correlated with *in vivo* plasma clearance rates and virulence. For the clones investigated, avirulence correlated *in vitro* with attachment pH optima close to physiologic pH and *in vivo* with a rapid clearance from plasma. Conversely, virulent clones had lower *in vitro* attachment pH optima and low plasma clearances *in vivo*.

Introduction

The virulence of some alphaviruses may be determined during the first interaction between virus and the reticuloendothelial system. In the course of hamster infection with Venezuelan equine encephalitis (VEE), equal quantities of virulent and avirulent virus have been found in most tissues (1, 7). While tissue quantities of virus were equal, the quantities of avirulent VEE virus in blood were lower than the quantities of virulent virus. This discrepancy in the amount of circulating virus suggested that a difference may exist in the adsorption of virulent and avirulent strains (11). Avirulent strains of VEE and Western equine encephalitis (WEE) viruses were found to be cleared from the circulation more rapidly than virulent strains (4, 6, 7). Hepatic endothelial and Kupffer cells selectively removed avirulent virus from plasma, but failed to remove virulent virus (6). To investigate these differences between virulent and avirulent alphaviruses we have examined their *in vitro* attachment to cultured cells.

The methods used herein to compare virulent and avirulent alphaviruses were previously shown to reflect an early interaction between virus and cells (8). A

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model of attachment between ionized receptors on the virus and cell surfaces was proposed following earlier work with these methods (8). We report here the *in vitro* attachment properties of six alphavirus clones and their correlation with *in vivo* plasma clearances and virulence.

Materials and Methods

Viruses

Large and small plaque VEE virus clones were derived from the virulent Trinidad donkey strain of VEE (5). Large and small plaque clones of WEE virus were derived from strain 72V1880 (3). These plaque size variants of VEE and WEE were picked from chicken embryo fibroblast (CEF) cultures for further study. The Eastern equine encephalitis (EEE) virus clones were derived from strain Arth 167 and were kindly provided by Dr. Philip Coleman, Medical College of Virginia, Richmond, VA. The six virus stocks were passed in duck embryo cell cultures (DEC) once prior to the radiolabeling procedures described below.

Cells for Adsorption Studies

Chicken embryo fibroblasts were prepared from 9- to 10-day-old embryos. Cells were seeded at 6×10^6 cells/well in 10-cm^2 wells of plastic 6-well plates (Linbro Chemical Co., New Haven, CT) and grown in medium 199, Earle's base, containing 10 percent calf serum (Armour Pharmaceutical Co., Chicago, IL). The monolayers were incubated at 37° C in 5 percent CO_2 for 3 to 4 days before use.

The J-111 human cell line was obtained from the American Type Culture Collection. Cells were seeded at a concentration of 5×10^5 cells/ml in 2 ml of Eagle's basal medium with Earle's salts containing 10 percent fetal calf serum in 10-cm^2 wells and were used after they had grown to confluency in 5—6 days.

Peritoneal macrophages were obtained from hamsters inoculated intraperitoneally (i.p.) with 5 ml of thioglycollate broth 48 hours prior to harvest. To obtain cells the peritoneal cavities of hamsters were washed with RPMI-1640 medium. Suspensions were centrifuged at $400 \times g$ for 10 minutes, washed twice, and seeded in 10-cm^2 wells at a concentration of 2×10^6 cells/ml in 2 ml of RPMI-1640 containing 10 percent calf serum. After incubation at 37° C for 1 hour, in a 5 percent CO_2 atmosphere, nonadherent cells were removed, monolayers were washed and used immediately.

To obtain Kupffer cells from guinea pigs, the procedure of MELLY (9) was employed. Briefly, livers of pentobarbital-anesthetized guinea pigs were perfused with Hanks' balanced salt solution (HBSS), followed by digestion medium containing 200 mg pronase and 1 mg DNAse/100 ml HBSS. Perfused livers were then minced with scissors, and incubated with digestion medium for 1 hour at 37° C. Digested liver suspensions were then filtered through gauze, centrifuged at $4500 \times g$ for 1 minute, washed 3 times in HBSS, resuspended in RPMI-1640 containing 10 percent fetal calf serum at a concentration of 2×10^7 cells/ml, and seeded in 10-cm^2 wells. Nonadherent cells were removed after 1 hour at 37° C. Medium was replaced and cells were used after incubation overnight at 37° C.

Preparation of Radiolabeled Virus

To prepare ^{3}H-labeled viruses, monolayers of CEF were grown in 800-cm^2 0.5 gallon roller bottles (Belleo, Vineland, NJ), seeded at 4.5×10^8 cells per bottle in 150 ml of medium 199, 10 percent calf serum, and rolled at 0.4 rpm for 2 days prior to use. The medium was then replaced with HBSS containing 0.025 M N-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid (HEPES) (pH 7.4). After 24 hours the HBSS was discarded and the roller bottles were infected with 10^9 PFU in 10 ml HBSS for 1 hour at 37° C. The inoculum was removed and replaced with 35 ml of amino acid-free medium 199 containing 10 μCi/ml [^{3}H] amino acid mixture (New England Nuclear Corp., Boston, MA), adjusted to pH 7.5 with 7 percent $NaHCO_3$, and rolled for 24 hours, 0.4 rpm, at 37° C.

The infectious supernatant was clarified at 10,000 rpm for 10 minutes (T-30 Spinco rotor) then pelleted at 25,000 rpm for 2 hours (T-30 rotor). The pelleted virus was suspended in a small volume containing 0.15 M NaCl, 0.05 M Tris (pH 7.4) and sedimented in a 10—30 percent sucrose (w/w) gradient, 0.15 M NaCl, 0.05 M Tris (pH 7.4) in a SW 25.1 Spinco rotor at 25,000 rpm for 2.5 hours. The opalescent virus band, harvested by puncturing the side of the tube, was stored at −70° C.

To prepare ^{32}P-labeled WEE large and small plaque viruses, BHK-21 cells were substituted for CEF in the above procedure and cells were incubated in phosphate-free medium containing 20 μCi/ml of ^{32}P as monopotassium phosphate as previously described (6).

Clearance of Virus From Hamster Plasma

Hamsters, anesthetized with sodium pentobarbital, were given a 1.0 ml intracardiac injection of virus containing 9 $\log_{10}$ PFU/ml. The animals were serially bled from the orbital sinus 1, 5, and 30 minutes after inoculation to obtain heparinized plasma samples which were titrated on DEC monolayers.

Attachment of Virus to Cell Monolayers

The six stock buffers for attachment experiments were 1 M sodium phosphate adjusted to 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 sterilized by passage through a 0.45 μ filter (Millipore, Bedford, MA). The six diluted, buffered solutions were used to dilute the viruses prior to the 1 hour adsorption period.

For the attachment studies, virus stock solutions were diluted with HBSS, 2 percent calf serum, and 0.025 M HEPES (pH 7.4) to contain 5×10^4 PFU/ml (titrated under optimal conditions, i.e. adsorption for 1 hour at 37° C in HBSS containing 10 percent calf serum) 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-hour attachment period. Unadsorbed virus was aspirated and the infected monolayers were overlaid with 2 ml of agar medium containing 1 percent agar (Ionagar, Difco, Detroit, MI) in EBME, 2 percent calf serum, 50 μg/ml gentamicin and 0.025 M HEPES (pH 7.4). After 2 days incubation at 37° C in 5 percent CO_2, 2 ml of 1:6000 neutral red (Grand Island Biological Co., Grand Island, NY) was added to each well for 3—5 hours at 37° C and the plaques were counted (5). The 100 percent attachment levels were calculated using virus titers obtained under optimal adsorption conditions of 1 hour at 37° C in HBSS containing 10 percent calf serum.

Attachment experiments with ^{3}H and ^{32}P-labeled viruses were performed as described for unlabeled virus; labeled virus was diluted 1:100 in the buffered solutions prior to the 1-hour adsorption period at 0° C. Unadsorbed virus was removed and discarded. The monolayers in each well were then dissolved in 0.4 ml 5 percent sodium dodecyl sulfate and transferred into a 10 ml scintillation vial with one 0.2 ml water rinse, and 6 ml Scintilute containing 20 percent Scintosol GP (Isolab Inc., Akron, OH) was added. ^{3}H or ^{32}P counts were corrected for background, quench, and counting efficiency.

Results

Biological Characteristics of Six Selected Alphavirus Clones

The strains of VEE and WEE viruses chosen for these investigations contained both large and small plaque variants. This plaque size heterogeneity was seen when the viruses were grown under agar on CEF monolayers. Large and small plaque virus clones were then isolated from these mixtures and used for all further experiments. These selected clones were tested for virulence by inoculating adult hamsters subcutaneously. The clone forming the larger plaques in each pair was the more virulent one.

The relative virulence of the VEE and WEE clones has been previously reported (4, 6). The avirulent WEE virus killed only 7 percent of hamsters inoculated subcutaneously with 4.5 $\log_{10}$ PFU, whereas the virulent WEE virus killed 74 percent of hamsters inoculated with 3.2 $\log_{10}$ PFU. The avirulent VEE virus killed only 15 percent of hamsters inoculated with 3.5 $\log_{10}$ PFU, but the virulent virus killed 100 percent of hamsters inoculated with 3.2 $\log_{10}$ PFU.

Large and small plaque clones of Arth 167 EEE virus had been picked from a plaque size mixture by Dr. Philip Coleman before we received them. Both the large and small plaque clones of EEE virus were avirulent for hamsters.

These six clones were then tested for their clearance from the plasma of hamsters after intracardiac injection. Both the large and small plaque EEE clones were relatively avirulent and had similar rapid clearances of more than 99 percent in 10 minutes as shown in Figure 1. Clearances for the VEE and WEE clones have been previously published (3, 5, 7), and graphs of their clearances are not repeated here. With both the VEE and WEE clones, the avirulent small plaque clone of each pair cleared rapidly and the large plaque clone cleared slowly. More than 99 percent of the avirulent VEE and WEE viruses left the circulation within 10 minutes. The virulent VEE and WEE viruses were not measurably removed from the circulation in 30 minutes.

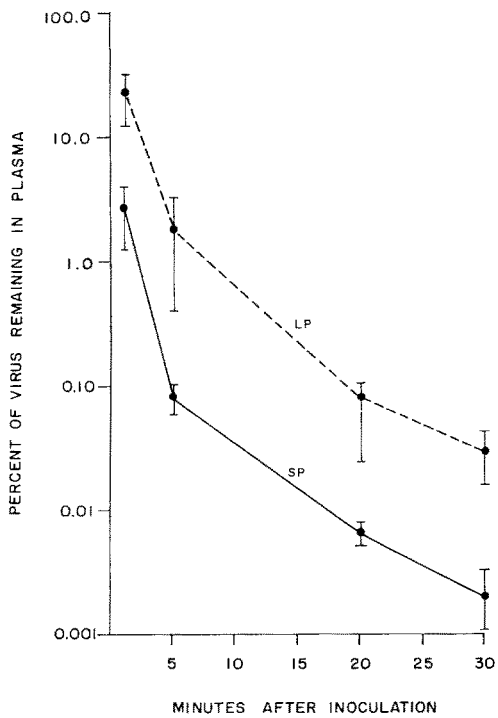

Fig. 1. Clearance of large and small plaque EEE clones from the circulation of hamsters inoculated via the intracardiac route with 9.2 $\log_{10}$ PFU of virus (expressed as percent of inoculum virus remaining in plasma, $\pm$SE)

Venezuelan Equine Encephalitis Virus Attachment

A comparison was made between the *in vitro* attachment characteristics of the virulent (Fig. 2A) and avirulent VEE clones (Fig. 2B). These two virus clones were diluted in buffered saline and allowed to remain in contact with monolayers of chicken embryo fibroblasts for only 1 hour at 0° C before the inocula were removed. The percent of virus attached to cell monolayer was then measured either by counting the plaques subsequently formed in the monolayer or by determining the amount of radiolabeled virus that remained on the monolayer. When attachment was measured as retention of radiolabeled virus on the monolayer, the avirulent small plaque VEE virus attached much more efficiently than the virulent, large plaque virus. This more efficient attachment of the avirulent virus correlated with its more rapid *in vivo* plasma clearance rate.

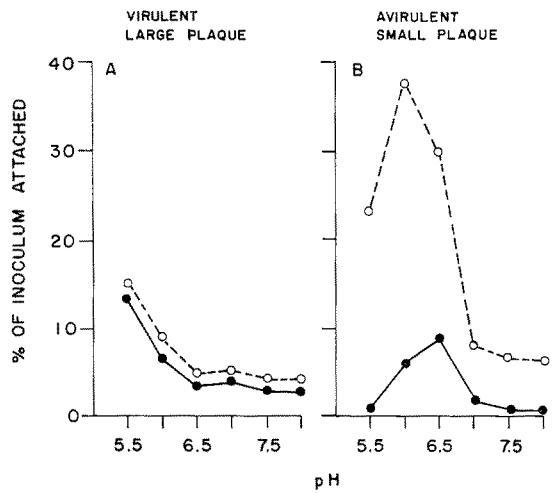

Fig. 2. Attachment of (A) virulent large plaque and (B) avirulent small plaque VEE clones to chicken embryo monolayers at 0° C. Attachment was measured with radiolabeled virus (○) and the formation of plaques (●)

These attachment experiments were performed at six different pHs to investigate the nature of this attachment phenomenon (8). The small plaque VEE clone attached most efficiently at pH 6.0 to 6.5, whereas the large plaque clone attached most efficiently at the lowest pH tested, pH 5.5.

In addition to the efficient attachment of the avirulent VEE clone a second distinct characteristic was seen with this virus (Fig. 2B). Only a fraction of the avirulent clone viruses that attached at 0° C subsequently formed plaques, 28 percent at pH 6.5. After the 1 hour attachment period, optimal conditions for virus growth were restored. The temperature was increased to 37° C and the media composition was changed to EBME, 2 percent calf serum and 1 percent agar. Thus this poor plaquing efficiency was a result of events that occurred during the attachment period. In contrast to the poor plaquing efficiency of the avirulent clone all the virulent VEE clone viruses that had attached under the experimental conditions formed plaques (Fig. 2A).

Western Equine Encephalitis Virus Attachment

Attachment experiments with the virulent and avirulent clones of WEE virus to chicken embryo monolayers are shown in Figure 3. These results were comparable to the results with the pair of VEE clones discussed above. The attachment of the avirulent WEE clone was slightly more efficient than of the virulent clone, and the optimal pH was between 6 and 6.5. The virulent clone attached best at pH 5.5, the lowest pH tested.

The attachment of the WEE clones was also studied at pH 6 and 7 with cells from three other species: guinea pig Kupffer cells, human J-111 cells, and hamster peritoneal macrophages (Table 1). At both pHs, attachment of the avirulent clone was more efficient than to the virulent clone.

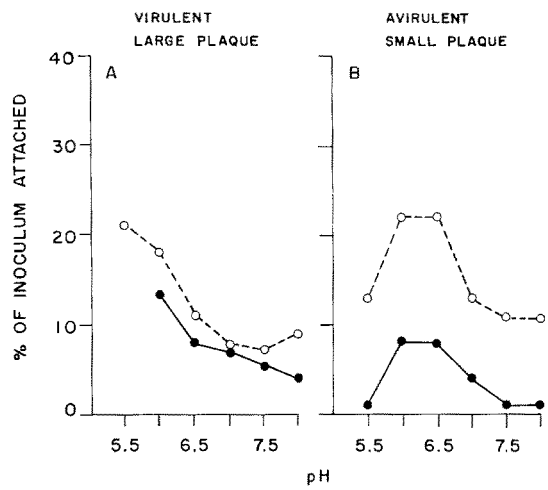

Fig. 3. Attachment of (A) virulent large plaque and (B) avirulent small plaque WEE clones to chicken embryo monolayers at 0° C. Attachment was measured with radiolabeled virus (○) and the formation of plaques (●)

Table 1. *Attachment of WEE virus clones to cell monolayers from 3 species*[a]

Clone	pH	Percent attachment (±SE)[b]		
		Guinea pig Kupffer cells	Human J-111 cells	Hamster macrophages
Avirulent	6	26.1±5.6	33.2±10.6	34.8±8.4
Small plaque	7	8.6±1.1	4.3± 0.6	6.7±1.3
Virulent	6	6.4±2.0	4.2± 0.5	3.8±1.2
Large plaque	7	0.3±0.2	1.6± 0.5	0.8±0.6

[a] Attachment is measured as the percent of radiolabeled virus remaining on the monolayer after 1 hour attachment at 0° C

[b] SE = standard error

The discrepancy between the percent of virions attached and the percent of virions that subsequently formed plaques was again seen only with the avirulent WEE clones (Fig. 3B). Only 36 percent of the attached avirulent virus formed plaques between pH 6 and 6.5. This abortive attachment was not seen with the virulent WEE clone (Fig. 3A). All the attached virulent WEE viruses replicated and formed plaques.

Eastern Equine Encephalitis Virus Attachment

Figure 4 shows attachment experiments with the two clones of EEE virus to chicken embryo monolayers. The percentage attachment of the small plaque EEE clone was only slightly more than of the large plaque clone, and with both clones the attachment pH optimum was at 6.5. The difference between the attachment percentages of these two avirulent clones was not nearly as marked as between the virulent and avirulent clones of WEE and VEE. However, the low percentage of attached small plaque EEE clone that formed plaques (Fig. 4B), 7 percent at pH 6.5, was similar to the low percentages seen with the avirulent, small plaque VEE and WEE clones (Figs. 2B and 3B).

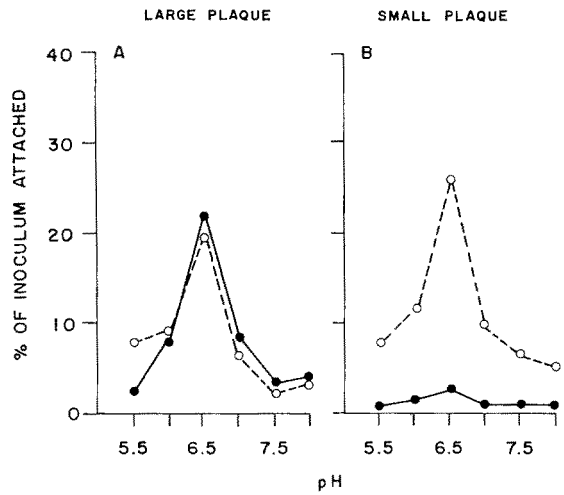

Fig. 4. Attachment of (A) avirulent large plaque and (B) avirulent small plaque EEE clones to chicken embryo monolayers at 0° C. Attachment was measured with radiolabeled virus (○) and the formation of plaques (●)

Discussion

It was our objective to determine if there were characteristics of virus attachment to cultured cells that correlated with *in vivo* plasma clearance and virulence. Earlier *in vivo* studies had shown a correlation between avirulence and rapid clearance of VEE and WEE viruses from plasma (6, 7). The rapid *in vivo* clearance of these avirulent viruses suggested that they may have a greater affinity for cells and that this property may even be observed *in vitro*. The *in vitro* cell attachment properties of six alphaviruses were tested. The most consistent difference we observed between virulent and avirulent viruses was a shift of the optimum attach-

ment pH of avirulent virus toward the physiologic pH range. This shift in optimal attachment pH was seen with one avirulent VEE clone, one avirulent WEE clone, and two avirulent EEE clones. Increased *in vitro* attachment of the avirulent alphaviruses to cultured cell monolayers was also observed, although this was not always striking and interpretation of the attachment data was complicated by the pH dependence of the process.

We chose readily reproducible conditions to study the *in vitro* attachment of these viruses to cell monolayers. Attachment of virus was performed in phosphate buffered saline to provide a stable pH and at 0° C to decrease the metabolic interaction of cell with virus. These conditions had previously been shown to measure only the earliest interaction between virus and cell. Such attachment conditions should reflect the surface structures on virus and cell and not more complex processes, such as pinocytosis.

The media and temperature used in these attachment experiments were necessarily artificial and dissimilar from *in vivo* attachment conditions. However, the correlations observed between *in vitro* attachment and *in vivo* clearance and virulence suggested we were observing an *in vitro* process that had important biological consequences, thus was worthy of further physiologic and biochemical investigation.

Viruses that attach more rapidly, firmly or completely to cells would be expected to be cleared from the circulation more rapidly than viruses that attach poorly to cells. A correlation between *in vitro* adsorption and serum clearance has been observed with Mengo viruses of varying virulence and with plaque size variants of Sindbis virus (2, 3, 10, 12). Clearly the avirulent VEE virus we studied *in vitro* attached more completely than the virulent VEE virus. Also, when the two WEE viruses were tested for their attachment to guinea pig Kupffer cells, human J-111 cells, and hamster macrophages, the avirulent virus attached more efficiently than the virulent virus. The greater attachment efficiency of the avirulent WEE virus was more pronounced at pH 6.0 than at pH 7.0. Although the complicating effects of pH and cell type must be taken into account even when simply measuring the attachment of virus to cultured cells, both avirulent VEE and WEE viruses attached more completely to cell monolayer than virulent viruses. Thus, VEE and WEE viruses demonstrated *in vitro* attachment preferences that could account for their *in vivo* plasma clearances.

It seemed that if enough time were allowed, all viruses would eventually have attached to the cell monolayer. This was not the case. Under the experimental conditions used, an equilibrium was reached before attachment of all virus. This equilibrium probably not only reflected the binding energy between virus and cell, but also the pKa's of ionized groups involved in the binding process. Similar complex equilibriums may well determine the rate of virus clearance from plasma.

The optimal pH's for *in vitro* virus attachment also appeared to be a function of these equilibriums. Avirulent viruses attached best between pH 6 and 6.5, whereas the virulent VEE and virulent WEE attached best at pH 5.5, the lowest pH tested. It was these attachment pH optima that appeared to have the most constant relationship with the *in vivo* plasma clearance and virulence. Those viruses with pH optima for attachment nearer to pH 7.0 were the avirulent viruses with rapid plasma clearances. There should be an *in vivo* attachment ad-

vantage for viruses with attachment pH optima nearer to the physiologic pH range than for viruses with more distant optima.

The correlation between rapid plasma clearances of alphaviruses and avirulence is common. A better understanding of this clearance mechanism should lead us to a virus structure or virus function that could explain one of the reasons some alphaviruses are virulent and others are avirulent. Presumably within each pair of alphaviruses that we studied there was a high degree of structural similarity. Each virus pair originated from the same initial isolate and within each pair the neutralization kinetics with single antisera were identical. The *in vitro* attachment data clearly showed differences between these virulent and avirulent viruses. These differences in *in vitro* attachment may reflect small differences in surface structures of these viruses. Because of the correlations seen between virulence, plasma clearance, and *in vitro* attachment, perhaps all are due to these small differences in virus surface structure. This hypothesis could be tested by comparing the exact amino acid sequence of the coat proteins in each virus pair. The ultimate test would be to compare amino acid sequences from a virus pair with a point mutation that resulted in a change in plasma clearance rate, virulence, and *in vitro* attachment.

An unexpected result of these experiments was that both the avirulent, small plaque VEE and WEE clones and the small plaque EEE clone exhibited a uniquely blocked replication after attachment at 0° C. Only a small fraction of the attached virus subsequently developed plaques. This was in marked contrast to the virulent large plaque Venezuelan and Western clones and the avirulent large plaque Eastern clones that had an almost 100 percent efficiency of replication with plaque formation for attached virus. These viruses are not defective in the classical sense, as they are fully infectious if the *in vitro* attachment is simply carried out at 37° C. This phenomenon of blocked replication resulting from a unique attachment seemed to correlate better with plaque size than virulence because it occurred with all the avirulent small plaque clones but not with the avirulent large plaque EEE clone.

Several observations suggested that this blocked replication may have resulted from a unique attachment of virus to cell. First, the blocked replication was not an effect of the buffers on the cells. Cells preincubated in these buffers at 0° C and subsequently infected at 37° C showed no ill effects from the buffers. Secondly, virus preincubated in the buffers at 0° C showed no deterioration after 1 hour when subsequently assayed at 37° C. Thirdly, the block in replication occurred during the 1 hour at 0° C that the virus was in contact with the cells because after the 1 hour attachment period the conditions were returned to 37° C and regular media. Thus, this block in the replication of small plaque viruses appeared to result from an unexplained feature of virus attachment.

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